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A Protein Associated with Axon Growth, GAP-43, Is Widely Distributed and Developmentally Regulated in Rat CNS

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Development or regeneration of axons in several systems is accompanied by 20-100-fold increases in the synthesis of an acidic, axonally transported membrane protein with an apparent molecular weight of 43-50,000 (Benowitz and Lewis, 1983; Skene and Willard, 1981a, b), which we designate GAP-43. We have proposed that some step(s) in axon growth require production of GAP-43, and perhaps a small number of other "growth-associated proteins," at rates much higher than those typical of mature neurons. This hypothesis predicts that virtually all neurons synthesize GAP-43 at elevated levels during normal CNS development. Here we show that a protein similar to GAP-43 from regenerating toad nerves is prominent among the newly synthesized (^{35}S-methionine-labeled) and total (Coomassie blue-stained) proteins in neonatal rat cerebral cortex and cerebellum, suggesting that synthesis of GAP-43 is indeed a common feature of many developing neurons. Synthesis and accumulation of the protein decline an order of magnitude as animals mature. Antibodies raised against the rat cortex GAP-43 also recognize electrophoretically similar proteins from regenerating toad optic nerves and from developing hamster sensorimotor cortex, indicating that structural features of GAP-43 are conserved in evolution. Cell-free translation of polyadenylated RNA from neonatal and adult cortex suggests that developmental regulation of GAP-43 synthesis is mediated largely through changes in mRNA abundance. These observations together suggest that developmental regulation of GAP-43 gene expression may be common to most vertebrate CNS neurons.

GAP-43 remains detectable at a low level in adult rat cortex, and it co-migrates on two-dimensional gels with B-50, a synaptic membrane protein which is a preferred substrate for protein kinase C in adult brains. Phosphorylation of the protein by endogenous kinase(s) *in vitro* is 4-7-fold greater in growth cone membranes than in mature synaptic membranes, which raises the possibility that local modification of the protein in axon terminals may be synergistic with regulation of GAP-43 synthesis in the cell body.

Elongation of axons is a fundamental process in nervous system development and in the regeneration of injured pathways. Although some important environmental influences on axon growth have been demonstrated (e.g., Benfey and Aguayo, 1982; Fallon, 1985; LeTourneau, 1975; Richardson et al., 1984; Rogers et al., 1985; Sanes, 1985), the critical molecular events within the neuron that regulate axon growth have scarcely begun to be elucidated. We suspect that, by analogy with the regulation of cell growth in non-neural systems (e.g., Bishop, 1985; Weinberg, 1985), a few axonal macromolecules might be key sites for the control of axon growth. It is upon these intraneuronal regulatory molecules that environmental elements must exert their influences.

Among the candidate molecules for regulation of axon growth are the "growth-associated proteins" (GAPs) whose synthesis increases one to two orders of magnitude during successful axon regeneration in several systems (Benowitz et al., 1981; Benowitz and Lewis, 1983; Heacock and Agranoff, 1982; Skene and Willard, 1981a, b). We have proposed that the individual growth-associated proteins are essential participants in some aspects of axon growth, and that neurons in the mammalian CNS that fail to regenerate their axons do not synthesize GAPs in sufficient abundance to support axon growth (Skene and Willard, 1981b). If GAPs are essential for axon growth, one would expect that virtually all neurons would synthesize substantial levels of GAPs during normal developmental axon outgrowth. Thus, at times when many neurons are extending axons, one would expect GAP synthesis to be a prominent feature of developing brains. Conversely, if most neurons in the mature mammalian CNS fail to synthesize GAPs at a level sufficient to sustain axon growth, one would expect that GAP synthesis would be much lower in mature brain. Here we show that both of these predictions are fulfilled for one "growth-associated protein," GAP-43.

Materials and Methods

Materials

^{35}S-methionine (approximately 1000–1400 Ci/mmol) was obtained from Amersham. Urea, sucrose, SDS, acrylamide, and bisacrylamide were obtained from BioRad.

Labeling of rat brain proteins in vivo

Sprague-Dawley rats were obtained from Simonsen. The age of the rats used ranged from immediately postnatal (0 d) to adult. The rats were anesthetized by cold (0–14 d) or by ketamine (100–150 mg/kg, i.p.). The calvarium was surgically exposed, and 20–100 μCi of ^{35}S-methionine was injected intracerebrally with a Hamilton syringe, either through a hole drilled in the skull or directly through the skull. After 3–5 hr survival, the animals were killed with CO_2, and their brains quickly removed and stored at −70°C.

Labeling of GAP-43 in regenerating toad nerves

Toads (*Bufo marinus*; Delta Biologicals, Vidalia, LA) were anesthetized on ice, and the left optic nerve crushed with forceps approximately 2–3 mm proximal to the optic chiasm. Two weeks later, ^{35}S-methionine was injected into the left eye. The toads were killed 12 hr after injection, and the portion of the optic nerve and optic tract distal to the crush site removed. The nerves were homogenized and particulate fractions prepared for electrophoresis, as described for rat brains.

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Tissue preparation and electrophoresis

The cerebral hemispheres or cerebellum were homogenized with 15 passes of a Teflon pestle in H buffer containing 10 mM Tris, pH 7.5, 5 mM EDTA, and 5 mM dithiothreitol (DTT). Soluble and particulate fractions of this homogenate were separated by centrifugation at 100,000 × *g* for 30 min. The pellet from this centrifugation was resuspended in a solubilizing buffer consisting of 0.5–1.0% SDS and 5 mM DTT, and incubated at 95°C for 5 min. Protein was determined by the method of Lowry et al. (1951), using BSA as a standard, and radioactivity was determined by trichloroacetic acid (TCA) precipitation on glass filters and subsequent liquid scintillation counting. The samples were then diluted 1:1 with a second solution containing 8 M urea, 5 mM DTT, and 10% nonidet P-40. Proteins from the soluble fraction were precipitated by addition of 0.5 volume of 21% TCA and centrifugation at 8000 × *g* for 15 min. The TCA pellet was washed twice with diethyl ether, dried thoroughly, and solubilized as described for the particulate fraction above.

Two-dimensional electrophoresis was carried out essentially according to the method of O'Farrell (1975). Isoelectric focusing was performed using tube gels (1.2 mm diameter) containing 4% acrylamide, 0.21% bisacrylamide, 9 M urea, and 2.4% ampholytes. The specific ampholytes employed were at pH 3.5–10 and either pH 5–7 or pH 4–6, at a ratio of 2:1. After focusing at 350–400 V for 18–22 hr, the tubes were overlaid on an SDS–acrylamide slab gel and electrophoresed in the buffer system of Laemmli (1970). The slab gels consisted of either a uniform acrylamide concentration (usually 12%), or a 5–15% linear gradient poured with a Beckman density gradient former.

After completion of the second dimension, gels were fixed and stained with Coomassie brilliant blue according to the method of Fairbanks et al. (1971) and then impregnated with xylenes and 2,2-diphenyloxazole using the APEX system of Jen and Thach (1982). After drying on filter paper, the gels were exposed at −70°C to Kodak XAR x-ray film that had been previously flashed to an O.D. of 0.1 (Laskey and Mills, 1975). The exposures were adjusted to approximately equalize the product of the total radioactivity loaded on the gel times the length of the exposure, expressed as CPM-days, among gels that were to be compared. The optical density of individual protein spots was determined using a scanning densitometer (Transidyne General Corp.).

Purification of rat brain RNA

Total RNA from rat brains was obtained by the method of Chirgwin et al. (1979). Brain tissue was homogenized in 9 volumes of 6 M guanidine thiocyanate, 0.5% sodium *N*-lauryl sarcosinate, 0.5% *p*-mercaptoethanol, and 50 mM Na-citrate, pH 7.0. Cell debris was removed by centrifugation at 5000 × *g* for 10 min, and the supernatant was layered onto a solution of 5.7 M cesium chloride and 100 mM EDTA. RNA was pelleted by centrifugation at 120,000 × *g* for 16 hr, redissolved in 6 M guanidine/25 mM EDTA, and precipitated by addition of 10% volume 3 M sodium acetate and 2 volumes ethanol. The ethanol precipitate was collected after incubation at −20°C by centrifugation, washed with 70% ethanol, and dried under N_2.

Poly-A$^+$ RNA was purified from the total RNA preparation by oligo-dT chromatography (Aviv and Leder, 1972). RNA was translated *in vitro* using a commercial wheat germ system (BRL wheat germ IVT system, nuclease-treated) according to the manufacturer's protocol; ^{35}S-methionine was the labeled amino acid used for translation. Translation products were recovered by TCA precipitation and solubilized for 2-D electrophoresis, as described above.

Phosphorylation of GAP-43

Growth cone particles were isolated from 3-d-old rat brains by the method of Pfenninger et al. (1983); synaptosomes from adult rat brain were isolated according to Cohen et al. (1977) and Ueda et al. (1979). Samples from the discontinuous sucrose gradients were diluted 1:1 with lysis buffer containing 1 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (TES), pH 7.3; 1 mM $MgCl_2$; and 0.5 mM $CaCl_2$. After stirring 15 min on ice, the lysed samples were centrifuged 30 min at 100,000 × *g*. The pelleted membranes were resuspended in lysis buffer. Aliquots of the resuspended membranes were assayed for total protein according to Lowry et al. (1951), using BSA as a protein standard.

Aliquots of growth cone or synaptosomal membranes containing 15 μg total protein were incubated in a reaction mixture containing 50 mM sodium acetate, 10 mM magnesium acetate, and 1 mM calcium chloride, pH 6.5. ^{32}P-ATP was added to a final concentration of 10 μM containing 1 μCi ^{32}P in a final reaction volume of 25 μl. Reaction mixtures were incubated 3 min at 30°C, and the reactions terminated by addition of 25 μl of 2% SDS/2.5 mM EDTA. Aliquots of each reaction mix were analyzed by one- and two-dimensional electrophoresis, and labeled proteins were detected by exposure to x-ray film.

Preparation of antibodies

GAP-43 was purified in denatured form from brains of rats 7–13 d of age. Brains were homogenized in H buffer (10 ml/gm wet weight) and a crude particulate fraction collected by centrifugation at 100,000 × *g* for 30 min. The pellets were dissolved in 1% SDS and aliquots removed and assayed for total protein. To the remainder of the sample, we added an equal volume of 10% nonidet P-40/8 M urea/5 mM DTT. Aliquots containing approximately 0.5 mg total protein were applied to each of 12 isoelectric focusing (IEF) gels prepared as for analytical 2-D gels, except that the preparative gels were poured in 0.5-cm-diameter tubes. Isoelectric focusing was carried out as described above, and one tube was removed and stained according to Malik and Berrie (1972). We aligned the remaining gels with the stained gel and cut out 0.5 cm segments corresponding to the position of GAP-43. These gel segments were minced, incubated for 1 hr with transfer buffer, and loaded onto a preparative SDS–polyacrylamide gel (10% polyacrylamide in a 1-cm-diameter tube). Electrophoresis was carried out and proteins eluted in the preparative electrophoresis chamber obtained from BRL, and 1 ml fractions were collected. Aliquots of each fraction were analyzed by analytical SDS-PAGE and detected by silver staining according to Morrisey (1981). Fractions containing a single band with a molecular weight appropriate for GAP-43 were pooled and analyzed by 2-D electrophoresis. The pooled GAP-43 fractions were dialyzed against 0.05% SDS and concentrated approximately 5-fold by vacuum centrifugation.

Approximately 200 μg of GAP-43 was emulsified in an equal volume of Freund's complete adjuvant, with the addition of 1 M NaCl as needed to promote emulsification (approximately 100 mM final concentration), and the emulsion injected into a rabbit subcutaneously at multiple sites. Three boosts of approximately 50 μg GAP-43 in incomplete Freund's adjuvant were made at 1 month intervals, and serum was collected 1 week after each boost.

For affinity purification of antibodies, 100 μg of GAP-43 was incubated overnight with CNBr-activated agarose beads (Sigma), according to Parikh et al. (1974). Serum was passed twice through a column of the GAP-43-containing beads, and the flow-through collected. The column was then washed with 10 volumes of 50 mM Tris, 200 mM NaCl, pH 7.5 (TBS) and the bound antibodies eluted with 0.2 M glycine, pH 2.4; 0.5 ml fractions were collected into tubes containing 55 μl of 2 M Tris, pH 8.5, to neutralize the samples. Samples containing protein (determined by A_{280}) were pooled, and these constitute the affinity-purified antibody.

Immunoblotting of GAP-43 requires special conditions (K. Meiri, personal communication). To transfer proteins from SDS–polyacrylamide gels to nitrocellulose for antibody staining, gels were soaked 1–2 hr in 10% trichloroacetic acid/10% acetic acid/20% isopropanol, then soaked 1–2 hr in 25 mM Tris base/192 mM glycine/1% SDS. Electrophoretic transfer of proteins to nitrocellulose was then carried out for 16 hr at 30–50 V at 4°C in a transfer buffer containing 20 mM Tris base, 150 mM glycine, 20% methanol, and 0.05% SDS. The resulting Western blots were incubated 1 hr with 1% BSA in TBS containing 0.05% nonidet P-40, and then incubated overnight with serum at a dilution of 1:100. After washing the filters in TBS/0.05% nonidet P-40, the bound antibody was detected by sequential incubation with biotinylated goat anti-rabbit IgG and an avidin–biotin complex of HRP. We used 4-chloro-1-naphthol as a chromophore in the peroxidase colorimetric reaction.

Results

We used 2-D electrophoresis to identify overall differences in protein synthesis between neonatal and adult rat cortex, and to look for GAP-like proteins. One protein in the membrane fraction (washed pellet from a 10^5 × *g*, 30 min spin) of neonatal cortex appeared to correspond to the growth-associated protein GAP-43 described in regenerating toad optic nerve (Skene and Willard, 1981a). The rat cortex protein has a pI of about 4.6 and an apparent molecular weight of about 47 kDa on a 12% polyacrylamide gel, similar to toad GAP-43 (the discrepancy in apparent molecular weight is discussed below). When samples

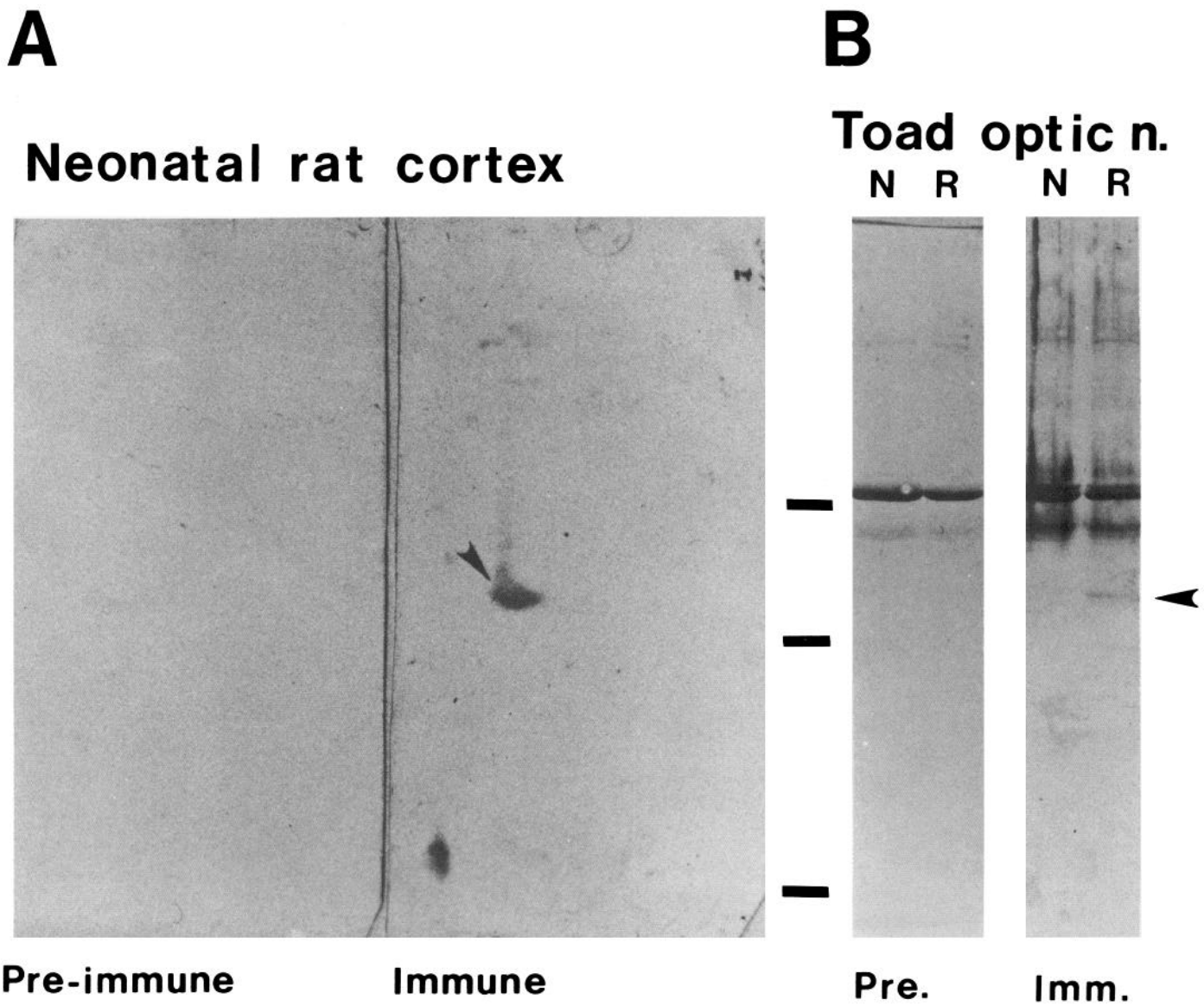

Figure 1. Immunologic cross-reactivity of rat and toad GAP-43. *A*, Immunostaining of Western blots of particulate fractions from 13-d-old rat cortex separated by 2-D electrophoresis (acidic half of gels shown, oriented with the acidic end to the left; the pH 4–6 range was expanded). *B*, Immunostaining of Western blots of particulate fractions from normal toad (*Bufo marinus*) optic nerves and optic tracts (*N*) and from regenerating nerve/tracts taken 10 d after optic nerve crush (*R*), separated on one-dimensional gels. In both *A* and *B*, the vertical gel dimension is SDS-PAGE on 10% polyacrylamide gels, and the blots are stained with antiserum raised against purified "GAP-43" from neonatal rat brain (1:100 dilution), or with pre-immune serum from the same rabbit. *A* confirms that the antiserum shows specific staining for rat "GAP-43"; *B*, shows that the antiserum cross-reacts with toad GAP-43, identified by its apparent molecular weight and induction during optic nerve regeneration (*arrow*). Coomassie staining of 2-D gels of regenerating toad optic nerves indicates that the samples in *B* contain much less GAP-43 than the rat samples in *A*, consistent with the lower intensity of immunostaining for the toad samples. *Horizontal bars* between *A* and *B* indicate the positions of molecular-weight markers run on parallel gels, transferred to nitrocellulose and stained with Amido black: BSA, 68,000; ovalbumin, 43,000; and trypsinogen, 25,000.

of rat cortex and regenerating toad optic nerve were run in the same series of gels, toad GAP-43 and the corresponding protein in rat cortex comigrated on 2-D gels with 12% polyacrylamide in the second dimension. When higher-resolution gradient gels were used in the second dimension, it was possible to separate the toad and rat proteins by a distance corresponding to a maximum difference in apparent molecular weight of 2 kDa. The isoelectric points of the 2 proteins were identical. The rat cortex protein also migrated in a very similar position to the GAP-43 identified in hamster corticospinal tract (Kalil and Skene, in press, data not shown).

To examine further possible homology between toad GAP-43 and the rat brain protein, we immunized rabbits with the rat protein purified from neonatal brains by preparative isoelectric focusing and SDS gel electrophoresis. Figure 1 shows that the resulting antiserum recognizes both the neonatal rat brain protein and a protein of nearly identical apparent molecular weight induced during regeneration of toad optic nerves. The antibody also recognizes a protein of similar molecular weight in neonatal hamster somatosensory cortex (see Fig. 9), which is also developmentally regulated (Kalil and Skene, 1986). Cross-reactivity of the anti-rat serum with hamster and toad proteins of similar molecular weights suggests that at least some structural features of the rat brain protein described here are phylogenetically highly conserved.

We found an additional property of this rat cortex protein

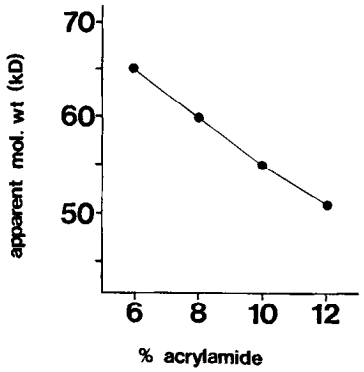

Figure 2. Variation of GAP-43's apparent molecular weight with gel concentration. Labeled 7 d rat cortex proteins were run on 2-D gels, with the second dimension slab gel consisting of the indicated acrylamide concentration. The gel was stained with Coomassie blue, and the apparent molecular weight of GAP-43 was calculated from the standard curve derived from the migration distances of the molecular-weight standards (68, 43, and 18 kDa).

Table 1. Estimated apparent molecular weights of GAP-43-like proteins from various sources[a]

Sample	12% Gels (kDa)	5–15% Gradient gels (kDa)
Toad optic nerve	47–48	56–57
Rat cerebral cortex	48–51	56–58
Rat cerebellum	50	58–60
B-50 (rat)	47–48	55–59
Translation product, rat cortex poly A⁺ RNA	48–50	59–60

[a] The values shown represent the apparent molecular weights calculated *independently* for each sample, using 2-D electrophoresis with the indicated concentration of acrylamide in the second (SDS) dimension. When the GAP-43-like proteins from different sources are compared directly on parallel gels or in mixed samples, all the rat proteins comigrate precisely. Toad GAP-43 precisely comigrates with the rat proteins on 12% gels and overlaps with them on the gradient gels.

that was shared with GAP-43 from both toad optic nerve and hamster pyramidal tract; its *apparent* molecular weight varies inversely with the concentration of acrylamide in SDS gels (Fig. 2). Although similar aberrations in SDS gel migration have been reported for some other proteins (Banker and Cotman, 1972; Segrest and Jackson, 1972), we have observed no other major proteins that exhibit this behavior in our samples. We have used this unusual electrophoretic behavior as an additional empirical criterion for identifying GAP-43-like proteins by comparing the relative mobilities of these proteins on 2-D gels with either 12% polyacrylamide or a gradient of 5–15% polyacrylamide in the second dimension. In a 5–15% gradient, GAP-43 migrates through low acrylamide concentrations during the run and exhibits an apparent molecular weight of 56–60 kDa, compared to 46–50 kDa on a 12% gel (Fig. 3 and Table 1). The degree of this apparent molecular-weight shift is similar for GAP-43 and the rat cortex protein.

The electrophoretic similarities between the rat brain protein and toad GAP-43, and the cross-reactivity of the anti-rat antiserum with a protein induced during toad optic nerve regeneration, strongly suggest that the rat protein described here is homologous to toad GAP-43. We will therefore refer to the rat protein as GAP-43.

Distribution and developmental changes of rat GAP-43

Rat GAP-43, identified on the basis of 2-D gel position and apparent molecular-weight shift, was found in the membrane fractions of neonatal cortex, cerebellum, and superior colliculus. The time course of GAP-43 synthesis and steady-state abundance was studied in detail in both cortex and cerebellum.

The relative amount of GAP-43 synthesized was determined from fluorographs of ^{35}S-methionine-labeled proteins from various postnatal ages separated by 2-D gel electrophoresis. Labeling of GAP-43 was normalized to total protein synthesis at each age by adjusting exposure of each fluorograph in series of gels so that the product of exposure time and total radioactivity loaded onto the gel was the same for all samples. This permitted us to quantify synthesis of GAP-43, relative to total protein synthesis at each age, by direct comparison of GAP-43 spot intensity on the fluorographs. Figure 4 shows one time series of fluorographs; similar results were obtained with 5 independent analyses. Two series of fluorographs were analyzed by densitometry to quantify relative GAP-43 synthesis as a function of age (Fig. 5). Synthesis of GAP-43 is relatively high in the cortex at birth, and increases about 30–40% in the first week. Thereafter, the synthesis declines sharply to about ⅛ of the 7 d level at 2 weeks. Over the next 4 weeks, the level of synthesis declines to the adult level, approximately 20-fold lower than the 7 d level. These results were not affected by the presence or absence of protease inhibitors during sample preparation, indicating that our observations reflected real changes in the synthesis of the protein. A similar pattern is seen in the cerebellum over the same time periods, except that maximum relative synthesis of GAP-43 appears to occur at or before the time of birth. GAP-43 synthesis, relative to total protein synthesis, is approximately 10× greater in a newborn rat cerebellum than in adults.

The steady-state abundance, shown by Coomassie staining, of rat GAP-43 also declines with increasing age (Fig. 4). However, the abundance of the protein remains relatively constant for several weeks after GAP-43 synthesis begins to decline, indicating that degradation of GAP-43 in the older animals is slow, with a half-life on the order of weeks.

Developmental changes in translatable GAP-43 mRNA

The decrease in GAP-43 synthesis with maturation could be due to a decrease in the level of specific mRNA, or to a decrease in the translational efficiency of the message, or to some combination of the 2 factors. To try to determine which factor is dominant, we analyzed the labeled products of *in vitro* translation of rat cortex poly A⁺ RNA in a wheat germ extract, using 2-D electrophoresis. The wheat germ system translated protein products effectively up to about 85 kDa. Among the products we found a protein with acidic pI and an apparent molecular weight of about 57 kDa on a 5–15% gradient gel. The apparent molecular weight of this protein (and no other protein in the translation products) shifted to about 47–50 kDa on a 12% gel (Fig. 6). We concluded on this basis that this protein is identical with GAP-43 labeled *in vivo* in rat cortex.

When equal amounts of poly A⁺ RNA from neonatal and adult rat cortex are translated, the relative amounts of GAP-43 are substantially different (Fig. 6). There is significantly less GAP-43 in the products of adult RNA than in those of neonatal RNA. The overall pattern of the *in vitro* synthesized proteins did not appear to change from neonate to adult, and the changes in other identifiable proteins appeared similar to those seen in *in vivo* labeled proteins (this was not quantified). The decrease in *in vitro* GAP-43 synthesis from 10 d to adult was estimated by densitometry to be about 10-fold. Since the translation conditions were identical and excluded any brain-specific factors that might have influenced translational efficiency, we conclude that this decrease represented an actual decrease in the abun-

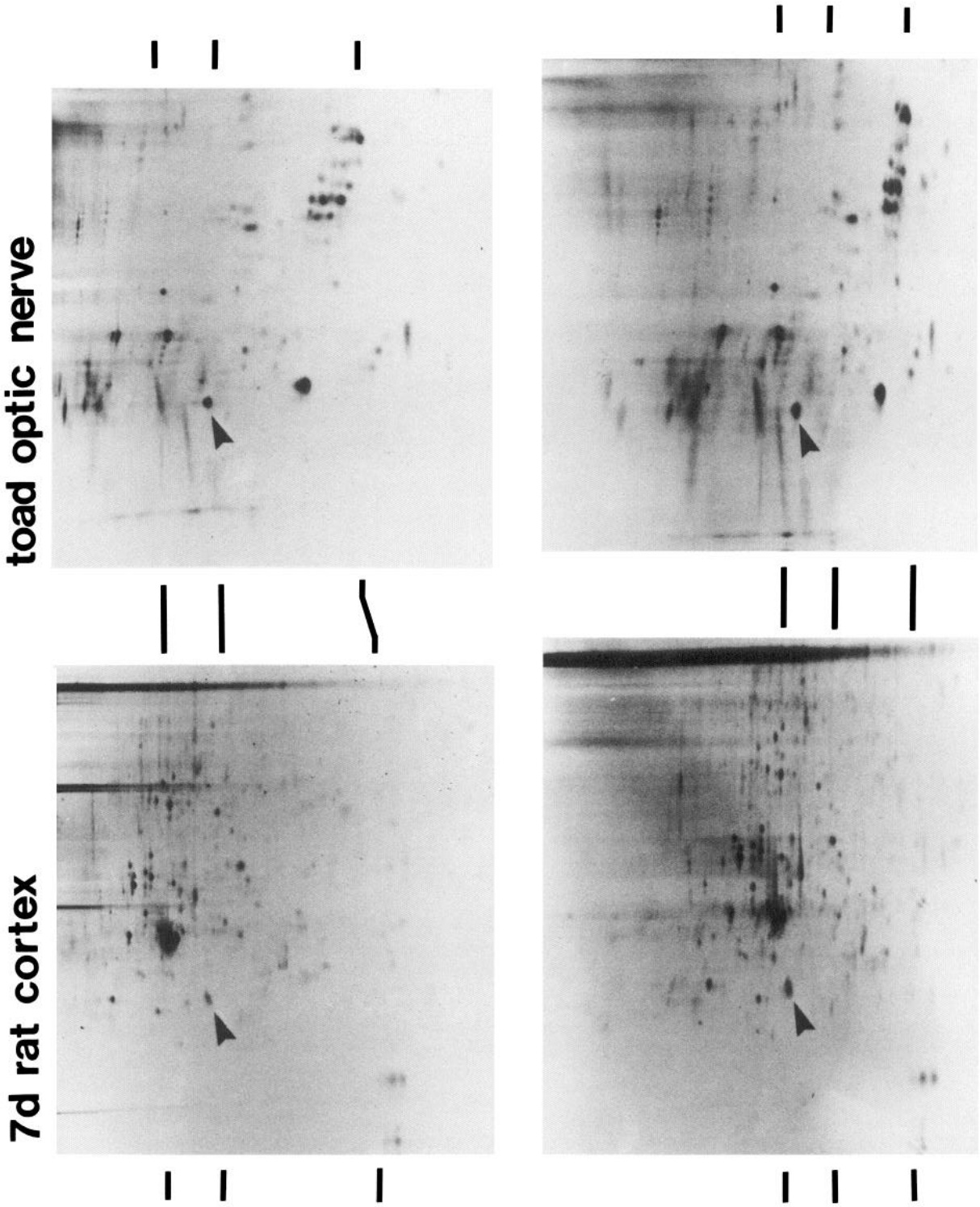

Figure 3. Presence of a GAP-43-like protein in the membrane fraction of neonatal rat cortex. ^{35}S-methionine labeled membrane proteins from 7-d-old (*7d*) rat cerebral cortex and rapidly transported proteins in toad optic nerve 10 d after crush were subjected to 2-D electrophoresis and visualized by fluorography as described in Materials and Methods. In all of the gels shown, the *horizontal axis* represents the direction of isoelectric focusing, with the acidic end on the left, and the *vertical axis* represents the direction of SDS electrophoresis. The isoelectric focusing was expanded in the range of pH 5–7. The top 2 gels are 12% acrylamide, and the bottom 2 are 5–15% acrylamide gradients. The *arrows* indicate GAP-43 in the toad, and a protein in the rat cortex sample that migrates to the same position on both kinds of gels. Approximately 7000 cpm were loaded on each gel; exposure time was 23 d. The *black bars* mark the molecular-weight standards: BSA, 68 kDa; ovalbumin, 43 kDa; and *b*-lactoglobulin, 18,000.

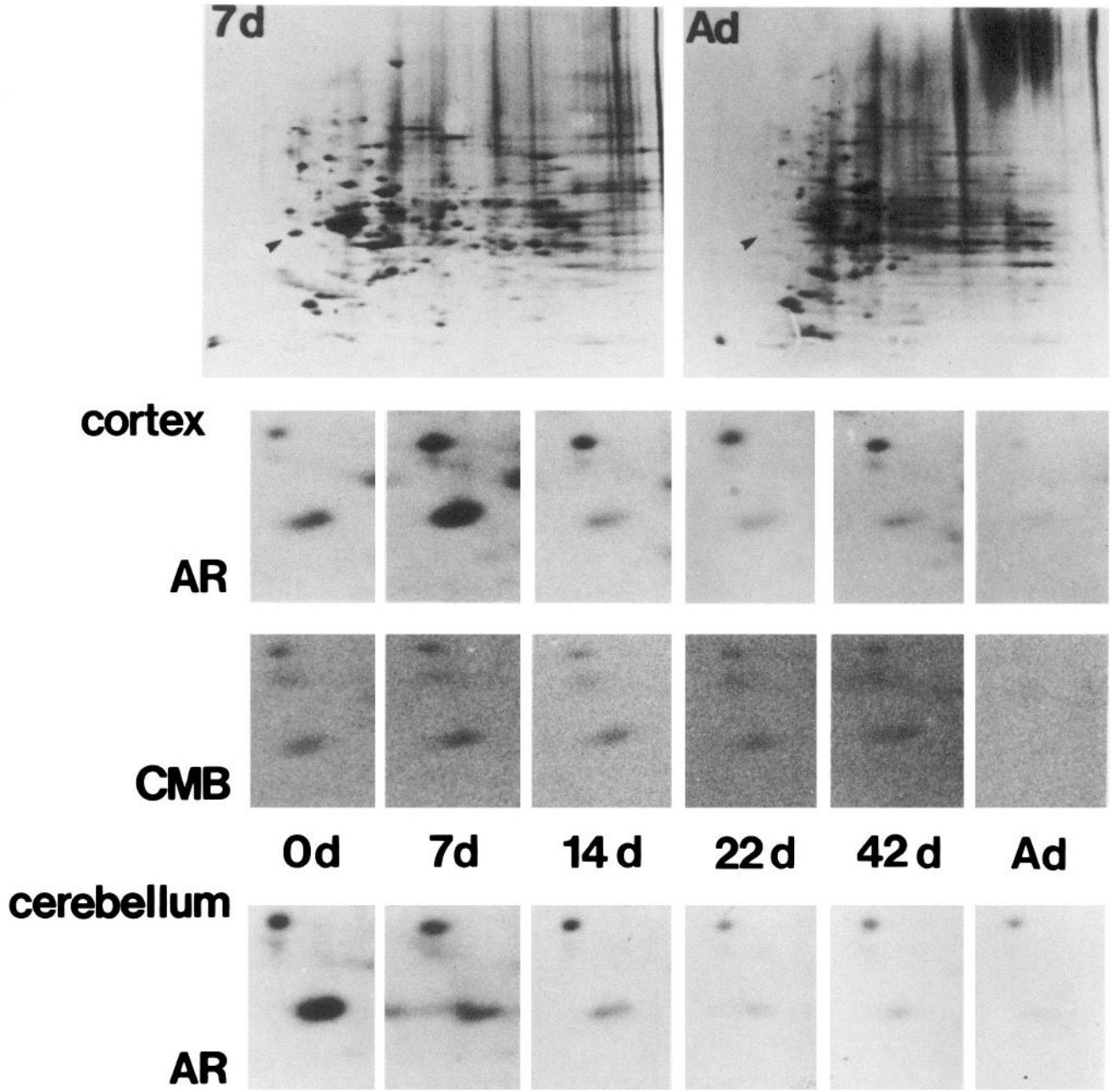

Figure 4. Developmental changes in GAP-43 synthesis in rat brain. The 2 fluorographs at the *top* are from 2-D gels of 7-d-old (*7d*) and adult (*Ad*) rat cerebral *cortex* membrane proteins. The pH 4–6 range was expanded in isoelectric focusing, and the second (SDS) dimension was run on 5–15% polyacrylamide gradient gels. 100 μg of protein were loaded on each gel; the 7 d gel was exposed for 260,000 cpm-d (corrected for decay), and the adult gel was exposed for 350,000 cpm-d, to clearly show the faint GAP-43 spot. Below these whole gels are 3 series of close-ups showing the GAP-43 region by fluorography (*AR*) and Coomassie staining (*CMB*). Samples were obtained from neonatal and adult rats on the days indicated. 100 μg of protein were loaded per gel in each case, and each exposure was approximately 260,000 cpm-d, with the exception of the 22 d (*22d*) gels, which for both cortex and cerebellar series were exposed for 220,000 cpm-d.

dance of the specific GAP-43 message, relative to the total mRNA population in the cortex.

Phosphorylation of GAP-43 and similarity to a kinase C substrate, B-50

The electrophoretic similarities between mature GAP-43 and the primary translation product suggest that GAP-43 does not undergo extensive post-translational modifications that would substantially alter its apparent molecular weight or isoelectric point, but our evidence does not exclude all post-translational modification of the protein. Indeed, Katz et al. (1985) have reported that a particulate-fraction protein electrophoretically similar to GAP-43 is among the major substrates for endogenous kinases in isolated growth cones from neonatal rat brains. We have shown that GAP-43 is a major component of these isolated growth cones (J. H. P. Skene, R. D. Jacobson, G. J. Snipes, C. B. McGuire, J. J. Norden, and J. A. Freeman, unpublished observations). When we exposed isolated growth cone membranes to a pulse of ^{32}P-ATP *in vitro*, one of the most heavily labeled proteins exhibited the acrylamide-concentration-dependent molecular-weight shift characteristic of GAP-43 (not shown) and comigrated with Coomassie-stained GAP-

43 upon 2-D electrophoresis (Fig. 7). *In vitro* phosphorylation of GAP-43 was 4–7-fold greater in growth cone membranes than in synaptic membranes isolated from adult rats (Fig. 8).

The position of GAP-43 on 2-D gels and its phosphorylation in axon terminals are reminiscent of a protein designated "B-50" by Gispen and colleagues (Zwiers et al., 1978, 1980). B-50 is a prominent substrate for protein kinase C in synaptic terminals (Aloyo et al., 1983) and appears to influence phosphoinositide metabolism in synaptic membranes (Oestreicher et al., 1983). Recently, B-50 has been found in growth cones from fetal rat brain (de Graan et al., 1985). To test the possibility that the B-50 protein in adult synaptic membranes is the same protein as GAP-43, we partially purified B-50 protein from adult rat brains by the "new procedure" of Oestreicher et al. (1983) through the penultimate step. The final step in the purification of B-50 is preparative IEF. Therefore, authentic B-50 protein is easily identified by 2-D electrophoresis of the partially purified material, using analytical scale IEF in the first dimension. Figure 7 shows that the B-50 protein from adult rat brains exactly comigrates with GAP-43 from neonatal rat cortex on 2-D gels. In addition, the apparent molecular weight of the B-50 protein shows the same aberrant dependence on acrylamide concentration as GAP-43 (Table 1 and Oestreicher et al., 1984). Thus the B-50 protein is identical to neonatal cortex GAP-43 in its isoelectric point, apparent molecular weight, aberrant behavior in SDS electrophoresis, and in its localization to membranes of axon terminals.

The antiserum prepared against GAP-43 from neonatal rat brain also recognizes the partially purified B-50 (not shown). This evidence, however, does not rule out the possibility that GAP-43 and B-50 are distinct proteins that comigrate on 2-D gels, because our antigen was purified by preparative electrophoresis. To examine this possibility further, we subjected the anti-GAP-43 antiserum to affinity chromatography with the original antigen preparation. We reasoned that, if B-50 and GAP-43 were different proteins, B-50 would not be expected to be developmentally regulated in the same way as GAP-43. It follows that the "GAP-43" antigen prepared from neonatal brains should contain substantially more GAP-43 than B-50. Immunoaffinity purification with limiting amounts of antigen, then, should yield an antibody fraction proportionately enriched for anti-GAP-43 antibodies. We applied equal amounts of the partially purified B-50 to 2 gel lanes and probed Western blots of these lanes with "preabsorbed" serum (the flow-through from affinity chromatography) and with the affinity-selected antibodies. Figure 9 shows that the preabsorbed antiserum is partially depleted of anti-B-50 antibodies, while the affinity-purified material is enriched for antibodies recognizing the B-50 band. The affinity-purified antiserum also stains the GAP-43 band in samples of neonatal brain (hamster sensorimotor cortex—lane 3; and rat growth cones—lane 4), and the ratio of antibody staining to total protein in this band (determined from Coomassie staining) is similar to that of the B-50 band in the B-50 preparation. Thus, the purified GAP-43 must be selecting relatively similar amounts of anti-B-50 and anti-GAP-43 antibodies. The implication of these observations is that B-50 is present in the neonatal rat brain in amounts comparable to GAP-43. This further similarity strongly suggests that B-50 and GAP-43 share antigenic determinants and are likely to be the same protein.

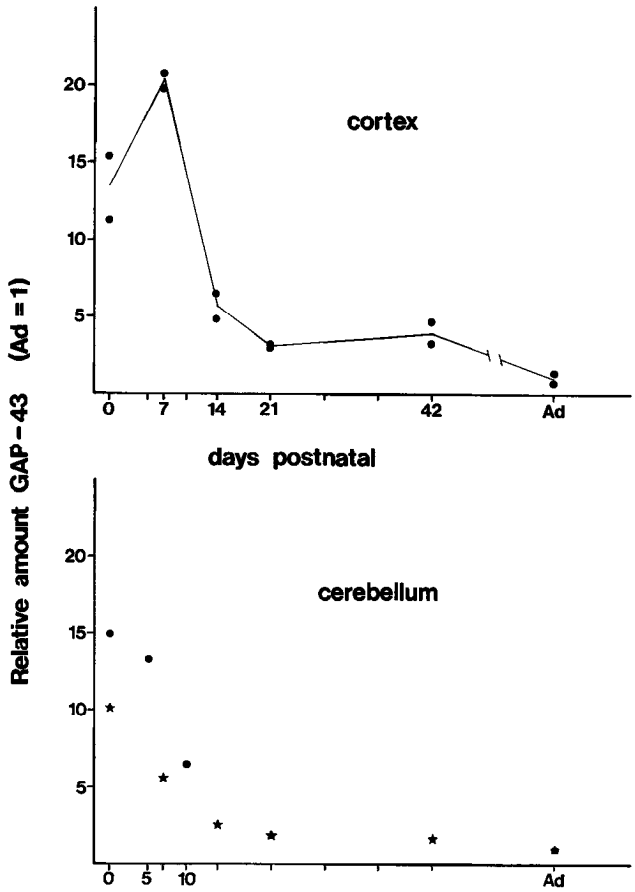

Figure 5. Quantitation of rat GAP-43 synthesis during postnatal development. Fluorograms of 2-D gels were scanned with a densitometer using a 0.1 mm slit whose length encompassed the entire GAP-43 spot. The area under the GAP-43 peak was determined and normalized to the value from the adult (*Ad*) gel. *Top,* Cortex series; data from two series. *Mean* of the 2 adult *levels* set equal to 1. *Bottom,* Cerebellar series; data from 2 series (● and ★). Adult level from *each series* set equal to 1.

Discussion

Identification of GAP-43

A prominent developmentally regulated protein expressed throughout neonatal rat brains appears to be a rat homolog of GAP-43 from regenerating toad optic nerves. The rat protein is nearly identical to toad GAP-43 in its migration on 2-D gel electrophoresis, and an antiserum raised against the rat protein cross-reacts with toad GAP-43. Subcellular fractionation shows that GAP-43 in neonatal rat brain is concentrated primarily in growth cones and immature synaptic terminals (J. H. P. Skene, R. D. Jacobson, G. J. Snipes, C. B. McGuire, J. J. Norden, and J. A. Freeman, unpublished observations), indicating that the rat protein, like toad GAP-43, is an axonally transported neuronal protein.

Toad GAP-43 and the rat protein described here also share a very distinctive aberration in their behavior during SDS gel electrophoresis. The apparent molecular weight of these proteins depends on the acrylamide concentration (increasing apparent molecular weight with decreasing gel concentration). Regardless of the structural basis of this aberration, it is sufficiently unusual among proteins to serve as one criterion for identifying GAP-43. The electrophoretic similarities, axonal transport and antigenic cross-reactivity of GAP-43-like proteins from toad, rat, and hamster, strongly suggest that the major structural features of GAP-43 are highly conserved in evolution.

The unusual behavior of GAP-43 on SDS gels confounds our previous estimate of the protein's true molecular weight (Skene and Willard, 1981a). For a different class of proteins with a somewhat similar aberration in SDS gel electrophoresis (glycoproteins), the apparent molecular weight approaches the true molecular weight asymptotically with increasing gel concentration (Banker and Cotman, 1972; Segrest and Jackson, 1972). We do not think GAP-43's aberrant gel migration is due to glycosylation, because the putative primary translation product

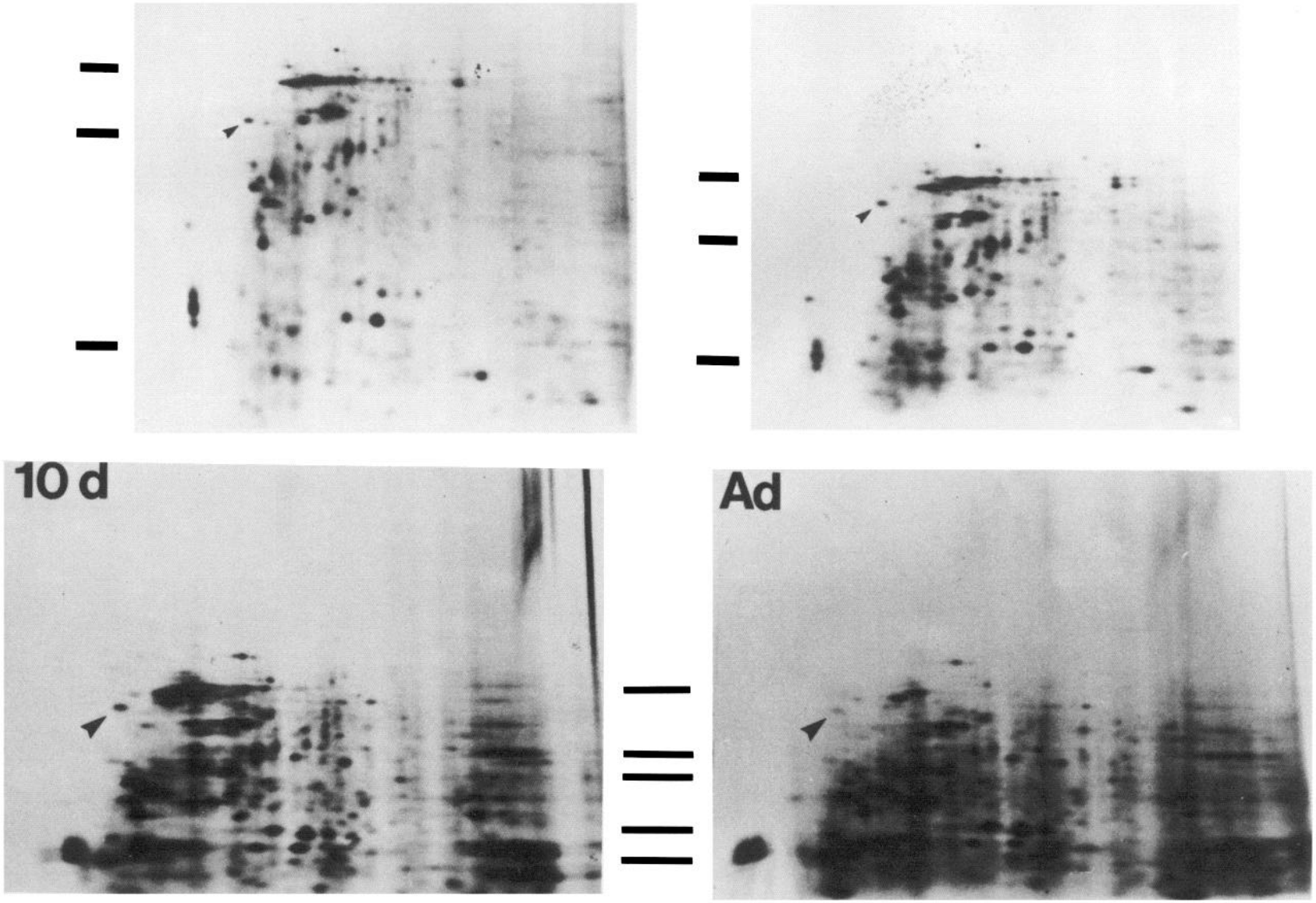

Figure 6. Developmental regulation of GAP-43 mRNA. *Upper panels,* Identification of GAP-43-like protein in products of *in vitro* translation. Two day rat cortex poly A^+ RNA (1.3 μg) was translated with a wheat germ system. One-fourth of the total reaction was TCA-precipitated and run on 2-D gels (pH range, 5–7, expanded). *Arrows,* A protein spot identified as GAP-43 on the basis of its position on 2-D gels and its characteristic shift in apparent molecular weight with different gel concentrations (see text). *Left,* 12% acrylamide gel; *right,* 5–15% gradient gel. *Bars,* Molecular-weight standards: 68, 43, and 18 kDa.
Lower panels, Developmental change in GAP-43 mRNA. Poly A^+ RNA (1 μg) from 10-d-old (*10d, left*) and adult (*Ad, right*) rat cerebral cortex was translated *in vitro* and the products analyzed by 2-D electrophoresis, with a 5–15% polyacrylamide gradient in the second dimension. One-quarter of each reaction was loaded per gel; exposures were 558,000 cpm-d for the 10 d, and 992,000 cpm-d for the adult sample.

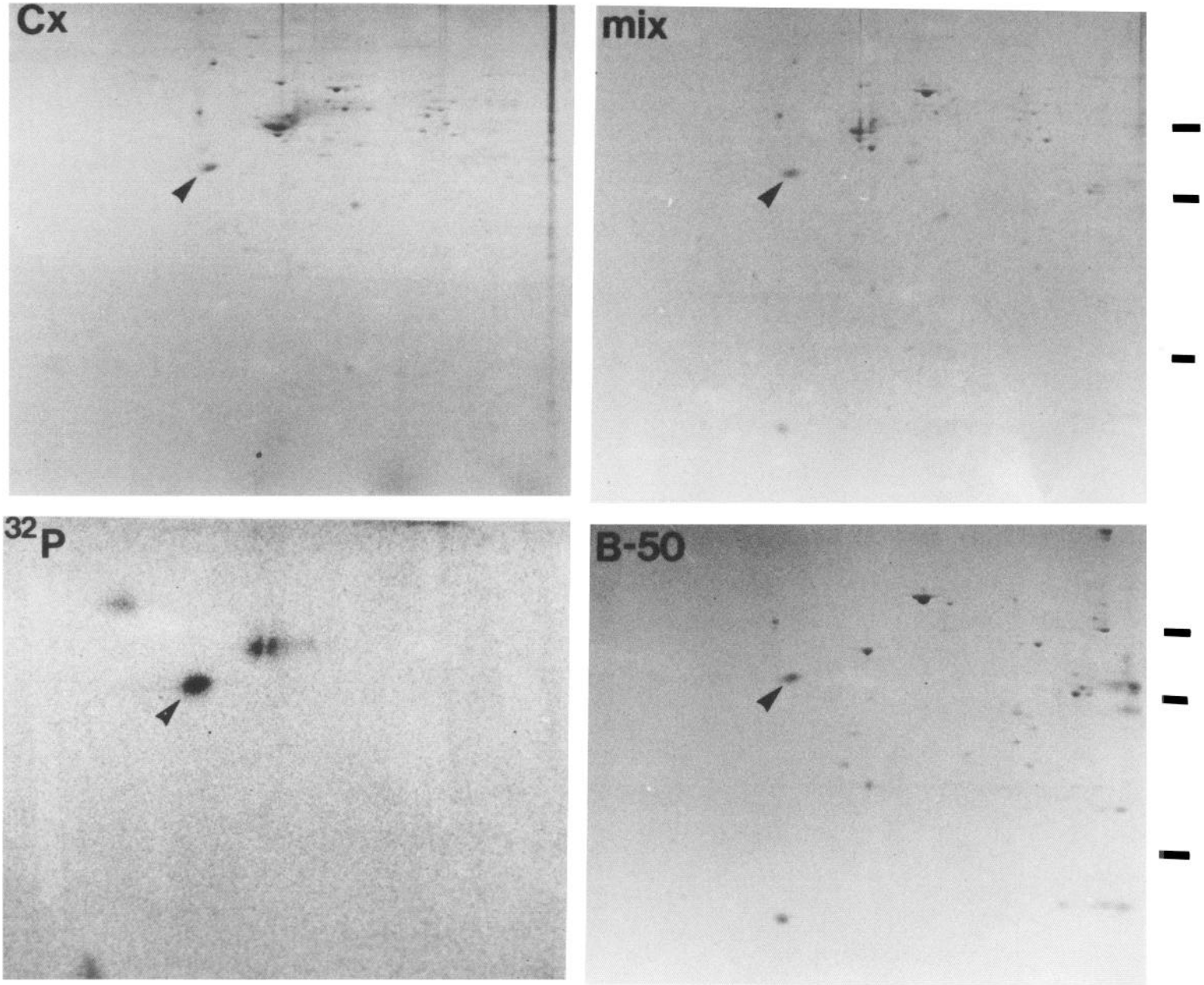

Figure 7. Electrophoretic similarity of GAP-43 and B-50 proteins. Particulate fractions from 7-d rat cortex (*Cx*) and a partially purified preparation of B-50 protein from adult rat brain (*B-50*) were prepared for 2-D electrophoresis (pH 4–6 range expanded in isoelectric focusing; 12% polyacrylamide in the second dimension). Aliquots of the separate samples were loaded onto separate gels; a second aliquot, containing half as much protein as the first aliquot, was removed from each sample and the combined second aliquots loaded onto a single gel (*mix*). After electrophoresis, the gels were stained with Coomassie blue. *Arrows* indicate positions of GAP-43 and B-50. The mixed sample shows a single spot in this position, with a staining intensity equal to that of the separate samples, indicating that the B-50 and GAP-43 proteins precisely comigrated. *Lower left quadrant,* An autoradiograph of a 2-D gel of ^{32}P-labeled proteins, labeled by endogenous kinases in a growth cone membrane fraction prepared from whole 3-d-old rat brain (see Materials and Methods). Labeled protein (*arrow*) comigrates with stained GAP-43. Marks at right indicate positions of molecular-weight markers: BSA, 68,000; ovalbumin, 43,000; and trypsinogen, 25,000.

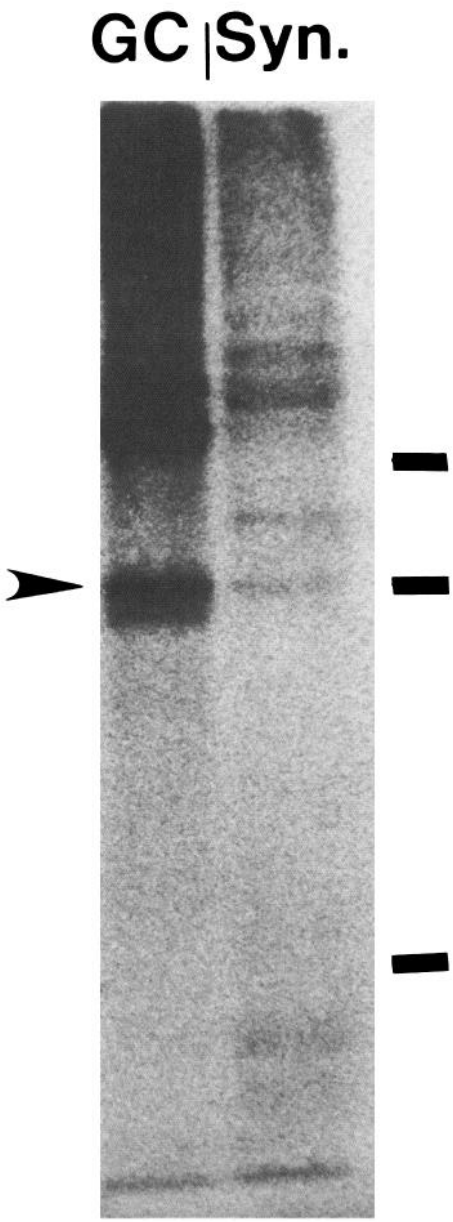

Figure 8. Comparison of GAP-43 phosphorylation in growth cone (*GC*) and mature synaptic (*Syn*) membranes. Growth cone membranes from 3-d-old rats and synaptic membranes from adult rats were prepared as described in Materials and Methods, and incubated with ^{32}P-ATP for 3 min. The labeled proteins were separated by SDS/urea electrophoresis on a gel containing 12% polyacrylamide and 8 M urea, pH 9.3. *Arrow,* The position of GAP-43. *Bars,* The positions of molecular-weight markers: BSA, 68,000; ovalbumin, 43,000; and trypsinogen, 25,000.

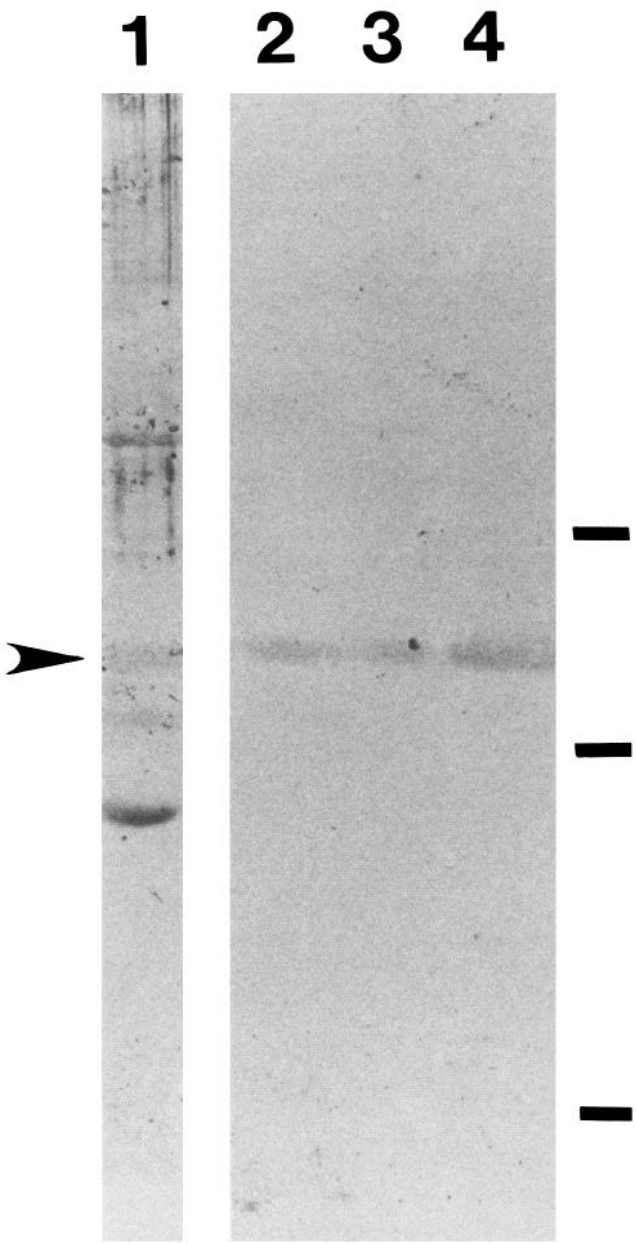

Figure 9. Immunological similarity of GAP-43 and B-50 proteins. Western blots of partially purified B-50 protein (*lanes 1* and *2*), 12-d-old hamster somatomotor cortex (*lane 3*), and growth cone membranes from 3-d-old rats (*lane 4*) were immunostained with the flowthrough (preabsorbed) serum from a "GAP-43" affinity column (*lane 1*) or with the affinity-purified antibodies (*lanes 2–4*). Electrophoresis was on 10% polyacrylamide gels. The preabsorbed serum is partially depleted of, and the affinity-selected material enriched for, antibodies that stain the B-50 band (*arrow*). The relative intensity of immunostaining of the B-50, hamster, and rat growth cone samples is proportional to the total amount of GAP-43 plus B-50 (determined by Coomassie staining of the GAP-43/B-50 spot on 2-D gels), indicating that the antibody is not recognizing simply a small amount of contaminating GAP-43 in the B-50 preparation. *Bars,* The positions of molecular-weight markers: BSA, 68,000; ovalbumin, 43,000; and trypsinogen, 25,000.

shows similar behavior. Nevertheless, the aberrant electrophoretic behavior of GAP-43 might be explained by resistance to SDS denaturation, as in the case of glycoproteins. This would suggest that the true molecular weight of GAP-43 is in the vicinity of 40–45,000, as indicated by its relative mobility on high-percentage acrylamide gels. Our original estimate of the protein's molecular weight (43,000) was made using high-percentage acrylamide SDS gels containing 8 M urea, where the dependence of the protein's apparent molecular weight on acrylamide concentration is less pronounced. To avoid introducing additional names for an already polynominous protein, we will continue for now to use the designation "GAP-43," with the caution that the protein's true molecular weight remains to be established.

GAP-43's aberrant electrophoretic behavior is important for our purposes in that it permits us to confirm the identity of GAP-43 in a variety of circumstances. For example, Szaro et al. (1985) have studied the changes in axonally transported pro-

teins during regeneration of the optic nerve in *Xenopus*, and did not find any protein homologous to GAP-43 in their rapid transport group. However, inspection of their 2-D gel fluorographs reveals an acidic protein with an apparent molecular weight of 56 kDa on a gradient poured from 4 and 17% solutions, which they have labeled No. 23. This protein is rapidly transported and substantially increased during regeneration. We believe that it may be the homolog of GAP-43 in *Xenopus*. This observation points out the importance of analyzing proteins of interest in a variety of gel systems.

The apparent molecular-weight shift has also permitted us to infer the presence of GAP-43 in primary translation products of rat cortex mRNA. We further infer that GAP-43 is not post-translationally modified to an extent that its electrophoretic properties are noticeably altered. This argues against the possibility that decline in GAP-43 in the adult is due to a modification that moves it to another part of the gel.

The *in vitro* translations also indicate that the difference between neonate and adult with respect to GAP-43 synthesis is mirrored in the relative amount of specific GAP-43 message in the poly A^+ RNA pool. Our quantitation of GAP-43 mRNA in neonatal and adult rat cortex, and comparison with the developmental decline in *in vivo* synthesis of the protein, indicates that developmental regulation of GAP-43 synthesis is mediated largely at the level of mRNA abundance. Our quantitative estimates of mRNA abundance, however, are not precise enough to rule out additional regulation at the level of translational efficiency or sequestration in the large poly A^- fraction found in adult brain RNA (Chaudhari and Hahn, 1983). More precise information about the level(s) of GAP-43 regulation can be obtained using specific cDNA probes for GAP-43 mRNA. Evidence for differential abundance of GAP-43 mRNA in neonatal and adult brains, and identification of a putative cell-free translation product, should provide the basis for isolation of an appropriate cDNA clone.

Correlation with axon growth

The rat brain protein that we have identified with toad GAP-43 is prominent among the newly synthesized proteins in several regions of the developing CNS, and Coomassie staining (a measure of steady-state abundance) shows that it is among the most abundant proteins in the brain during this period. This suggests that many different neurons synthesize GAP-43 during development. The marked decline in GAP-43 synthesis and abundance during maturation—apparent in all of the CNS regions we studied—indicates that GAP-43 is developmentally regulated in most neurons. The widespread occurrence of GAP-43 suggests that the protein participates in an activity common to all or most neurons, and its selective enhancement during development is consistent with a role in axon growth.

Participation of GAP-43 in axon growth is also suggested by subcellular localization of the protein. GAP-43 is a remarkably abundant component of growth cone membranes isolated from fetal and neonatal brains (Fig. 9; de Graan et al., 1985; J. H. P. Skene, R. D Jacobson, G. J. Snipes, C. B. McGuire, J. J. Norden, and J. A. Freeman, unpublished observations). Immature synaptosomes contain somewhat less GAP-43, and the small amount of GAP-43 in adult rat brains is concentrated in synaptic membranes. Immunohistochemical localization at the light-microscopic level (McGuire et al., 1985; Skene et al., unpublished observations) shows high levels of GAP-43 immunoreactivity in areas known to contain growth cones and immature synapses. We conclude that, like its homolog in regenerating toad neurons, GAP-43 in developing rat brain is a neuronal protein that is axonally transported and accumulates at the terminals of both growing axons and axons that have formed synaptic connections.

Because we have examined very large CNS regions, containing many different classes of neurons, we cannot precisely correlate the timing of GAP-43 synthesis with axon growth during development. Nevertheless, maximal GAP-43 synthesis does correspond roughly to periods of profuse axon outgrowth and synaptogenesis. In the cerebral cortex, GAP-43 synthesis follows a broad time course, peaking early in the second postnatal week. During the first and second weeks of postnatal life, there is extensive elongation of callosal axons, which pause and then reinitiate growth into deeper cortical layers (along with thalamocortical and association axons; Valentino and Jones, 1982). Elongation of corticospinal axons also continues through the second postnatal week (Schreyer and Jones, 1982). In the cerebellum, GAP-43 synthesis is most prominent during the first postnatal week and then declines to about twice the adult level at 3 weeks. During this early phase of postnatal development, granule cell precursors in the premigratory layer are extending processes that will eventually become the parallel fibers of the molecular layer (Altman, 1972a, b, c). The decrease in GAP-43 synthesis occurs at a time when fiber growth is still occurring, e.g., continued parallel fiber growth, outgrowth of Purkinje cell axons along with those of other intrinsic cerebellar neurons, and ingrowth of afferents. This decline may reflect the proliferation of other elements, such as neuronal and glial cell bodies and dendritic processes, relative to the bulk of growing axons. The time course of GAP-43 synthesis in the cerebellum is similar to that shown by the microtubule-associated protein MAP1; this pattern is the basis for the argument that MAP1 may play a particularly important role in axon growth (Calvert and Anderton, 1985). The appearance of high levels of GAP-43 immunoreactivity in dentate gyrus and superior colliculus also corresponds to times of exuberant axon ingrowth (McGuire et al., 1985). Thus, at the crude level of temporal resolution possible for large brain regions studied as a whole, maximal expression of GAP-43 is correlated with periods of axonal growth.

"Residual" GAP-43 in the mature CNS

The presence of significant amounts of GAP-43 in mature neurons is not predicted by the hypothesis that GAP-43 is a simple ON/OFF regulator of axon growth, and any complete explanation of the protein's role in neuronal life must take into account this "residual" GAP-43. It may be that the residual GAP-43 in mature brains is distributed homogeneously among synapses, each synapse in the brain containing the same small concentration of GAP-43 as any other synapse. In that case, we would expect that a low level of GAP-43 activity (whatever that activity might be) contributes to some aspect of normal synaptic function, while a greatly elevated level of GAP-43 activity might subserve some step(s) in axon growth. An alternative possibility is that the residual GAP-43 in mature brains is distributed heterogeneously among different neurons. In an extreme case, one can envision a minority of neurons synthesizing GAP-43 and accumulating the protein in their axon terminals at levels similar to growth cones, while the majority of neurons contain little or no GAP-43. The population of "high-GAP-43" neurons might comprise a discrete set of neurons that synthesize GAP-43 at high levels throughout life; or the "high-GAP-43" population might represent the set of neurons in which GAP-43 synthesis has been activated transiently at the time of sampling. In either case, we would expect the neurons with high levels of GAP-43 to be more susceptible to axon sprouting or synaptic plasticity.

The "residual" GAP-43 in mature brains is particularly interesting in light of the proposed identity of GAP-43 with B-50. B-50 was first described in the adult rat brain and was selected as a particularly interesting protein because modulation of its phosphorylation by ACTH corresponded closely to the effects of ACTH on learning (Zwiers et al., 1978, 1980). Phosphorylation of an apparently identical protein (F1) has been related to long-term potentiation in the rat hippocampus (Nelson and Routtenberg, 1985; Nelson et al., 1985), a phenomenon often taken as a model of learning and memory. The correlations

between B-50/F1 phosphorylation and effects on memory suggest that the function of GAP-43/B-50/F1 is related to synaptic plasticity. Synaptic plasticity comprises a spectrum of cellular events—from changes in the coupling of depolarization to neurotransmitter release, to synaptic and axonal sprouting, to actual fiber outgrowth—many of which share requirements for changes in cytoskeletal and membrane organization. It is therefore tempting to speculate that GAP-43 mediates some event(s) common to many components of plasticity.

The influence of GAP-43 on axon terminals is potentially regulated at 2 levels. First, changes in the *abundance* of GAP-43 can be brought about by changes in mRNA levels in the cell body. Changes in GAP-43 synthesis in development and regeneration appear to occur with a time course in the range of days to weeks. Second, the *specific activity* of the protein might be regulated in response to local conditions via endogenous GAP-43 kinase(s). Experiments with B-50 and F1 in synaptic membranes have suggested that the residual GAP-43 in synaptic terminals represents a pool of the protein that can be activated by phosphorylation in response to short-term stimuli. Our experiments and the more detailed study by Katz et al. (1985) suggest that GAP-43 may be much more highly phosphorylated in growth cones than in mature synapses. Katz et al. reported a much higher rate of *in vitro* phosphorylation of an acidic, 46 K protein in a particulate fraction growth cones than in a comparable synaptic fraction. A recent report (Nelson et al., 1985) indicates that this pp46 is identical to protein F1, and that the protein can be phosphorylated by both calcium/calmodulin-dependent kinase and kinase C. We have no evidence indicating whether the higher rate of GAP-43 phosphorylation in growth cones represents constitutive activation of a kinase, or whether the kinase(s) can be regulated by interactions of the growth cone with its environment (Berridge and Irvine, 1984; Nishizuka, 1984). Kinase C has been detected in significant amounts in the developing brain, using ^{3}H-phorbol ester binding (Nagle et al., 1981), and its distribution is suggestive of a presence in differentiating, rather than proliferating, neural tissue (Murphy et al., 1983). It is likely that phosphorylation of GAP-43 regulates the protein's activity, either by activating the inactive unphosphorylated protein or by modifying the substrate or binding specificities of the protein. In either event, the level of growth-related GAP-43 activity would be regulated synergistically by regulation of GAP-43 synthesis in the cell body and local control of physphorylation at axon terminals.

Implications for CNS regeneration

Although the small pool of GAP-43 in mature synaptic terminals might be activated under appropriate conditions to permit various elements of plasticity, this pool of GAP-43 is not available in the case of axon injury. Physical interruption of an axon by trauma or disease deprives the proximal axon stump of the synaptic bolus of GAP-43. A net increase in GAP-43 to replace the lost bolus and elevate the protein to the level typical of growth cones (J. H. P. Skene, R. D. Jacobson, G. J. Snipes, C. B. McGuire, J. J. Norden, and J. A. Freeman, unpublished observations) would require either increased synthesis of GAP-43 or a decrease in the protein's degradation. Since turnover of GAP-43 in mature neurons appears to occur on the order of weeks (Fig. 4), changes in protein degradation alone could not cause rapid increases in GAP-43 abundance. If degradation of GAP-43 in injured axons continued at least at the low level typical of uninjured neurons, then no net increase in the protein could occur without increased GAP-43 synthesis. In contrast to successfully regenerating systems, the mammalian CNS neurons examined so far fail to increase GAP-43 synthesis after axotomy (Kalil and Skene, in press; Skene and Willard, 1981b). "Growth cones" formed by these injured axons therefore may be deficient in at least one major membrane protein, which might limit the axons' ability to regenerate.

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