The Journal of Neuroscience, December 1988, 8(12): 4797-4808

Expression of Two Developmentally Regulated Brain-Specific Proteins Is Correlated with Late Outgrowth of the Pyramidal Tract

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The regulation of axon outgrowth is not well understood. In previous studies, however, axon elongation has been well correlated with expression of a small number of growth-associated proteins (GAPs). To identify other proteins whose expression could be correlated with axon outgrowth during development of CNS pathways, monoclonal antibodies were raised against growth cone particles isolated from neonatal hamster brains. Two of these antibodies recognized a brain-specific 33 kDa protein associated with intracellular membranes of axons and growth cones. Immunoblotting demonstrated a sharp developmental decline in levels of the protein in hamster brain during the first postnatal week and a more gradual decline thereafter. Immunocytochemical studies with the antibodies revealed ubiquitous staining of the neuropil during the first several days, which by the end of the first week became restricted to a few later-maturing pathways. Staining was most intense in the pyramidal tract and was well correlated with axon outgrowth, which continues until 14 d in this pathway. These results suggest that the 33 kDa protein may, like previously identified GAPs, play a role in axon elongation.

Late outgrowth of the hamster pyramidal tract is also correlated with expression of another developmentally regulated protein, the high-molecular-weight neurofilament subunit (NF-H). Immunostaining with a monoclonal antibody that recognized phosphorylated NF-H demonstrated that this subunit does not begin to appear in the late-maturing pyramidal tract fibers until several weeks after birth, in striking contrast to intense immunoreactivity of other spinal cord pathways from postnatal day 1. This finding suggests that specific pathways may have a highly idiosyncratic time course for expression of neurofilament subunits.

A major event in the development of the nervous system is the outgrowth of axons toward their targets. The regulation of axonal elongation during the development or regeneration of the nervous system is poorly understood. Thus, in recent years there has been considerable interest in identifying proteins whose developmental expression can be correlated with axon outgrowth. One such growth associated protein, designated GAP-43 (Skene and Willard, 1981a, b), is synthesized by many populations of neurons in the mammalian CNS (Jacobson et al., 1986). Synthesis and transport of this protein in the mammalian central and peripheral nervous system is highly elevated during developmental and regenerative axonal growth (Skene and Willard, 1981b; Kalil and Skene, 1986), and the protein constitutes a major component of growth cone membranes (Meiri et al., 1986; Skene et al., 1986). Similar proteins expressed in a variety of species during neural development, regeneration, or phenomena involving synaptic plasticity have recently been shown to be equivalent to GAP-43 (see Benowitz and Routtenberg, 1987, for a review).

We were interested in studying other developmentally regulated proteins expressed during axon outgrowth in the mammalian CNS. A useful model system for such studies is the hamster pyramidal tract. Its protracted period of postnatal outgrowth from the sensorimotor cortex to the spinal cord (Reh and Kalil, 1981) made possible a precise temporal correlation between axon outgrowth and elevated synthesis of a GAP (Kalil and Skene, 1986). Given the evidence that GAP-43 and identical proteins are highly enriched in growth cones (Jacobson et al., 1986; Meiri et al., 1986; Skene et al., 1986) and in synaptosomal membranes (Benowitz and Lewis, 1983; Katz et al., 1985), we used recently developed subcellular fractionation techniques to isolate growth cone particles from neonatal hamster brains (Pfenninger et al., 1983). These could then be used as an antigen to raise monoclonal antibodies (mAbs). In the present study we describe a 33 kDa protein associated with intracellular membranes of axons and growth cones. Staining of developing hamster CNS with 2 mAbs that recognize this protein reveals that early in development the protein is widely distributed but that after the first postnatal week it becomes restricted to late-developing pathways, particularly the pyramidal tract.

Another important aspect of axon outgrowth during development and regeneration is the formation of the axonal cytoskeleton, major components of which are the 3 subunits of the neurofilament (NF). Recently, development of mAbs specific to both phosphorylated and nonphosphorylated forms of the low- (68 kDa), middle- (150 kDa), and high- (200 kDa) molecular-weight subunits (NF-L, NF-M, and NF-H) of the rat NF triplet made possible a study of the sequential expression of the 3 subunits during the embryonic development of the rat (Carden et al., 1987; Lee et al., 1987). In agreement with earlier studies (Shaw and Weber, 1982, 1983; Willard and Simon, 1983; Pachter and Liem, 1984), it was found that NF-H develops later in

Received Mar. 15, 1988; revised Apr. 22, 1988; accepted Apr. 26, 1988.

We thank Cheryl Adams for excellent technical assistance, Dr. E. Schweitzer for providing PC 12 cells, Dr. R. K. Liem for bovine neurofilament protein, Dr. G. Perdew for ^{125}I-streptavidin and for many helpful suggestions during the course of this study, and Dr. S. Puszkin and E. Floor for comments on the manuscript. This work was supported by NIH Grant NS14428 (K.K.).

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embryonic life than the 2 smaller subunits and NF-H, particularly in axons, appears to undergo a prolonged time course of phosphorylation. However, differential development of NF subunits in pathways with different time courses of development has been little studied. We were interested in whether a late-growing pathway that arises entirely postnatally would show a more prolonged period of neurofilament development, particularly with respect to phosphorylated axonal forms of NF-H, than observed in other pathways of the spinal cord that develop embryonically. The hamster pyramidal tract provided an excellent model system for this comparison. Using an antibody that recognizes the phosphorylated form of hamster NF-H, we found that NF-H appears surprisingly late in the pyramidal tract in contrast to other spinal cord pathways that are intensely immunoreactive by 1 day postnatal. However, since the pyramidal tract is incompletely stained with the mAb to NF-H even at 3 weeks postnatal, the late appearance of NF-H may not be attributable solely to the late outgrowth of these fibers.

Materials and Methods

Antigen preparation. To generate mAbs against proteins specific to developing axonal membranes we immunized mice with a preparation of growth cone particles (GCPs) prepared from 3 d postnatal hamster brain tissue. Discontinuous sucrose density centrifugation was carried out according to the method of Pfenninger et al. (1983). Briefly, the brain tissue from 3-d-old hamsters was homogenized in 8 vol of 0.32 M sucrose with 1 mM TES (pH 7.3) and 1 mM $MgCl_2$. The homogenate was centrifuged, and the resultant low-speed supernatant was loaded onto a discontinuous sucrose density gradient. Following centrifugation, the band containing the GCPs was collected, recentrifuged, resuspended in PBS, and after assaying for amount of total protein, emulsified with Freund's adjuvant for immunization. An additional immunogen was obtained by excising the ventral region of the medulla, including the pyramidal tract, from 3-d-old hamster brains fixed in 4% paraformaldehyde. The tissue was homogenized in PBS (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.5) in a Dounce homogenizer and then frozen at −70°C.

Generation of monoclonal antibodies. Nine- to twelve-week-old BALB/c mice were immunized over a period of 2–3 months with either the growth cone particle preparation or the pyramidal tract homogenate emulsified with Freund's adjuvant and injected intraperitoneally. The final boost was administered either intravenously or intraperitoneally and contained no adjuvant. Three to four days following the final injection, splenocytes were harvested, combined with NS-1 mouse plasmacytoma cells (in a 5:1 ratio), and treated with polyethylene glycol (Kodak 1450) essentially as described by Gefter et al. (1977) for fusion. Following fusion the cells were plated out in 6–7 96-well plates, and hybridomas were selected by addition of HAT to the medium after 4–12 hr. Hybridoma culture supernatants were screened histochemically on frozen cryostat sections of hamster brain tissue (see below) and hybridomas producing desirable antibodies were subcloned by limiting dilution. Monoclonal antibodies were obtained as hybridoma culture supernatant or ascites fluid from hybridoma-injected mice and were subisotyped using the Bio-Rad Mouse-Typer subisotyping kit (Bio-Rad Labs, Richmond, CA).

Immunohistochemistry. For localizing antibody labeling on tissue sections, hamsters were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.5) and the brains postfixed in the same fixative at 4°C overnight. The brain was then equilibrated in 30% sucrose/PBS, frozen in OCT compound, cut at 10–15 μm on a cryostat microtome, and mounted on gelatin-subbed slides. Indirect immunohistochemistry was performed on these sections at room temperature using hybridoma supernatant or ascites diluted in 3% BSA, 2% NGS, 0.5–2.0% Triton X-100 (depending on the age of tissue) in PBS (blocking solution). In some experiments, a commercial mAb to the middle-molecular-weight (NF-M) subunit (Amersham, Arlington Hts., IL) was used at recommended concentrations for purposes of comparison. Tissue sections were incubated for 1–2 hr in these antibody solutions in a humidified chamber and then rinsed in PBS 3×5 min. Controls were incubated with nonproducer hybridoma supernatant. The tissue was then incubated for 30–60 min in FITC-conjugated goat anti-mouse IgG (Hyclone, Logan, UT) diluted 1:40 in the above blocking solution and rinsed 3×5 min in PBS. Tissue sections were coverslipped in 80% glycerol/10 mM Tris-HCl, pH 8.6, and viewed by epifluorescence with a Leitz Orthoplan microscope.

For localizing antigens in NGF-stimulated PC 12 cells (kindly provided by Dr. Erik Schweitzer), the cells were fixed on coverslips in buffered Bouin's fixative, washed in 50 mM NH_4Cl in HEPES buffered saline (HBS) and permeabilized with 0.1% Triton X-100 prior to application of the antibodies diluted in 1% non-fat dry milk/HBS. Cells were incubated in diluted antibody solutions for 1 hr at 37°C and washed 3×5 min in HBS. The cells were then incubated in FITC-goat anti-mouse IgG, diluted in milk/HBS, for 30 min at 37°C, followed by rinsing 3×5 min in HBS. PC 12 cells were mounted on slides in 80% glycerol/Tris-HCl and viewed as above.

Gel electrophoresis and Western blot analysis. To characterize 2 of the mAbs, 6-4E and 3-11G, obtained from a fusion in which GCPs had served as the antigen, crude synaptosomes were prepared from hamster brain tissue, in lieu of GCPs, essentially as described by Cotman (1974) and DeRobertis et al. (1963), up to the discontinuous sucrose density gradient step. In brief, hamsters at postnatal ages 3, 6, 9, 12, 15, 21, and 37 d were anesthetized and sacrificed by either an overdose of Nembutal (Abbott Labs, IL) or hypothermia on ice for infant animals. To look for possible variations in levels of the 33 kDa protein in different areas of the CNS, brains were separated into 3 regions—cerebral cortex, cerebellum, and brain stem—prior to homogenization. Tissues were homogenized in 0.32 M sucrose, 2 mM TES-NaOH, 1 mM $MgCl_2$ (pH 7.4) and centrifuged at 1000 × g for 10 min. The supernatant was collected and centrifuged at 15,000 × g for 15 min. The final pellet was resuspended at a concentration of 1–3 mg/ml and stored at −70°C. Pyramidal tract membranes and cytosol were isolated according to the method of Jacobson et al. (1986) from homogenate of pyramidal tracts excised from the ventral region of the medulla. Protein was assayed by the Pierce Bicinchoninic Acid (BCA) method (Pierce Chem. Co., Rockford, IL).

To localize the antigen with respect to membrane versus cytosol, some crude synaptosomal fractions were subjected to osmotic shock by modifications of the methods of Cotman (1974) and Perdew et al. (1986). Pellets were resuspended in either 5 mM KPO_4 buffer, 1 mM EDTA (pH 8.0) or 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) diluted to 1 mg/ml protein, allowed to sit 15 min on ice, then incubated at 37°C for 1 min and quickly cooled to 4°C. Samples were then centrifuged at 25,000 × g for 40 min to pellet osmotically shocked membranes. These membranes were then resuspended in 150 mM Tris-HCl (pH 8.0) to a protein concentration of 1 mg/ml, to wash off any electrostatically bound proteins, and then centrifuged as before. Both the pellet and supernatant fractions were stored at −70°C. All steps were carried out at 4°C, except where specified. Following osmotic shock and analysis by immunoblotting, some synaptosomal membrane preparations were then further treated with 0.5% Triton X-100 according to the methods of Cotman et al. (1971) and Zanetta et al. (1985) to determine whether the 3-11G protein could be solubilized from the membrane with a mild detergent treatment. Briefly, membrane fractions from previously osmotically shocked crude synaptosomes were resuspended in 10 mM Tris-HCl (pH 6.7) containing 0.5% Triton X-100 to a 1 mg/ml protein concentration. This was allowed to sit for 30 min at 4°C and subsequently centrifuged for 1 hr at 200,000 × g. The resultant Triton X-100-solubilized proteins in the supernatant and those not solubilized in the pellet were both analyzed by immunoblotting, as described below.

Samples described above, including growth cone particles and pyramidal tract fractions, were combined with 2X Laemmli SDS-sample buffer and proteins separated by 10 and 12% SDS-PAGE according to the procedure of Laemmli (1970). Following electrophoresis, the proteins were either stained with Coomassie Brilliant blue R250 or transferred to nitrocellulose (NC) sheets by electroblotting for 5 hr at 60 V, 210 mA in a Bio-Rad Transblot Apparatus, according to the method of Towbin et al. (1979). NC sheets containing the transferred proteins were then either stained with Amido black, for demonstration of total protein, or incubated in PBS containing 3% BSA, overnight at 4°C, for immunolabeling.

After a brief wash in 0.05% Tween-20/PBS (T-PBS), the sheets were incubated 1–2 hr at room temperature (RT) with mAb ascites diluted 1:500 in PBS containing 3% BSA, 2% NGS, and 0.2% Tween-20. After washing in T-PBS 3 × 5–10 min, the NC sheets were incubated for 1 hr at RT with biotin-conjugated goat anti-mouse IgG (Hyclone, Logan, UT) diluted 1:250 in the same buffer used for the primary antibody.

Following the T-PBS wash, the NC sheets were then incubated 30 min with avidin conjugated to HRP (Hyclone, Logan, UT) diluted 1:125 in the same buffer used above. Western blots were developed using 1% diaminobenzidine (Sigma, St. Louis) and 0.02% H_2O_2 in 50 mM Tris-HCl (pH 7.5). Control blots were incubated in nonproducer hybridoma supernatant or dilute ascites, in place of the primary antibody. To quantify antigen levels during development, ^{125}I-streptavidin (200,000 cpm/ml; kindly provided by Dr. Gary Perdew) was used instead of the avidin-HRP above. ^{125}I-labeled blots were exposed to preflashed Kodak X-OMAT X-ray film for 2 d, and the film was developed and used as a template to localize the protein bands on the NC sheet so they could be excised and counted in a gamma counter. A standard response curve was determined for crude synaptosomal fractions applied to a gel within the 50–200 μg protein range.

Digestion of NF-H with alkaline phosphatase. Since the immunocytochemistry suggested that mAb 3-8G recognized axonal neurofilaments (NFs), immunoblotting with bovine NF triplet protein (kindly provided by Dr. Ronald Liem) was carried out as described above to determine which of the subunits 3-8G recognized. To determine whether 3-8G recognized the NF-H subunit in its phosphorylated form the bovine NF triplet was subjected to alkaline phosphatase digestion prior to separation by SDS-PAGE and transfer to NC, as described by Carden et al. (1985). Briefly, bovine NF at 6 mg/ml in 6 M urea, 0.3 M $NaPO_4$ buffer were diluted in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $ZnSO_4$ and dialyzed overnight against the same buffer. Alkaline phosphatase from *E. coli* (Type III-N, Sigma) was then added at 2–20 units/mg NF protein and the reaction allowed to proceed for 18 hr at 37°C. The reaction was terminated by addition of excess phosphate buffer and EDTA and protein isolated by addition of ice-cold acetone and centrifugation. The methods of Carden et al. (1985) were also used to dephosphorylate NFs after separation by SDS-PAGE and transfer to NC. NF protein bound to nitrocellulose was rinsed in 50 mM Tris-HCl, pH 8.0, 1 mM $ZnSO_4$, 150 mM NaCl. *E. coli* alkaline phosphatase was then added to a final concentration of 2 units/ml and the blot incubated overnight at 37°C. Western blots of both types were subsequently incubated in 3-8G mAb ascites diluted in blocking solution and immunolabeled as described earlier.

Immunoprecipitation with Sepharose IgG. Since the 6-4E mAb did not bind to the SDS-denatured form of its antigen on conventional immunoblots, we immunoprecipitated the native form of the antigen bound to 6-4E and analyzed this complex by Western blotting to determine whether 3-11G and 6-4E mAbs recognized the same protein. First, aliquots of Sepharose CL-4B coupled to goat anti-mouse IgG (200 μg IgG/antibody, Cappel, Cooper Biomedical, Inc., Malvern, PA) were incubated separately with 3-11G and 6-4E ascites diluted in PBS for 1 hr at RT with mild agitation. Controls were first incubated in the 3-8G (NF-H) mAb and then treated the same as the 3-11G and 6-4E Sepharose-IgG bound samples to account for possible nonspecific binding of proteins to both the Sepharose and/or to the mAbs themselves. The Sepharose-IgG mAbs were then centrifuged, the excess mAb aspirated off, and then PBS added for 3 washes, 5 min each. The Sepharose-IgG bound mAbs were then incubated for 2 hr at RT in an appropriate solution that we had determined, by immunoblotting, to contain the 3-11G antigen, i.e., for 6-4E the supernatant from osmotically shocked, Tris-washed crude synaptosomes containing only the native form of the antigen or, in the case of 3-11G, supernatant from 0.5% Triton X-100-solubilized, osmotically shocked synaptosomes, in which the protein might be partially denatured. Following this second incubation, the Sepharose-IgG bound mAb–protein complex was washed with T-PBS 3 × 10 min, to remove some nonspecifically bound protein. To the final washed Sepharose–mAb–protein complex was added an equal volume of 2X Laemmli sample buffer. These samples were then separated by 10 or 12% SDS-PAGE and immunoblotted with the 3-11G mAb as described above.

Results

From a large number of mAbs obtained from 5 successful fusions, we chose 3 for further study. Two of these, 3-11G and 6-4E, generated by immunization with GCPs, recognized proteins that appeared to be developmentally regulated. Immunofluorescence revealed ubiquitous staining on brain and spinal cord sections up to 1 week after birth. Thereafter, immunostaining became restricted to late-developing regions of the brain stem and spinal cord, particularly the pyramidal tract and fiber systems within the cerebellum. A third mAb, 3-8G, obtained from a fusion in which the immunogen was the fixed medullary pyramid from 3-d-old hamster brains, appeared to recognize a cytoskeletal component of developing and mature axons in the brain and spinal cord. Upon immunoglobulin subisotyping, the 3-8G mAb was identified as an IgG 1, 6-4E as an IgG2b, and 3-11G as an IgM.

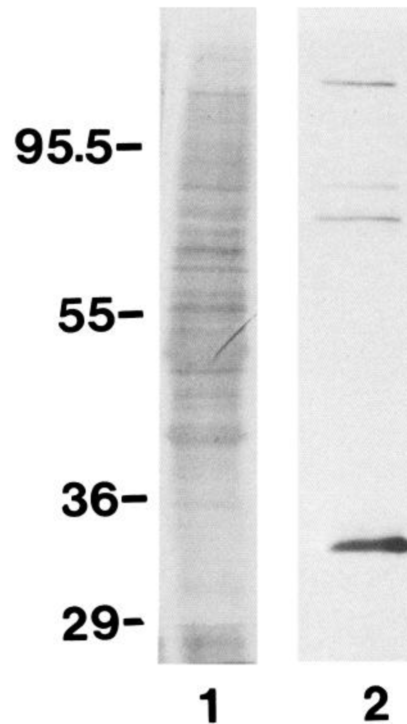


Figure 1. Comparison of total protein with 3-11G mAb immunolabeling in crude synaptosomes from 9 d cerebral cortex. The Amido black stain (*lane 1*) shows low levels of protein within the 33 kDa region. Immunoblotting (*lane 2*) demonstrates intense labeling of the 3-11G protein.

Antigen characterization

The 3-11G antigen was found on immunoblots to recognize a protein with an apparent molecular weight of 33 kDa on 10% SDS-PAGE. This protein is likely to be brain specific since immunoblots with liver, lung, and thymus of young hamsters did not reveal any cross-reactivity (data not shown). Comparison of total protein with 3-11G antibody labeling on NC transfers of growth cone or synaptosomal preparations revealed this protein to be a relatively minor component of these membranes from brain tissue (Fig. 1) and thus highly antigenic. As shown in Figure 2 for pyramidal tract, 3-11G recognizes a membrane-associated protein, as opposed to a component of the cytosol (lane 2), which is enriched in a crude synaptosomal preparation from the brains of young hamsters. Control blots incubated with nonproducer hybridoma ascites or culture supernatant labeled the same 3 higher-molecular-weight proteins seen in addition to the 33 kDa band, and thus this labeling was attributed to nonspecific binding. For further assessment of the membrane association of the 33 kDa protein, the crude synaptosomal preparations were subjected to osmotic shock coupled with a high-molarity, high-pH buffer wash, to remove the water-soluble

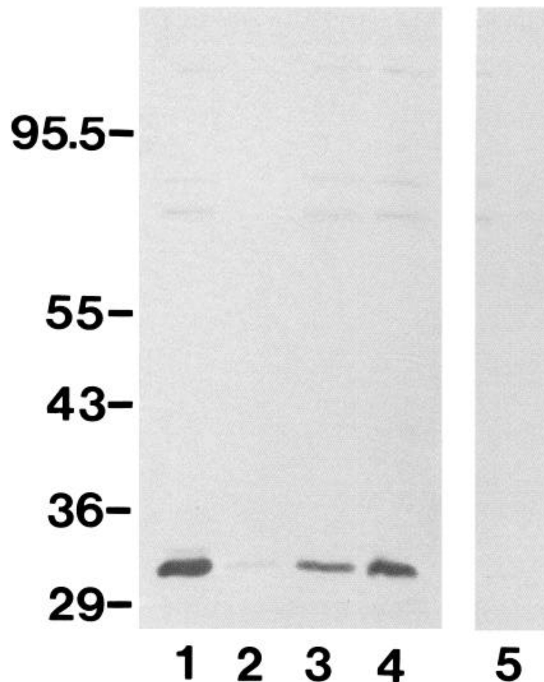


Figure 2. Immunoblot analysis comparing levels of 3-11G reactivity in crude synaptosomes, pyramidal tract membranes, and pyramidal tract cytosol. All lanes contained 100 μg protein separated by 10% SDS-PAGE, transferred to nitrocellulose. *Lanes 1–4* were stained with the 3-11G mAb (1:500 dilution of ascites) and *lane 5* with dilute nonproducer hybridoma ascites, as described in Materials and Methods. Lanes 1 and 4 contain crude synaptosomes from 6 and 12 d brain stem, respectively. Lanes 2 and 3 contain cytosol and membrane fractions, respectively, from a homogenate of 12 d pyramidal tract. Lane 5 contains 12 d brain stem crude synaptosomes incubated in dilute nonproducer hybridoma ascites, showing that the 3 high-molecular-weight bands observed in the immunoblots are due to nonspecific binding of some component of the reaction system other than the 3-11G mAb. Reference proteins (Diversified Biotech, Newton Centre, MA) indicated on the *left* are phosphorylase B (95,500 Da), glutamate dehydrogenase (55,000 Da), ovalbumin (43,000 Da), lactate dehydrogenase (36,000 Da), and carbonic anhydrase (29,000 Da).

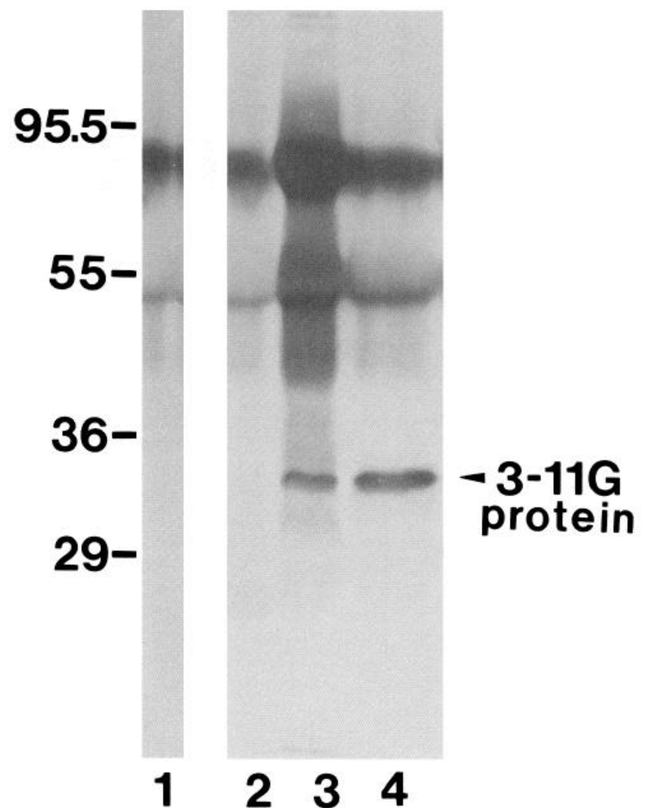


Figure 3. Immunoblot analysis comparing (*lane 3*) 6-4E and 3-11G (*lanes 1* and *4*) immunoprecipitated protein. A control (*lane 2*) contained 3-8G immunoprecipitated protein (NF-H). The immunoprecipitated proteins were separated by 12% SDS-PAGE, incubated with 3-11G mAb ascites diluted 1:500 (*lanes 2–4*) or dilute nonproducer hybridoma ascites (*lane 1*) and then stained as described in Materials and Methods. In the control lanes 1 and 2, the majority of the heavy staining above the 33 kDa protein did not result from 3-11G mAb incubation but was due to binding of the biotinylated goat anti-mouse IgM to both IgM and IgG heavy chains from both Sepharose IgG and the mAbs attached during the course of the immunoprecipitation.

proteins that could be packaged in vesicles and any loosely adhering membrane proteins (Glaumann and Dallner, 1968; Perdew et al., 1986). Immunoblotting with the resultant membrane fractions showed that the majority of the antigen remained in the membrane fraction (data not shown). Synaptosomes were also treated with 0.5% Triton X-100, which was sufficient to solubilize all of the 3-11G protein from the membranes, as determined by immunoblotting. Immunoblots of the membrane and supernatant fractions from this mild detergent-treated preparation showed that, in contrast to many of the proteins that remained with the membrane, the 3-11G polypeptide was totally solubilized and now found only in the supernatant. Results from the osmotic shock and detergent treatments confirm that the 3-11G polypeptide is membrane bound (Cotman et al., 1971). However, more detailed studies are necessary to determine whether it is a peripheral or integral membrane protein.

The 6-4E antigen could not be localized by conventional immunoblotting procedures, apparently because the antibody did not recognize the denatured form of the antigen. Nor were attempts to use nondenaturing (native) gel electrophoresis successful. However, by using immunoprecipitation strategies with Sepharose-goat anti-mouse IgG, we investigated the possibility that 6-4E might be recognizing the same antigen as 3-11G. We first immunoprecipitated both the 6-4E and 3-11G antigens in their native forms with their respective antibodies. Western immunoblot analysis of the immunoprecipitated protein with the 3-11G antibody (Fig. 3) showed that, indeed, both antibodies appear to recognize the same protein, a protein with an apparent molecular weight of 33 kDa.

As suggested by its distribution in tissue sections, 3-8G recognizes an element of the axonal cytoskeleton but does not stain neuronal cell bodies. We therefore carried out immunoblotting with a purified NF preparation and found that this antibody specifically recognizes the 200 kDa, high-molecular-weight (NF-H) subunit of the NF triplet (Fig. 4). The localization of antibody staining in axons suggested that 3-8G recognizes the phosphorylated form of NF-H. We therefore treated the NF preparation with alkaline phosphatase prior to separation by SDS-PAGE. Results from this experiment showed, first, an increased mobility of both the 200 and 150 kDa NF subunits, indicative of dephosphorylation (Carden et al., 1985). Second, 3-8G no longer recognized this dephosphorylated form on immunoblots. This result was confirmed by additional alkaline phosphatase treatment directly on the NC sheet containing bound

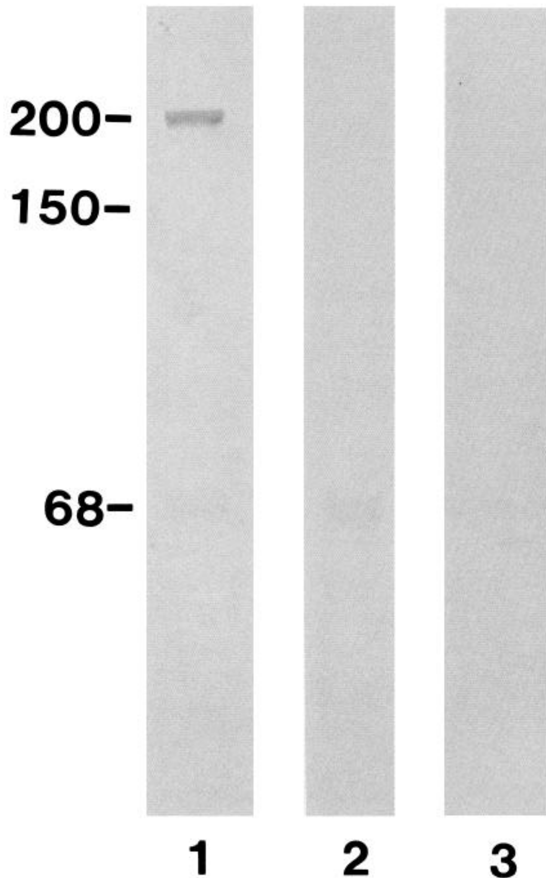


Figure 4. Immunoblots of undigested (normal; *lane 1*) and alkaline phosphatase-digested (*lanes 2* and *3*) bovine NF triplet protein labeled with 3-8G mAb to determine possible specificity for a phosphorylated epitope of the 200 kDa (NF-H) NF subunit. In *lane 2,* NF protein was treated with alkaline phosphatase prior to separation by 7.5% SDS-PAGE and transfer to nitrocellulose. In *lane 3,* the NF protein was phosphatase-digested directly on the nitrocellulose sheet, following SDS-PAGE and transfer to nitrocellulose but before incubation with the 3-8G mAb.

NFs, since subsequent immunoblotting with 3-8G again failed to show labeling (Fig. 4, lane 3).

Immunocytochemical localization of 3-11G and 6-4E antigens

In general, the 3-11G and 6-4E antibodies have almost identical labeling patterns in tissue sections. Both recognize a protein widely distributed within the neuropil of most regions of the brain. In tissue sections it was difficult to localize the antigens specifically within neuronal cytoplasm. The heaviest staining was localized in a fine filamentous network that either surrounds neuronal cell bodies or is closely associated with axons. For example, in axon pathways, staining is particularly intense and usually follows the pattern of fascicles within the white matter. This staining pattern is particularly striking in the pyramidal tract. To further demonstrate the association of this antigen with axons, we eliminated pyramidal tract axons with a unilateral lesion of the medullary pyramid in neonatal animals. Figure 5 shows 6-4E antibody labeling of the normal pyramidal tract in the right-hand dorsal column in a staining pattern reflecting axon bundles within this pathway. In contrast, in the absence of pyramidal tract fibers on the left side, antibody labeling com-

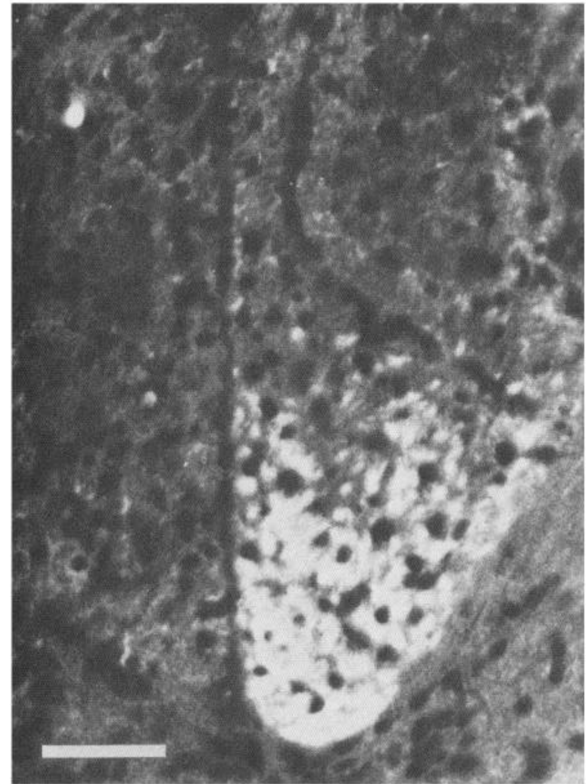


Figure 5. Photomicrograph of 12 d hamster cervical spinal cord stained with 6-4E. Immunofluorescence is confined to the pyramidal tract in the dorsal column on the right. A neonatal lesion of the medullary pyramid has eliminated all staining in the left dorsal column in which the pyramidal tract is absent. Scale bar, 50 μm.

pletely disappears. Similar results were obtained with 3-11G. In addition, pyramidal tract axons were anterogradely labeled with injections of rhodamine (RITC) and sections through the forebrain and medulla counterstained with 3-11G. The results showed FITC immunofluorescence localized precisely to RITC-labeled corticofugal axons within the caudate nucleus, internal capsule, corpus callosum, and the pyramidal tract (Fig. 6).

The staining patterns of the 3-11G and 6-4E mAbs showed striking changes during development. Whereas in 1-d-old tissue, immunofluorescence is both ubiquitous and intense within the gray and white matter of most regions of the brain, by day 3 staining becomes more restricted. The gray matter shows a decline in staining intensity at this age, although fiber pathways in the white matter are still well labeled. By 6 d (Fig. 7), staining in the brain stem, cerebellum, and spinal cord is restricted to a small number or fiber tracts. Thereafter, staining in the cerebellum and spinal cord is localized to a few specific neural systems, which have in common their late development. Most striking is the intense labeling of the pyramidal tract in the medulla and spinal cord, in which staining of the pyramidal tract continues until about 3 weeks of age with either of the antibodies. For example, at 12 d postnatal, intense staining of the pyramidal tract with 3-11G within the dorsal columns of the cervical cord is observed against a completely unstained background. At 14 d, 6-4E labeled pyramidal tract fascicles are present within the dorsal columns, while all other spinal pathways are completely unstained (Fig. 8). This restricted staining pattern continues until the third week but has diminished in intensity by 23 d.

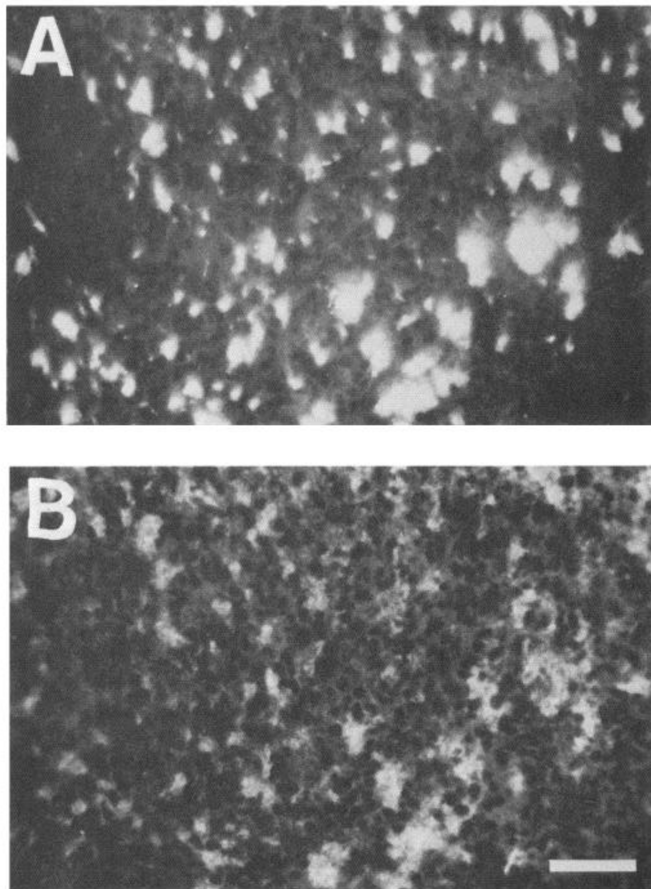


Figure 6. Comparison of axon labeling in the caudate nucleus of 2 d hamster brain after cortical injection of RITC (*A*) and immunofluorescent labeling with the 3-11G mAb (*B*). Scale bar, 50 μm.

Staining within the cerebellum with the 6-4E and 3-11G antibodies also shows dramatic alterations during development. For example, staining within the cerebellar cortex at 9 d postnatal is intense in the fiber stratum ventral to the Purkinje cell layer (Fig. 9). However, by 17 d this fiber layer is no longer stained. Instead, Purkinje cell apical dendrites are brightly fluorescent within the molecular layer and remain so until 23 d of age.

Developmental decline of the 3-11G protein

As described above, the immunocytochemistry showed that both 3-11G and 6-4E recognize a developmentally regulated protein, since immunostaining on tissue sections with 3-11G and 6-4E decreased during development of most regions of the brain and spinal cord. Since the mAbs were generated by immunization with GCPs, we decided to quantify this developmental decline in crude synaptosomal preparations from brain stem, cerebellum, and cerebral cortex at various ages, using ^{125}I-streptavidin immunoblotting and autoradiography. As illustrated in the autoradiogram in Figure 10*A*, crude synaptosomes from cerebellum show a large decrease in amounts of the 3-11G protein between 3 and 21 d of age. However, as shown in Figure 10*B*, this developmental decline is not uniform in all regions of the brain. For example, in the cerebral cortex, levels of 3-11G drop most precipitously between 9 and 12 d. Further, in the brain stem, relatively high levels of the protein (45% of the level at 3 d of age) are maintained until as late as 37 d postnatal.

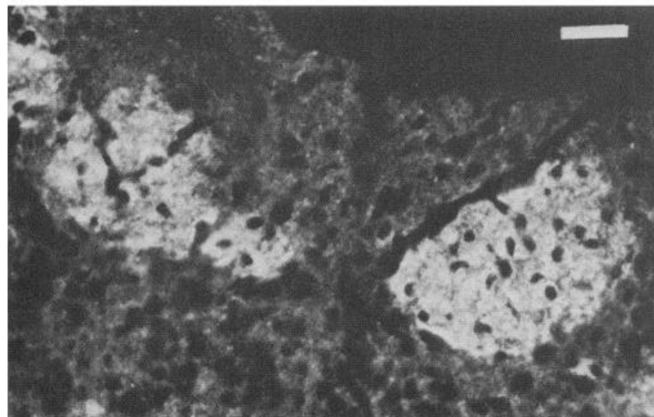


Figure 7. Photomicrograph of immunofluorescence in pyramidal tracts of 6 d hamster cervical spinal cord stained with the 3-11G mAb. Scale bar, 50 μm.

Localization of 6-4E and 3-11G antigens in PC 12 cells

To determine whether the 6-4E and 3-11G antigens are localized in the neuronal plasma membrane or within the cell cytoplasm, we stained PC 12 cells with these mAbs. When PC 12 cells were stimulated with NGF, the cytoplasm of the outgrowing neurites, as well as that of the cell bodies, was uniformly stained and showed an overall granular fluorescence (Fig. 11). This staining pattern extended to the tips of the growing neurites and was intensified during neurite outgrowth.

Developmental expression of the 200 kDa (NF-H) subunit

The development of NF-H in its phosphorylated form was observed in 3-8G-stained tissue sections from the brain and spinal cord of hamsters from 1 to 23 d of age. With respect to the pyramidal tract, the expression of NF-H occurs surprisingly late in development compared with many other fiber populations. At day 1 (Fig. 12), when the pyramidal tract has not yet arrived in the spinal cord (Reh and Kalil, 1981), the entire white matter of the cord is intensely fluorescent. By 3 d of age, when pyramidal tract axons first invade the dorsal columns of the cervical spinal cord, all the major ascending and descending pathways of the cord are brightly labeled with the exception of the pyramidal

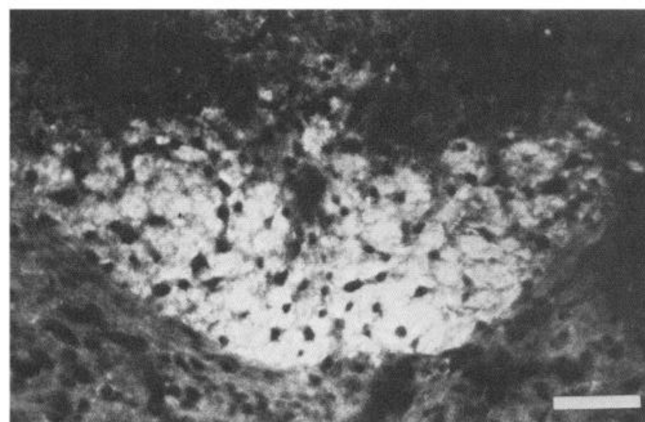


Figure 8. Photomicrograph of immunofluorescence in pyramidal tracts of 14 d hamster cervical spinal cord stained with the 6-4E mAb. Scale bar, 50 μm.

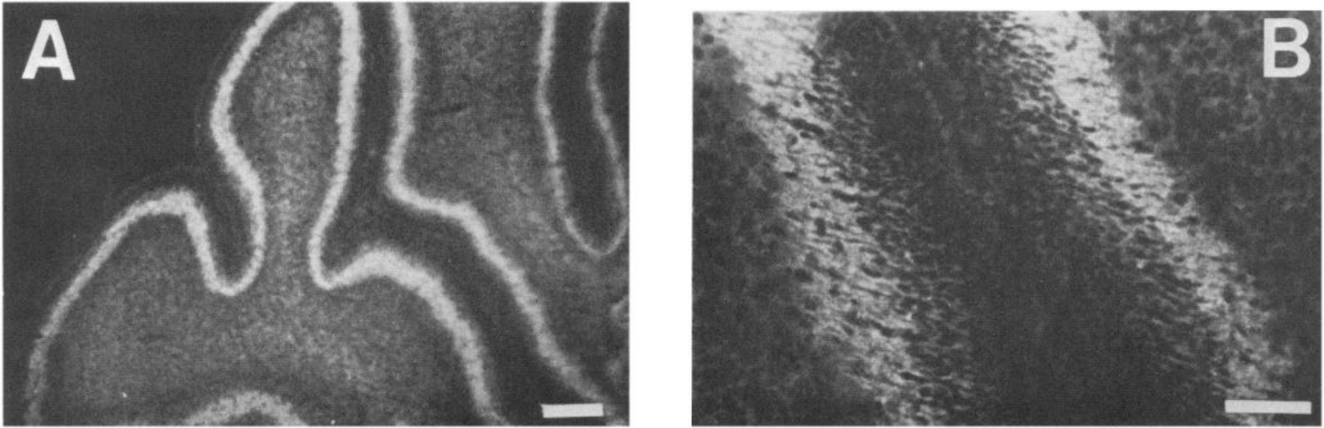


Figure 9. Immunofluorescence in 9 d cerebellum stained with the 6-4E mAb. Low-power view (*A*) shows intense labeling in the neuropil ventral to the Purkinje layer, shown at higher power in *B*. Scale bar, 100 μm in *A*, 50 μm in *B*.

tract, which appears as an unstained area within the ventral region of the dorsal column. Local fibers within the cord, as well as afferent dorsal root axons, are also well stained. At 6 d of age, within the cervical cord, the contrast between the unstained pyramidal tract and the brilliantly fluorescent ascending axons lying dorsal to the tract is even more striking (Fig. 13). The punctate staining within the pyramidal tract at 3 and 6 d results not from labeling of the tract itself but from labeling of afferent fibers to the spinal cord that ascend through this region to the dorsal column. At 10 d of age the pyramidal tract remains virtually unlabeled. As shown in Figure 14, the medullary pyramid contrasts sharply with the bright staining of the medial lemniscal fibers above it. By 14 d, whereas other major pathways of the forebrain, such as the corpus callosum, optic nerve, and afferent fibers within the cerebral cortex, are well labeled, the pyramidal tract continues to lag well behind these, as well as fibers of the cerebellum, brain stem, and spinal cord, in the developmental expression of the 200 kDa NF subunit. Interestingly, within the caudate nucleus, corticofugal axons are brightly stained, in contrast to the corticospinal axons, which remain almost completely unstained in sections through the medulla and spinal cord. Thus, the late appearance of NF-H, or at least this phosphorylated epitope, is not a general feature of cortical efferent axons but appears to be specific to the pyramidal tract. Even at later ages, up to 23 d, the pyramidal tract failed to achieve the intense staining characteristic of other fiber systems. For example, at 19 d of age the tract is characterized by intermittent punctate staining rather than the uniform bright labeling of the lemniscal pathway dorsal to it. This is illustrated in Figure 15, which shows a section through the dorsal columns of the cervical spinal cord of a 19-d-old hamster in which an early lesion of the pyramidal tract eliminated these fibers to the left of the midline, emphasizing the obvious lack of staining in the intact pyramidal tract on the right side.

To determine whether late development of NF-H is specific to the high-molecular-weight subunit, we also stained sections throughout development with a monospecific antibody for the middle-molecular-weight (150 kDa) NF-M polypeptide. Within the 6-d-old spinal cord, staining with the antibody to the NF-M subunit resulted in bright labeling of the white matter, with the exception of the pyramidal tract. However, several days later, labeling of the pyramidal tract begins to appear and increases steadily, such that, by 23 d, staining of the pyramidal tract is indistinguishable in intensity from neighboring fiber tracts of the spinal cord.

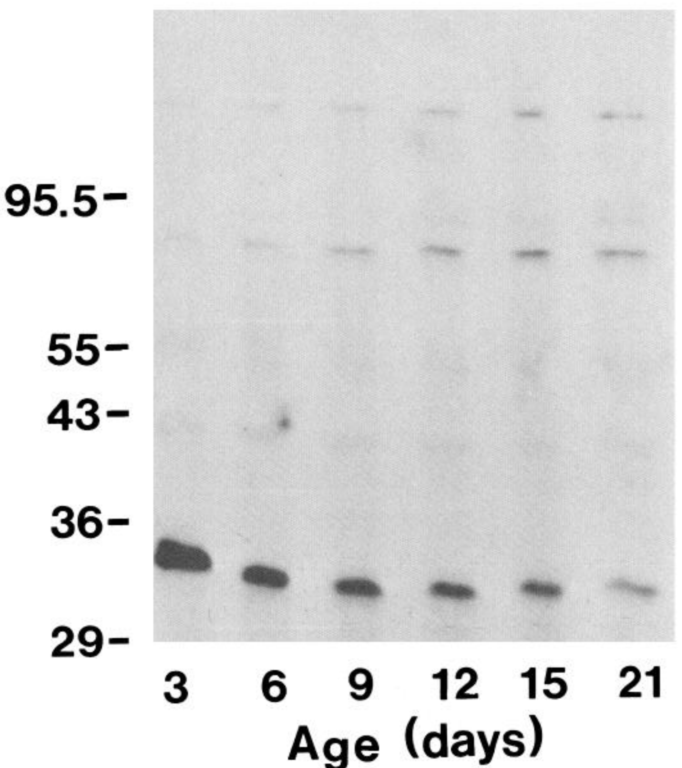
Discussion

We have described 2 brain specific proteins in the hamster CNS whose developmental expression correlates with the late outgrowth of the pyramidal tract. One is a novel 33 kDa membrane protein that is ubiquitous in the neuropil of infant hamster brains but declines precipitously during the first 2 postnatal weeks in all CNS regions except the late-maturing pyramidal tract and some fibers of the cerebellum. The other is the 200 kDa NF subunit (NF-H), which is present at birth in many fiber tracts of the brain and spinal cord but is conspicuously absent in the pyramidal tract; there, NF-H does not appear until several weeks after birth.

Developmental expression of the 33 kDa protein

As suggested by immunoprecipitation and subsequent immunoblotting, the 33 kDa protein is recognized by both mAbs 6-4E and 3-11G, raised against growth cone particles from young hamster brains. Because the 6-4E mAb recognizes only the native form of the antigen, whereas 3-11G recognizes both native and denatured forms, it is likely that if these mAbs recognize the same protein they are specific for different epitopes.

Several results suggest that the 33 kDa protein is membrane bound. First, osmotic shock treatments of crude synaptosomal preparations failed to release the protein into the supernatant. Second, detergent treatments were necessary to solubilize the 33 kDa polypeptide. Staining of PC 12 cells revealed a granular cytoplasmic fluorescence, suggesting that the 33 kDa protein is most likely associated with intracellular membranes as opposed to the plasma membrane. Moreover, staining patterns with both mAbs suggest that the protein is localized primarily in axons since the ubiquitous staining early in development was observed in neuropil as opposed to cell bodies, and early axotomy of pyramidal tract neurons with a lesion of the medullary pyramid eliminated staining of this pathway in the spinal cord distal to the lesion. Nevertheless, in the absence of electron microscopic evidence, it is not possible to attribute immunoreactivity to staining of axonal membranes alone. The early ubiquitous meshlike staining of the neuropil surrounding neuronal cell bod-



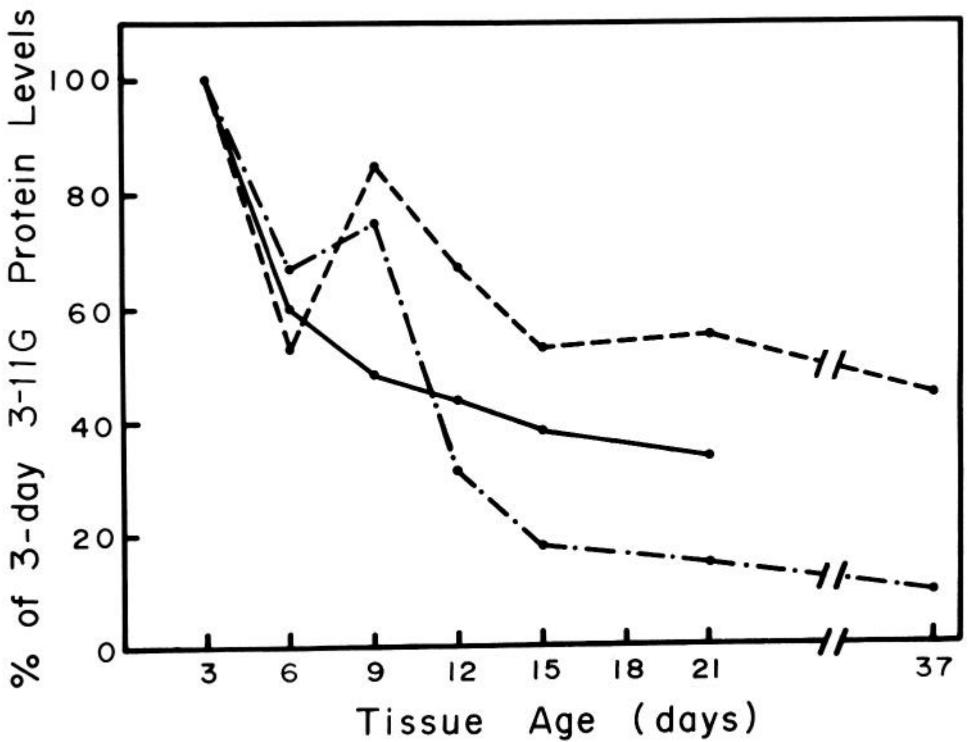


Figure 10. Developmental decline of the 33 kDa protein in hamster brain. The autoradiogram (*A*) of an immunoblot of cerebellar crude synaptosomes (75 μg/lane) labeled with the 3-11G mAb and ^{125}I-streptavidin shows the steepest decline between 3 and 6 d. In *B*, plots of gamma counts of 33 kDa bands excised from nitrocellulose sheets expressed as a percentage of total radioactivity in the 33 kDa band at 3 d show nonuniform declines in crude synaptosomes from cortex (-·-·-), brain stem (– – –), and cerebellum (——).

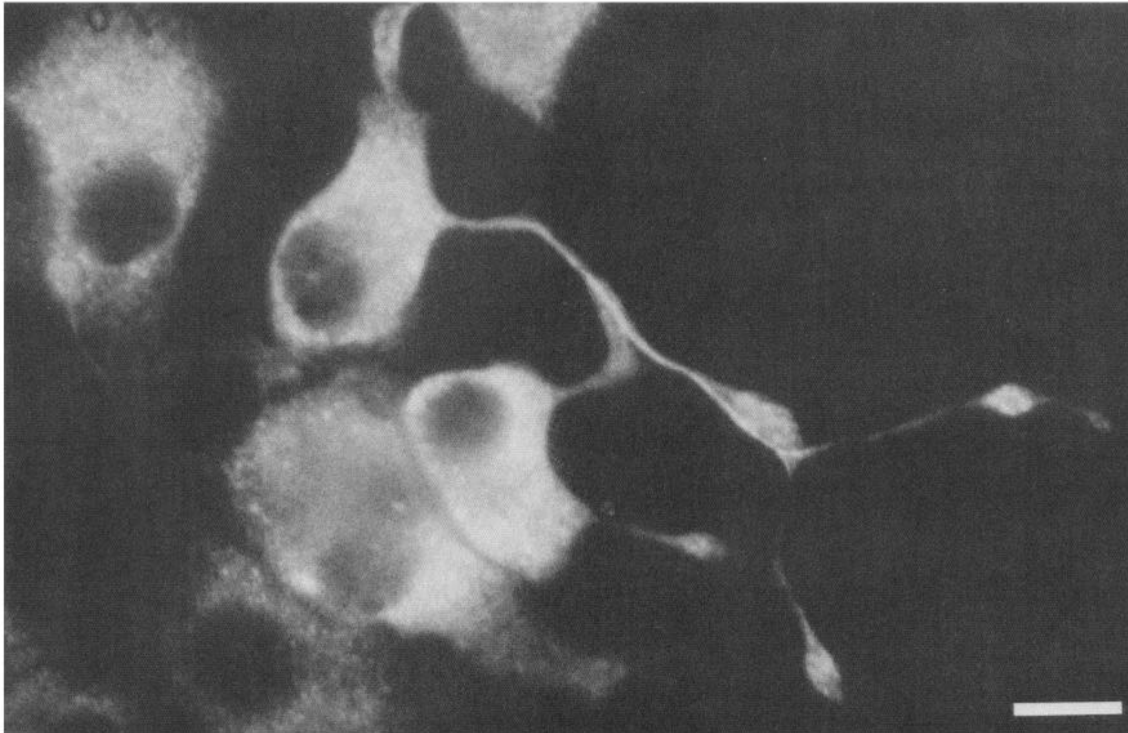


Figure 11. NGF-stimulated PC 12 cells showing immunofluorescence with 6-4E mAb staining.

ies suggests that, at early stages of development, the 33 kDa protein may also be expressed by extracellular matrix or by glial processes.

Gel analysis suggests that the 33 kDa protein is not a major constituent of growth cone membranes or synaptosomes. Nevertheless, immunoreactivity on tissue sections shows the ubiquitous distribution of the protein during the first postnatal week. After this time, both the immunoblots and the immunocytochemistry reveal a developmental decline in levels of the protein. Immunostaining continues in a few neural systems that have in common their late development. The best correlation between a prolonged time course of development and the continued expression of the 33 kDa protein was observed in the pyramidal tract, which in hamsters continues to elongate until 2 weeks postnatal (Reh and Kalil, 1981). Intense staining with mAbs to the 33 kDa protein is also apparent in the cerebellum during the second postnatal week. However, in the cerebellum it is difficult to localize the immunostaining to a single fiber system or to correlate this with axonal outgrowth, since the heavily stained region of the neuropil ventral to the Purkinje cell layer contains axons of basket and Golgi cells as well as the terminals of afferent axons to the granule cells. Further evidence for the specific association of the 33 kDa protein with process outgrowth comes from the observation that PC 12 cells are much more intensely stained after NGF-stimulated neurite outgrowth.

Several features of the developmental distribution of the 33

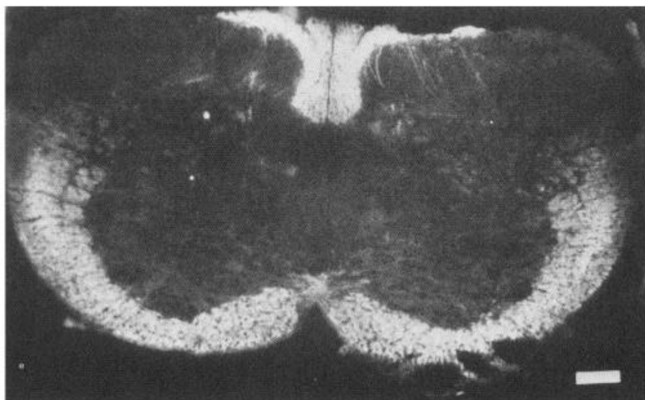


Figure 12. Photomicrograph of 2 d spinal cord immunostained with the 3-8G mAb to NF-H. Scale bar, 100 μm.

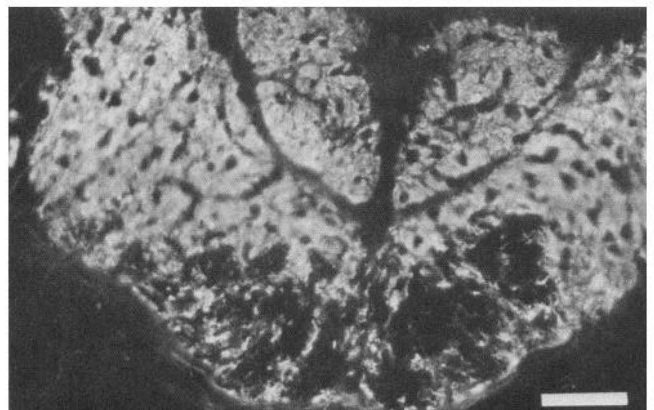


Figure 13. Photomicrograph of the dorsal columns in 6 d cervical spnial cord showing lack of NF-H immunoreactivity in the pyramidal tracts after staining with 3-8G. Scale bar, 50 μm.

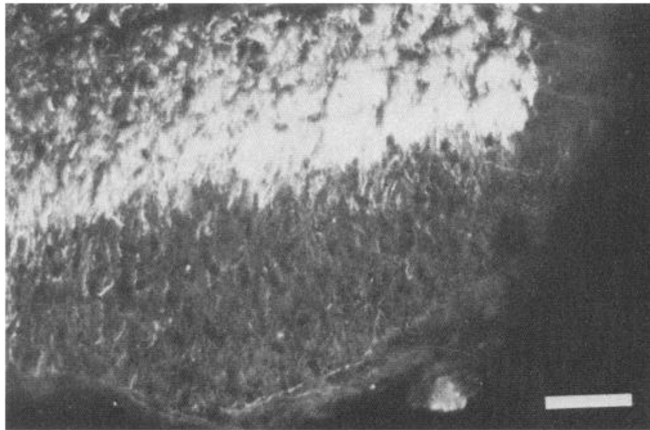


Figure 14. Photomicrograph of 10 d medullary pyramid contrasts intense fluorescence medial lemniscus (dorsal) with unlabeled pyramidal tract (ventral) after staining with the 3-8G mAb to NF-H. Scale bar, 50 μm.

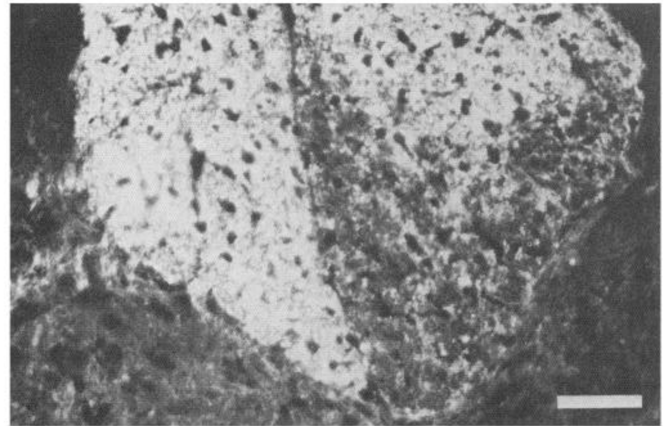


Figure 15. Photomicrograph of dorsal columns in 19 d cervical spinal cord stained with 3-8G. Immunoreactivity is still sparse in the pyramidal tract at right. On the *left,* NF-H immunofluorescence extends through the entire dorsal column because a lesion at 5 d eliminated the pyramidal tract on this side.

kDa protein resemble those described for GAPs, a group of developmentally regulated proteins thought to be important during axon outgrowth and synaptogenesis (Skene and Willard, 1981a, b; Benowitz and Lewis, 1983). Like GAP-43, the 33 kDa polypeptide is present in CNS pathways during periods of axon outgrowth, but it has not been determined whether the 33 kDa protein is rapidly transported as is GAP-43. Another feature shared by GAP-43 and the 33 kDa protein is the time course of its developmental decline. Levels of both proteins are highest in the brains of rodents during the first postnatal week. The steepest decline in synthesis of GAP-43 in the rat cerebral cortex and cerebellum occurs during the second postnatal week, after which there is a gradual decline to adult levels (Jacobson et al., 1986). A more precise temporal correlation between expression of GAP-43 and axon outgrowth was obtained by a study of GAP-43 synthesis in the hamster pyramidal tract (Kalil and Skene, 1986). The developmental decline of this protein begins at 8 d postnatal and continues until 3 weeks when adult levels are reached. This parallels the outgrowth of the tract since most of the axons in the medullary pyramid are still elongating within the spinal cord at 8 d but by 14 d have reached their caudalmost extension and are innervating spinal targets (Reh and Kalil, 1981). The 33 kDa protein also begins to decline early in postnatal life, and the time course for this decline does not appear to be uniform for all regions of the brain. For example, in synaptosomes from the cerebellum, cerebral cortex, and brain stem there is an approximate 40% decline in levels of the protein between 3 and 6 d of age. In the cerebral cortex a further decline to about 20% of 3 d levels occurs during the second postnatal week. In contrast, the levels of the protein in brain-stem synaptosomes do not show further decline below the level at 6 d, and in cerebellum the developmental decline is very gradual after 6 d. As with GAP-43, relatively high levels of the 33 kDa protein can be correlated in time with axon outgrowth of specific pathways. The protracted period of axon elongation in the pyramidal tract, for example, correlates well with intense immunostaining with the 6-4E and 3-11G mAbs that persists until the third week after birth. GAP-43 is abundant in growth cone membranes (DeGraan et al., 1985; Jacobson et al., 1986; Meiri et al.,1986; Skene et al., 1986) and also in nerve terminal membranes of the adult nervous system (Benowitz and Lewis, 1983). The presence of GAP-43 in selected regions of the adult brain suggests that, in addition to its roles in axon outgrowth and synaptogenesis, the protein may also have a function in synaptic plasticity (Akers et al., 1986; Benowitz and Routtenberg, 1987; Benowitz et al., 1988). In contrast to GAP-43, the 33 kDa protein appears to be of relatively low abundance in growth cone membranes. Its continued presence in adult synaptosomes, however, suggests that it, too, may play a role in the activity of the mature nervous system.

Although the 33 kDa protein shares several features with GAPs, it will be important in future studies to determine whether the protein is rapidly transported, whether it accumulates in growth cones of cultured neurons, and where it is localized at the ultrastructural level in growth cones and synapses. More precise correlations with neurite outgrowth will also be necessary to establish a growth-associated function for the 33 kDa protein.

Late expression of NF-H in the pyramidal tract

A number of recent studies have shown that the 3 NF protein subunits appear at different times in development, such that early in development NF-H is absent or expressed at very low levels in comparison with NF-L and NF-M (Shaw and Weber, 1982, 1983; Willard and Simon, 1983; Hirokawa et al., 1984; Pachter and Liem, 1984). In the rat optic nerve, for example, NF-H is not present until almost 3 weeks postnatal (Pachter and Liem, 1984). Most recently, the development of a large panel of mAbs to phosphorylated and nonphosphorylated forms of all 3 subunits (Lee et al., 1987) has made possible a careful study of their time of expression in the embryonic rat nervous system (Carden et al., 1987). In the latter study, the delayed NF-H expression noted in previous work was confirmed, and the conclusion was drawn that during development of the cytoskeleton in mammalian neurons there is an early immature form of NFs characterized by a lack of or low levels of NF-H. Moreover, it was found that accumulation of phosphorylated NF-H in maturing axons takes place over a prolonged time course (Carden et al., 1987). In developing rat embryos prior to E15 NF-H could not be detected with mAbs to phosphorylated or nonphosphorylated types. Gradually, staining increased over time during embryonic development, but NF-H immunoreactivity did not reach adult levels in some regions of the

nervous system until several weeks postnatal. Phosphorylation of NF-H, while incomplete in the embryonic rat nervous system, appears in its adult pattern early in development, i.e., in a gradient of relatively low phosphorylation in the cell body to increasing levels in the axon.

The present results confirm previous observations on the delayed appearance of NF-H during development but also emphasize that specific pathways may have a highly idiosyncratic time course for expression of NF subunits. In the present study, staining of newborn hamster spinal cord with a mAb (3-8G) specific for phosphorylated NF-H revealed a pattern of intense immunoreactivity in the white matter of the cord similar to that shown by Carden et al. (1987) for late embryonic rat spinal cord. The hamster pyramidal tract, however, which just begins to enter the spinal cord at 3 d postnatal, remains completely unstained with 3-8G until several weeks postnatal. The prolonged delay in the appearance of NF-H in the pyramidal tract, in contrast to other spinal pathways, is correlated to some extent with the late outgrowth of pyramidal tract axons. However, the time course of development of this pathway may not completely account for the late maturation of NFs. Although it takes almost 2 weeks for the tract to complete its longitudinal extension into the cord, many of the axons terminating in rostral segments are establishing synapses during the first postnatal week (Reh and Kalil, 1981). Thus, it would be difficult to argue that the appearance of NF-H is correlated with synaptogenesis as suggested by Carden et al. (1987), especially since ascending fibers in the dorsal column and descending axons arising from the brain stem are stili establishing synapses in the newborn hamster but are already intensely immunoreactive with the 3-8G antibody. Another possible correlate of the slow accumulation of phosphorylated NF-H in maturing axons might be radial growth in axons, which in the pyramidal tract continues until 3 weeks postnatal (Reh and Kalil, 1982). Again, the disparity between NF-H expression in the hamster pyramidal tract and other spinal pathways would be difficult to explain simply on the basis of radial axonal growth, which would undoubtedly continue in the postnatal development of most spinal pathways.

The present study does not rule out the possibility that NF-H is present in newly growing pyramidal tract axons but in a nonphosphorylated form undetectable with our antibody. Since we did not employ a panel of antibodies to differentially phosphorylated forms of NF-H, we cannot rule out this possibility. However, previous results generally show that in adult as well as developing neurons NF-H is present in nonphosphorylated form in the perikaryon, becomes increasingly phosphorylated in more distal regions of the axon (Sternberger and Sternberger, 1983; Carden et al., 1985, 1987; Lee et al., 1986; Oblinger, 1987), and can continue to be phosphorylated during axoplasmic transport (Nixon et al., 1987). It therefore seems unlikely that NF-H is present in pyramidal tract axons early in development but persists for a long time in an unrecognizable nonphosphorylated state. Rather, NF-H appears to be exceedingly slow to develop in the pyramidal tract and may never be a major cytoskeletal element in these axons. This is consistent with the conclusions of Shaw and Weber (1982), who postulated that certain NFs in adult brain may never express NF-H.

Why pyramidal tract axons are slow to express NF-H or, in the case of some fibers, may never express the mature form of NFs remains an open question. Given the putative crosslinking function of NF-H (Hirokawa et al., 1984; Glicksman et al., 1987), the late development of this subunit may confer an extended period of plasticity on the pyramidal tract (Kalil and Reh, 1982). Further studies on the time course of appearance of NF subunits in specific pathways with well-characterized developmental histories may help to elucidate the temporal relationship between cytoskeletal maturation and the ability of axons to undergo age-related plastic changes such as sprouting, remodeling, or further growth after injury.

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