0270-6474/84/0408-2080$02.00/0
Copyright © Society for Neuroscience
Printed in U.S.A.

The Journal of Neuroscience
Vol. 4, No. 8, pp. 2080–2094
August 1984

INITIAL EXPRESSION OF NEUROFILAMENTS AND VIMENTIN IN THE CENTRAL AND PERIPHERAL NERVOUS SYSTEM OF THE MOUSE EMBRYO *IN VIVO*[1]

PHILIPPE COCHARD[2] AND DENISE PAULIN*

*Institut d'Embryologie du Centre National de la Recherche Scientifique et du Collège de France, 94130 Nogent-sur-Marne, France and *Laboratoire de Génétique Cellulaire, Institut Pasteur, 75015 Paris, France*

Received December 9, 1983; Accepted February 9, 1984

Abstract

The appearance of neurofilaments (NFs) and vimentin (Vim) in the nervous system of the mouse embryo was documented using immunohistochemical techniques. The three NF protein subunits appear early and simultaneously in central and peripheral neurons at 9 to 10 days of gestation. The onset of NF expression is concomitant with axon elongation and correlates extremely well with neurofibrillar differentiation and, in the case of autonomic ganglia, with the expression of adrenergic neurotransmitter properties. In the central and peripheral nervous system, NF expression is preceded by that of Vim, and both types of intermediate filaments coexist within the same cell for a short period of time.

Specific markers of neuronal and glial cell types are powerful tools for studying the mechanisms generating cellular diversification in the nervous system. In recent years, intermediate filaments (IFs), a particular class of cytoskeletal proteins, have attracted much interest, largely because of their polymorphism; their constitutive units are specifically expressed in various cell types and, thus, should represent excellent differentiation markers (see, for references, the reviews by Lazarides, 1980; Franke et al., 1982b; Holtzer et al., 1982). So far, three different types of IFs have been identified in the nervous system. (*1*) Neurofilaments (NFs), which take their name from their ubiquitous neuronal distribution, consist of a triplet of polypeptides, 65,000 to 70,000 daltons, 140,000 to 160,000 daltons, and 200,000 to 210,000 daltons (Hoffman and Lasek, 1975; Liem et al., 1978; Schlaepfer and Freeman, 1978). (*2*) Glial fibrillary acidic protein (GFA) is an IF of 50,000 to 55,000 daltons present in astrocytes (reviewed by Eng and Bigbee, 1978; Bignami et al., 1980). (*3*) Vimentin (Vim), a protein of 52,000 to 58,000 daltons, is found in glial cells but has also a widespread distribution in mesenchymal derivatives and established cell lines (see Franke et al., 1979; Paulin et al., 1982).

Conventional antisera, as well as monoclonal antibodies, have been prepared against each of these IF proteins. In some cases, they have revealed common epitopes shared by the various classes of IF (Pruss et al., 1981; Dellagi et al., 1982; Gown and Vogel, 1982 and, in the NF class, by the various NF subunits (Willard and Simon, 1981; Lee et al., 1982; Goldstein et al., 1983).

Recently, the neuronal specificity of the 70,000-dalton component of NF has been questioned (Granger and Lazarides, 1983). Furthermore, it appears that the restriction of each IF class to a specific cell type, the adult situation, is not always the rule in immature systems. For example, the transient or continuous expression of Vim in certain neurons, glial cells, and their precursors has been demonstrated (reviewed by Lazarides, 1980; Bennett et al., 1982).

Therefore, it has become necessary to investigate the validity and limits of IF as cell type-specific markers during ontogeny. Mammalian embryos at an early pre-implantation stage lack detectable IF; cytokeratins are the first to appear, concomitantly with cellular differentiation, in the outer cells of the blastocyst (Paulin et al., 1980). After implantation, co-expression of Vim and cytokeratins occurs in some cells of the parietal endoderm (Lane et al., 1983; Lehtonen et al., 1983). Subsequently, at the primitive streak stage, Vim is found in primary mesenchymal cells (Franke et al., 1982a). In neuronal systems, *in vivo* studies of IF expression have mostly focused on the central nervous system (CNS) of chick (Bignami et al., 1982; Tapscott et al., 1981a, b), rat (Dahl et al., 1981; Raju et al., 1981; Bignami et al., 1982), and mouse embryos (Schnitzer et al., 1981; Houle and Fedoroff, 1983). The main findings indicate that NFs appear early in development and progressively replace Vim, which is expressed before NF in most, if not all, dividing neuroepithelial cells.

In the peripheral nervous system (PNS), most studies have been conducted *in vitro*. The development of IF apparently follows a scheme similar to that found in the CNS. Vim is

[1] We wish to express our gratitude to Professor N. Le Douarin for her constant support throughout this work. We thank Dr. J. Smith for critical reading of manuscript. This work was supported by the Centre National de la Recherche Scientifique, the Délégation Générale à la Recherche Scientifique et Technique, the Association pour le Développement de la Recherche sur le Cancer, the Ministère de l'Industrie et de la Recherche (Grant 82 E 1145), and the National Institutes of Health (Grant 2 RO1 DEO 4257-06).

[2] To whom correspondence should be addressed.

present in neural crest cells, the progenitors of most peripheral neurons (Ziller et al., 1983), and coexists for some time with NF in neurons as the latter IF develops (Jacobs et al., 1982; Ziller et al., 1983).

However, no systematic study on the *in vivo* expression of NF and Vim in neural crest- and placode-derived ganglia has been performed. We have focused on the early appearance and development of NF and Vim in both CNS and PNS of the mouse embryo, with special reference to the expression of the neuronal phenotype through neurofibrillar differentiation and the acquisition of certain neurotransmitter properties. Using indirect immunofluorescence on sections obtained from specimens embedded in polyethylene glycol, a technique which allows excellent preservation of young embryonic tissues and optimal retention of antigenicity (see Cochard et al., 1978, 1979), we have asked specific questions: Is NF expression, from the onset, strictly related to nervous structures? At which particular stage of neuronal differentiation do NFs appear, and how does their expression relate with other events in neuronal ontogeny? Do the three NF subunits occur simultaneously or sequentially in CNS and PNS neurons? Are there differences among neuronal types, especially in the PNS, with respect to NF expression? Is Vim present in neural crest derivatives, and is it coexpressed with NF in peripheral neurons *in situ*?

Materials and Methods

Preparation of specific antibodies against individual NF proteins. NF proteins were isolated and purified from bovine spinal cord using a procedure reported by Delacourte et al. (1980). Further purification of individual triplet proteins of 68,000, 160,000, and 210,000 daltons was carried out by preparative electrophoresis gels.

Each purified polypeptide preparation was injected to rabbits to obtain polyclonal antibodies. The antibodies were screened (*1*) on bovine and mouse NF proteins transferred to nitrocellulose paper and (*2*) by indirect immunofluorescence on primary cultures of 13-day mouse embryo mesencephalon. These antibodies were purified by affinity technique on polypeptides coupled to Sepharose 4B.

Immunological detection of NF proteins on nitrocellulose paper. NF proteins were separated on 8% SDS/polyacrylamide gels. The transfer to nitrocellulose paper was performed as described by Towbin et al. (1979) with Tris/glycine/methanol buffer, modified as follows: The transfer was conducted at 10°C for 3 hr with a current of 350 mA. The transferred polypeptides were revealed with 3% red Ponceau solution in 0.3% trichloroacetic acid. After 3 hr of incubation in 3% bovine serum albumin and 0.2% Tween 20, the paper strips were incubated for 18 hr at room temperature with the antibodies, washed with three changes of phosphate-buffered saline (PBS) and 0.2% Tween 20 for 15 min, and then incubated with peroxidase-conjugated goat anti-rabbit antibodies. Finally, the washed strips were reacted with diaminobenzidine and H_2O_2 in Tris buffer, pH 7.4.

As controls, fibroblast extracts and crude preparations from mouse brain, including tubulin, GFA, and actin polypeptides, were transferred to nitrocellulose paper and incubated with the same antibodies as described above.

Each antibody selected for this study, directed against the 70,000-, 160,000-, and 210,000-dalton bovine NF proteins reacted strongly with the corresponding mouse NF-subunits of 68,000, 145,000 and 200,000 daltons, respectively. No reaction with other IF or cytoskeletal proteins or other nervous tissue constituents was observed. There was no cross-reactivity either between the anti-70,000-dalton antibodies and the 150,000- or 200,000-dalton NF proteins, nor between the anti-150,000-dalton antibodies and the 70,000- or 200,000-dalton NF subunits.

Antibody screening on cultured cells. Mesencephalic cells from 13-day-old mouse embryos were dissociated and cultivated on coverslips in a medium with or without serum. After 8 days, glial cells are predominant in serum-containing medium, whereas in the absence of serum virtually pure neuronal cultures are obtained (for details, see Prochiantz et al., 1982).

After fixation with methanol at −20°C for 6 min, cells were incubated with the antibodies (dilutions 1:10 to 1:500) for 1 hr at 37°C, extensively rinsed in PBS, and incubated for 30 min at 37°C with fluorescein-conjugated goat anti-rabbit antibodies (Pasteur, France). Cultures of mouse fibroblasts were used as controls in the same conditions. In some instances, antibody screening was also performed on cryostat sections of sciatic nerve.

All of the NF antibodies used in this study reacted strongly with nervous structures but did not stain glial cells and fibroblasts. Differences in the distribution of the fluorescence detected on cultured nerve cells were noticed among the various reagents; the anti-70,000-dalton antibody stained strongly neuronal cell bodies and neurites, whereas with the anti-150,000- and -200,000-dalton antibodies, staining was primarily found on neurites.

Monoclonal antibody against Vim. A human monoclonal IgM antibody reacting specifically with Vim was obtained from a patient with Waldenström macroglobulinemia. The characteristics of this antibody have been described in detail by Dellagi et al. (1982).

Preparation of embryos. Embryos of Swiss mice were used. Animals were mated at night, and the following morning was designated gestational day 0.5 (E 0.5). Pregnant mice were sacrificed between E 8.5 and E 14.5. Developmental stages were checked by referring to the data of Rugh (1968). They were defined by the gestational age and, in early embryos, by the number of somite pairs. Embryos were dissected free of extraembryonic membranes and fixed either *in toto* (from E 8.5 to E 11.5) or after severing the head (from E 12.5 to E 14.5), in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Fixation times varied between 4 and 8 hr. Longer periods of fixation resulted in a decrease of the immunocytochemical reaction for IF. Embryos were then thoroughly rinsed in 0.1 M phosphate buffer, pH 7.4, for 12 hr.

Immunohistochemical techniques. Although most previous studies on the immunocytochemical demonstration of IF *in situ* have been performed on frozen sections, it proved quite difficult to obtain cryostat sections of early embryos with satisfactory preservation of embryonic tissues, thus rendering hazardous the identification of positive structures. For this reason, we preferred to use specimens embedded in polyethylene glycol (PEG) according to Drews (1975) and Mazurkiewicz and Nakane (1972). We wish to stress here the usefulness of this method, now routinely employed in our own and several other laboratories, which enables one to perform histochemical and immunohistochemical reactions easily on free-floating or mounted serial sections of early embryos. Tissue morphology is very well preserved with no apparent shrinkage, allowing observation of cellular details, and the loss of antigenicity is minimal (see Cochard et al., 1978, 1979; Duband and Thiery, 1982; Thiery et al., 1982).

Embryos were dehydrated through a graduated series of alcohols, then embedded and stored in PEG 1000. Coons' (1968) indirect immunofluorescence method was used on batches of free-floating, 7-μm sections as previously described (Cochard et al., 1979) or on serial sections mounted on rubber-coated slides (Drews, 1975).

Sections were incubated overnight at 4°C with the various antisera, monoclonal antibodies, or, for control experiments, with appropriate nonimmune sera, all diluted in PBS containing 0.1% Triton X-100 (PBS-Triton). Working dilutions were: rabbit antisera 1:100 to 1:150 (for anti-NF 70,000 daltons, -NF 150,000 daltons, and -NF 200,000 daltons), human monoclonal anti-Vim antibody 1:1000. After three rinses in PBS-Triton, sections were incubated for 30 min at room temperature with fluorescein isothiocyanate (FITC)-conjugated antibodies (goat anti-rabbit/FITC, Nordic, dilution 1:40; affinity-purified goat anti-human IgM/FITC, KPL, dilution 1:20). Sections were rinsed three times in PBS-Triton, mounted on gelatin-coated slides, air dried, and coverslipped with 1:1 glycerin:bicarbonate (0.5 M, pH 8.6).

Double staining experiments. In some instances, the localization of NF was confronted with that of Vim on the same tissue preparation. Sections were first incubated with one of the rabbit antisera, as described above, then reacted with tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit Ig (Nordic), diluted 1:40 in PBS/Triton. Adequate staining and absence of nonspecific reaction were verified on a fluorescence microscope. Sections were subsequently incubated overnight with the anti-Vim monoclonal antibody and finally reacted with the anti-human IgM/FITC antiserum.

Specificity controls. Nonspecific immunohistochemical fluorescence was controlled by substituting pre-immune or nonimmune rabbit sera for the specific reagents, at the same working dilutions. For double staining experiments, the substitution was done either on one of the specific antibodies at a time or on both.

Fluorescence microscopy. Preparations were observed with a Leitz

Orthoplan epifluorescence microscope equipped with a Ploemopak 2 illuminator. I and N filter blocks were used, respectively, for excitation in the blue (FITC) and in the green (TRITC). Photographs were taken on Kodak Ektachrome 400 ASA color film and Kodak Tri X 400 ASA.

Results

Appearance of NF

Central nervous system (CNS)

Immunoreactivity for any of the three NF protein subunits was undetectable during neural tube closure, in 6- to 12-somite embryos (8.5 days of gestation, E 8.5). NF 70,000-, NF 150,000-, and NF 200,000-dalton immunoreactivities first appeared at E 9 to E 9.5 (15- to 18-somite embryos) in restricted areas of the neural primordium (Figs. 1 and 2). Antisera stained a small number of cells and processes at midbrain and hindbrain levels and not in more rostral and caudal levels of the neural anlage. Stained cells were consistently located ventrolaterally and midlaterally, near the outermost border of the neuroepithelium. They were never seen more internally, near the neurocele (Figs. 1 and 5). Fluorescence was localized in a narrow ring of cytoplasm surrounding a dark rounded nucleus and in cytoplasmic extensions of variable thickness which were mainly oriented in two directions, radially toward the neurocele and tangentially, running in dorsal and ventral directions along the pial surface (Fig. 2).

From this stage onward, the development of NF-immunoreactive structures was very rapid and occurred progressively in cranial and caudal directions, thus recapitulating the temporal sequence of closure of the neural tube. At E 10.5, numerous immunoreactive cells and their processes were observed almost throughout the CNS, including telencephalon, diencephalon, and spinal cord, except for the most caudal part of the latter structure. No attempts were made to identify the NF-positive areas in the embryonic brain. Again, on transverse sections, most positive cell bodies were found in the lateral and ventrolateral aspect of the neural primordium. Fluorescent processes accumulated mostly outside the mantle layer, thus forming the primordium of the marginal layer (Fig. 3). In the spinal cord, NF-immunoreactive structures were also mostly found in the ventrolateral region and, to a lesser extent, in the intermediolateral region. Stained cells were most numerous in the primitive ventral horn, giving rise to a meshwork of disorganized processes (Fig. 4). Already at this stage, a thin bundle of NFs was seen crossing the ventral aspect of the neural tube, representing the early ventral commissure. Ventral roots were intensely stained and extended lateroventrally in the mesenchyme. Dorsal roots were also clearly visible at this stage, as described below. Intensely stained filaments, isolated or in bundles, presumably of central origin, extended in various directions within the mesenchyme, sometimes as far ventrally as the aortic arches, and they could already be found in the developing anterior limb buds.

Older developmental stages, from E 11.5 to E 14.5, were characterized by a progressive thickening of the NF-positive marginal layer, separated into ventrolateral and dorsal white columns, the latter being the most intensely fluorescent structure in the spinal cord, and by an enlargement of areas containing NF-reactive cell bodies (Fig. 6).

Peripheral nervous system (PNS)

Neural crest cells, the progenitors of most peripheral ganglia, did not react with NF antisera during their migration, between E 8.5 and E 9.5. NF-immunoreactive cells first appeared in the periphery between E 9 and E 10.5 in the following structures: cranial and dorsal root ganglia, sympathetic ganglia, and gut mesenchyme.

At E 9.5, NF-positive cells and short processes were localized only in the head, grouped in small clusters within the cephalic mesenchyme, lateral to the neural primordium, underneath the superficial ectoderm (Fig. 7). These cellular aggregates could be identified at E 10.5 as early cranial ganglia.

At E 9.5 to E 10, after a dorsoventral migration, trunk neural crest cells aggregate between neural tube and somites and along the aorta, thus forming, respectively, the primordia of dorsal root and sympathetic ganglia. NF immunoreactivity was clearly observed at E 10.5 in both ganglion primordia. In dorsal root ganglia, NF antisera decorated bipolar cells (Fig. 8). Fluorescence was found in the cytoplasm surrounding a dark rounded nucleus and in many processes which had already, at this early stage, reached the neural tube dorsally, forming the primordia of the dorsal roots and of small dorsal white columns (Fig. 4). Processes also extended ventrally near the ventral roots. In the sympathetic chain primordia, fluorescence was seen in the cytoplasm of apparently unipolar cells associated in small cellular aggregates (Fig. 10). NF-positive cells, morphologically similar to sympathoblasts and found at this stage within the gut wall, were, in contrast, dispersed among mesenchymal cells (Fig. 14).

As described in the CNS, the development of NF immunoreactivity in the PNS was extremely rapid. At E 11.5 and E 12.5, numerous bipolar cells were found in dorsal root ganglia. The dorsal roots and dorsal white columns appeared as well individualized structures, the latter exhibiting an intense immunoreactivity (Fig. 9). Similarly, the number of NF-positive cells in sympathetic ganglia increased rapidly (Fig. 11). From this stage, stained cells were also found aggregated along the ventral surface of the aorta, forming the primordium of aortic plexuses (Fig. 12). Between E 10.5 and E 12.5, intensely fluorescent nerve bundles of central origin reached the sympathetic structures, and, within ganglia, individual fibers closely surrounded the stained ganglion cells.

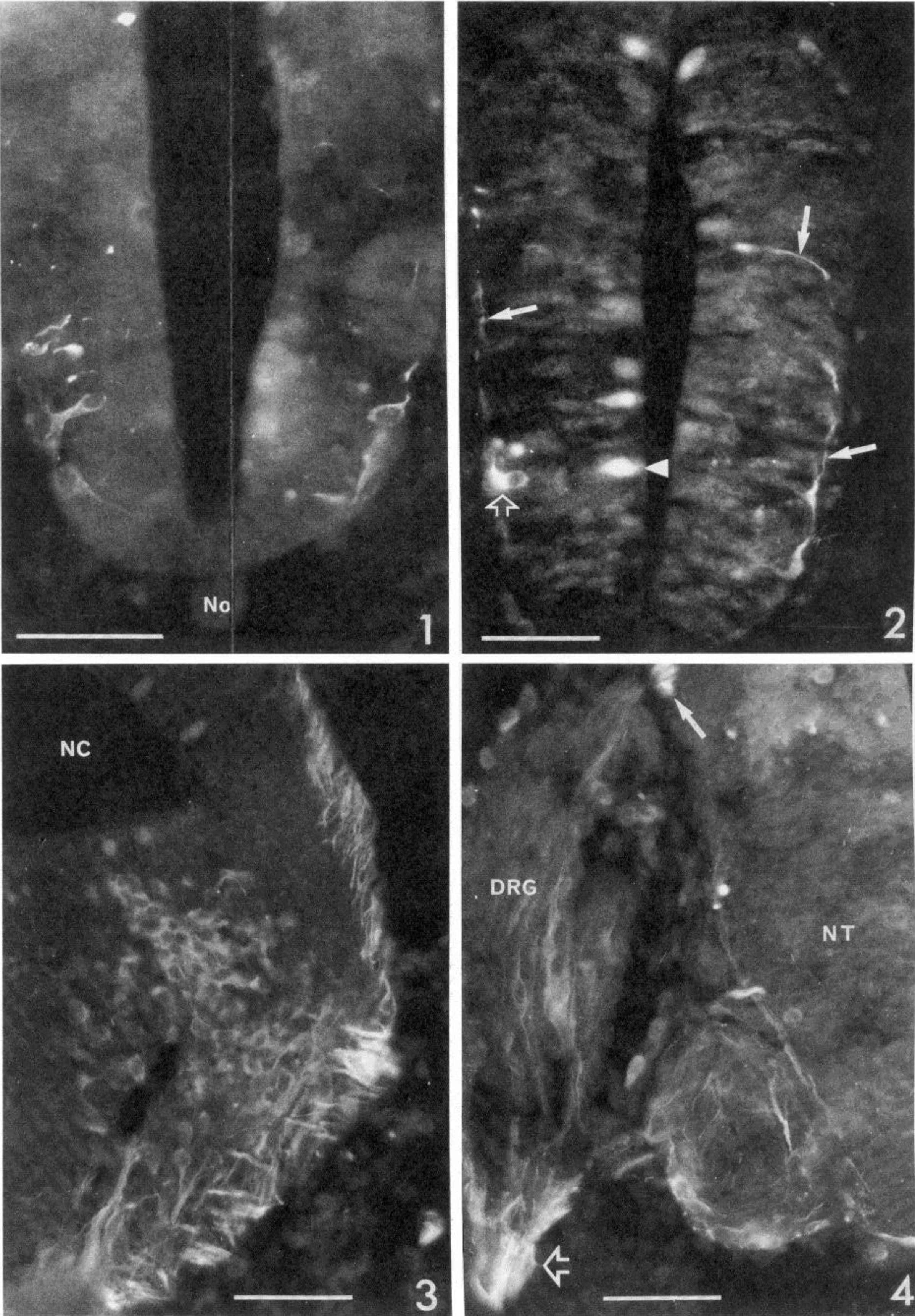
At E 12.5, NF immunoreactivity appeared in a large population of cells located more caudally, the early celiac ganglia (Fig. 13). At the same stage, NF-positive fibers and also very faintly stained cells were seen converging from sympathetic chains

Figure 1. Initial expression of NF-70,000-dalton immunoreactivity in the CNS; transverse section in the rhombencephalon of an 18-somite embryo (9 days of gestation). Immunoreactive cells and processes are only found at the ventrolateral aspect of the neural tube. Note that the antiserum stains the cytoplasm surrounding a dark, unstained nucleus. *No*, notochord. The *bar* represents 50 μm.

Figure 2. Initial expression of NF-150,000-dalton immunoreactivity in the CNS; transverse section in the rhombencephalon of a 9.5-day-old embryo. NF-immunoreactive structures include the cytoplasm of a cell body (*open arrow*), cell processes oriented radially and tangentially (*white arrows*), and mitotic cells in the germinal layer (*arrowhead*). Such staining of dividing cells was also found in all other embryonic tissues and was a characteristic of anti-150,000-dalton antibodies. The *bar* represents 50 μm.

Figure 3. NF-150,000-dalton immunoreactivity; rhombencephalon of a 10.5-day-old embryo. The number of NF-immunoreactive cells has considerably increased. Fluorescent processes already form a conspicuous marginal layer. Note that they are generally more intensely labeled than cell bodies. *NC*, neurocele. The *bar* represents 50 μm.

Figure 4. NF-200,000-dalton immunoreactivity; transverse section of the neural tube (*NT*) and dorsal root ganglion (*DRG*) of a 10.5-day-old embryo. In contrast with the staining observed with the anti-70,000- and anti-150,000-dalton antibodies, most cell bodies are unstained. Immunoreactive processes scattered in the DRG and in the ventrolateral part of the neural tube extend and join ventrally to form a conspicuously stained ventral root (*open arrow*). The dorsal projections of the DRG already reach the neural primordium, forming the initial dorsal white column (*white arrow*). The *bar* represents 50 μm.



Figures 1 to 4

and plexuses toward the mesenchymal thickening of the adrenal blastema. Some of these cells possibly represent precursors of chromaffin cells and/or intramedullary autonomic neurons. NF-positive enteric neurons also increased rapidly in number after E 10.5 and became localized around the differentiating circular muscle layer, thus forming the primordium of the myenteric plexus (Fig. 15). In addition, from E 11.5, strongly fluorescent fibers were seen in the digestive tract. At the esophageal level, they were aggregated in two large bilateral trunks, whereas only individual fibers were observed in more caudal parts of the gut. These fibers are presumably of vagal origin.

After E 12.5, NF immunoreactivity developed rapidly in all of the peripheral nervous structures described above. In sensory neurons, cytoplasmic fluorescence accumulated mostly at one pole of the cell, whereas no such regional distribution was noticed in sympathetic or enteric neurons. At E 14.5, the latter were regularly distributed at the outer surface of the developing muscle layer of the gut but were not yet found between the muscle layer and the mucosa at the level of the future submucosal plexus. Well individualized enteric ganglia were not apparent. In the adrenal gland, NF immunoreactivity was mostly confined to individual fibers, and stained cell bodies could not be identified with certainty.

Differences in the expression of the three NF subunits

From the earliest stages of NF expression, no major differences were found in the staining patterns obtained with the various antisera. However, cell bodies were consistently much more intensely labeled with the anti-70,000-dalton and anti-150,000-dalton antisera than with anti-200,000-dalton antibodies (compare Figs. 6 and 9 with Fig. 4), confirming the initial observations on mouse brain cell cultures (see "Materials and Methods"). Staining with the anti-200,000-dalton antisera gave a slightly different picture than that observed for the two other NF proteins. Initially fewer immunoreactive cells were found, and staining was essentially restricted to fibers (Fig. 4). Nonnervous tissue was always negative except with anti-150,000-dalton antibodies, which diffusely stained rounded mitotic cells scattered in all embryonic tissues (Fig. 2). Such staining was not found in control sections incubated with either nonimmune or pre-immune sera or with other antisera and was a constant feature of all of the anti-150,000-dalton antibodies, polyclonal and monoclonal, tested so far (P. Cochard and D. Paulin, unpublished results). The significance of this reactivity in dividing cells is unknown at the present time.

Relationships between the expression of NF and Vim

Development of Vim immunoreactivity

CNS. Vim immunoreactivity, which was already present in most mesenchymal cells of the earliest embryos examined (6- to 12-somite stage, E 8.5), appeared in the neuroepithelium of 15- to 18-somite embryos (E 9), first rostrally, at cephalic levels, then progressively more caudally. In contrast to what had been found for NF, Vim-specific fluorescence was not restricted to isolated cells but was distributed throughout the neural tube, although at initial stages the number of labeled filaments decreased in a ventrodorsal gradient (Fig. 16). Moreover, in most cases the fluorescence pattern indicated that the stained material was not evenly distributed in the cytoplasm but was condensed in elongated fibrillae that extended radially from the neurocele to the pial surface. At this level, the tips of the fibrillae were enlarged (Fig. 16). At E 10.5 and E 11.5, the density of fluorescent fibrillae was lower in the ventrolateral parts of the neural tube where, as described above, numerous NF-positive cells developed (Fig. 17).

Subsequently, Vim-positive cell processes became progressively more organized and adopted the typical pattern found in Golgi-stained preparations (see Ramón y Cajal, 1909), with a plexiform arrangement of fibers at the transition from mantle to marginal layer and a regular radial arrangement in this latter zone, thus indicating the restriction of Vim immunoreactivity to the glial compartment of the CNS.

PNS. As stated above, Vim immunoreactivity was a regular feature of most mesenchymal cells and was already present during crest cell migration in areas that are known to constitute crest cell migration pathways. This fact makes it difficult to assess *in situ* the presence or absence of this antigen in individual migrating crest cells scattered among Vim-rich elements. Nevertheless, the presence of this IF has been demonstrated in avian neural crest cells *in vitro*, using the same antibody (Ziller et al., 1983).

After crest cell migration was completed, Vim immunoreactivity was detectable in a variety of neural crest derivatives, almost as soon as they could be identified in their definitive locations. At E 10.5, numerous Vim-positive cell processes were found in dorsal root ganglia but were comparatively less conspicuous in sympathetic ganglion primordia. Subsequently, Vim immunoreactivity was still found in peripheral ganglia. However, at E 14.5, there was a striking difference in the pattern of Vim staining between dorsal root and sympathetic ganglia. Numerous Vim-positive small satellite cells closely surrounded each dorsal root ganglion neuron (Fig. 20), whereas only occasional short processes exhibited Vim in sympathetic ganglia (Fig. 21).

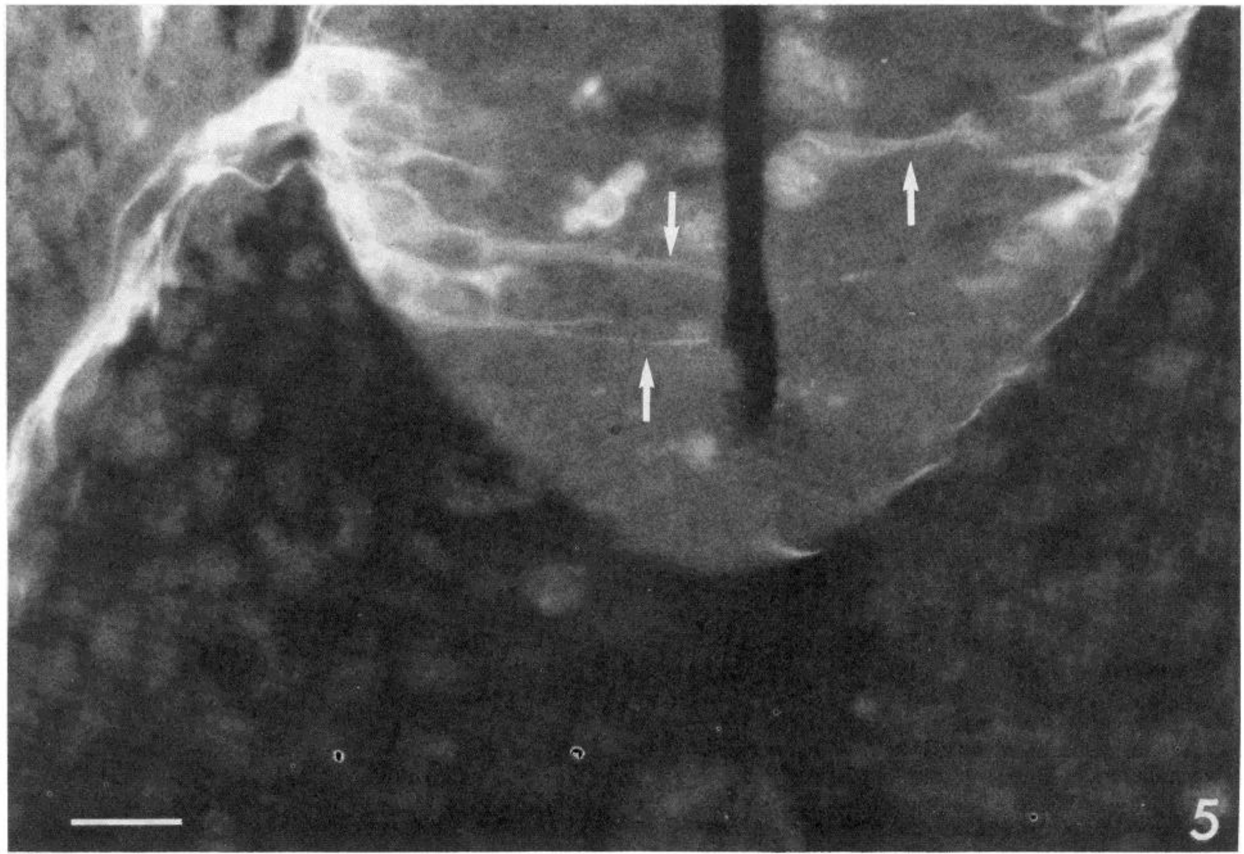
Double staining experiments

The coexistence of NF and Vim in developing neurons has been previously demonstrated in the CNS (Dahl et al., 1981; Tapscott et al., 1981a; Bignami et al., 1982) and in PNS neurons *in vitro* (Jacobs et al., 1982; Ziller et al., 1983). Nevertheless, it was of interest to reinvestigate this question, first to clarify the stage in neuronal differentiation at which this coexistence takes place and, second, to determine whether this also occurred in PNS neurons *in situ*, since it has been suggested that Vim expression *in vitro* might represent an adaptation to culture conditions (Franke et al., 1979).

Simultaneous detection of NF and Vim by double immunohistochemical staining revealed that in most cases their localizations were mutually exclusive. Cells and processes intensely stained for NF were Vim-negative and vice versa (Fig. 18). In some instances, however, double-staining was evident, both in the CNS and in the PNS. Vim and NF coexisted in fibers within the neural tube of E 9.5 and E 10.5 embryos. These fibers were always radially oriented, whereas NF-positive fibers elongating tangentially along the pial surface or in various directions in the mantle layer never expressed Vim immunoreactivity. This is not surprising, since, as reported above, Vim at this stage was mostly found in radial fibers. Thus, it appears that NF and Vim only coexist in the radial process of early bipolar neuroblasts, before this process is lost. It is interesting to note that immunofluorescence for each IF was always weaker in double-stained fibers than in processes positive for only one IF, which supports the contention of a progressive replacement of one by the other.

In the periphery, double staining was found at E 10.5 in a few cell bodies and in fibers running within the early differentiating dorsal root ganglia (Fig. 19) but was not found outside the ganglion primordia. For instance, Vim was totally absent from the dorsal white columns. This indicated that NF and Vim coexisted only in close proximity to the nerve cell bodies and for a short period of time.

Control sections, incubated with only one of the specific reagents and thereafter with both fluorochrome-conjugated



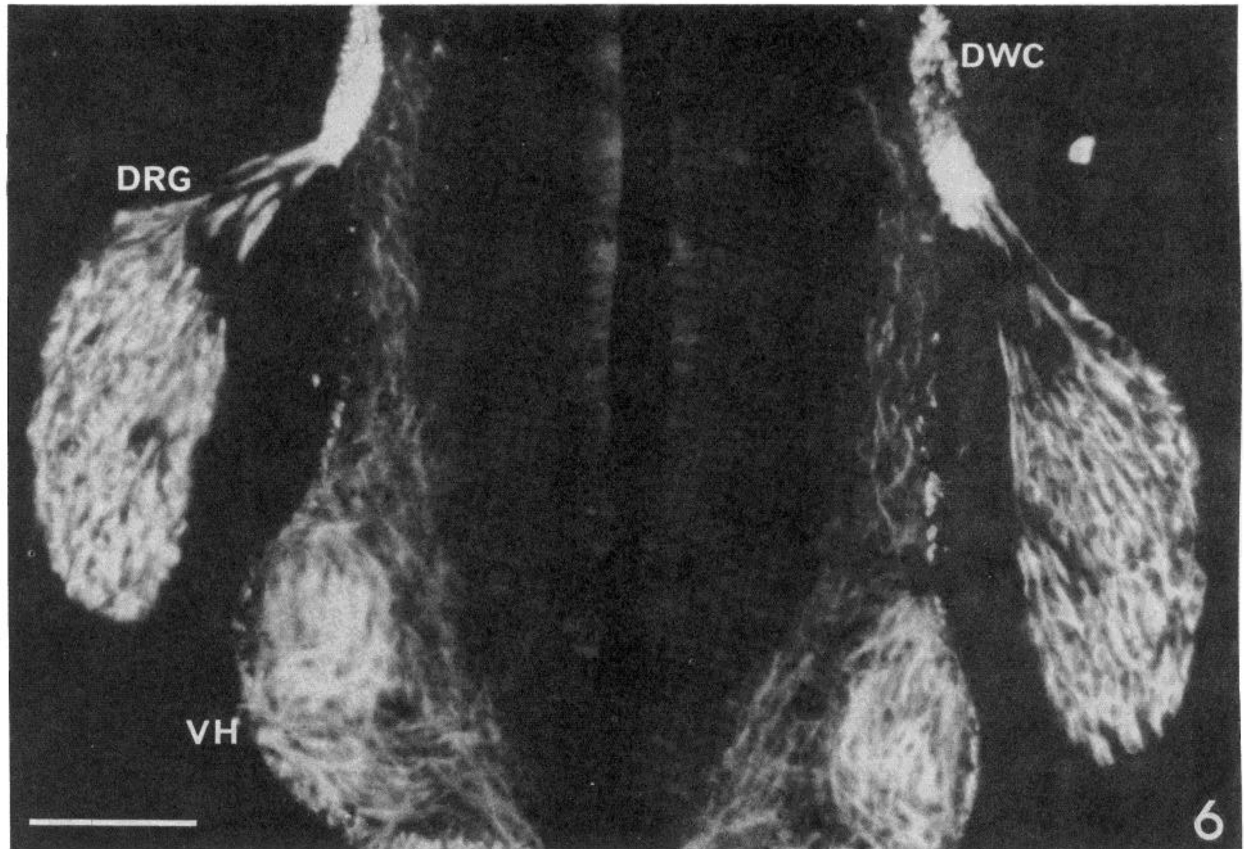
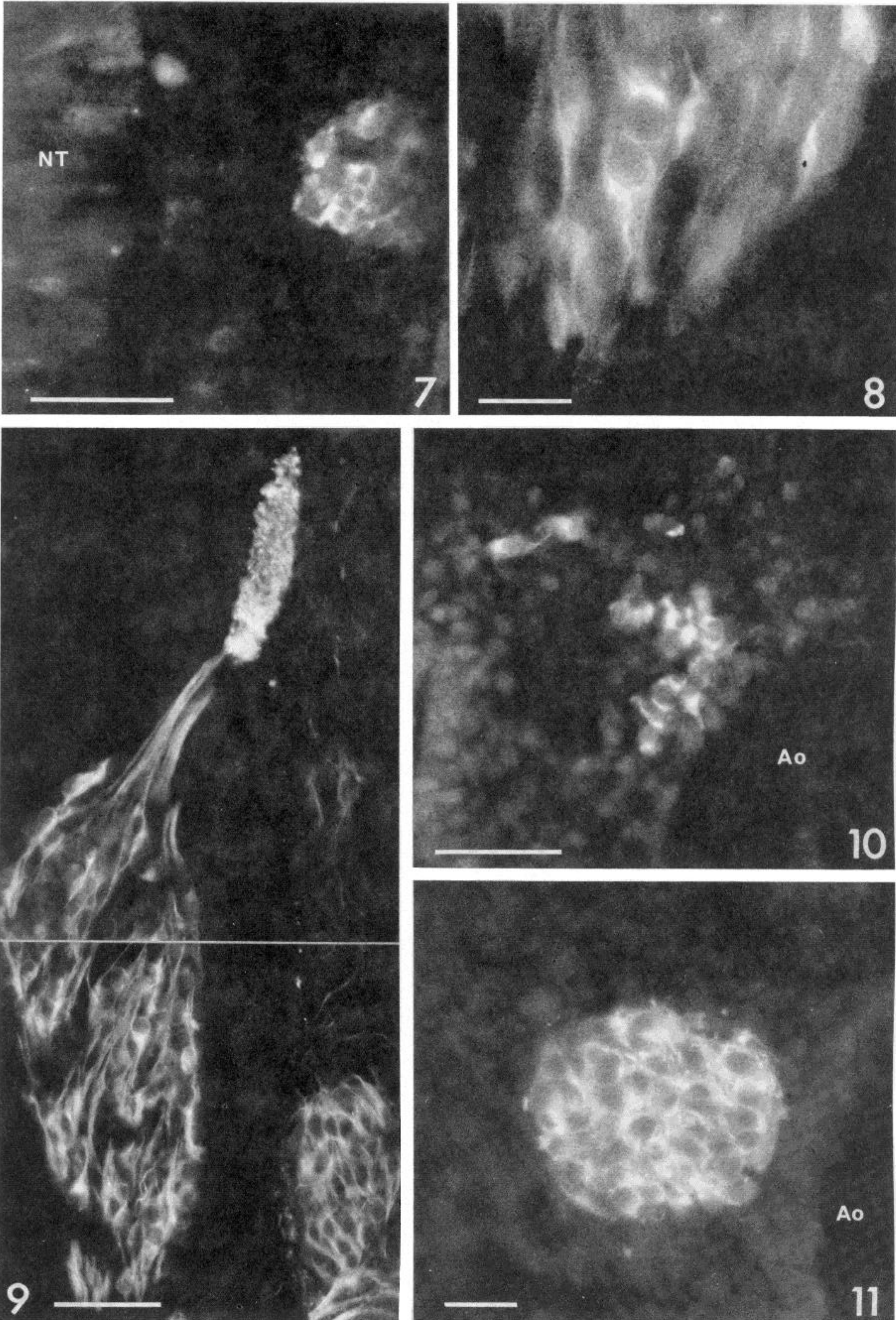


Figure 5. NF-70,000-dalton immunoreactivity; transverse section in the lumbar region of a 12.5-day-old embryo. NF-positive cells are aggregated at the ventrolateral aspect of the neural tube and, on the left, extend a small bundle of processes, the ventral root primordium. Some of these cells also extend radial processes (*arrows*) which are still connected with the neurocele. The *bar* represents 20 μm.

Figure 6. NF-70,000-dalton immunoreactivity; same stage as in Figure 5. This is a low power micrograph of a transverse section in the cervical region. At this rostral level, in contrast to Figure 5, numerous cell bodies in the dorsal root ganglia (*DRG*) and ventral horn (*VH*) of the spinal cord display intense immunofluorescence. In the spinal cord, positive fibers extend from the ventral commissure up to the dorsal white columns (*DWC*), which are very prominent at this stage. The *bar* represents 100 μm.



Figures 7 to 11

antisera, did not reveal cross-reactivity between the various antibodies used. However, bright staining with the TRITC conjugate was sometimes visualized through the I barrier filter used for FITC as a very faint yellow-orange fluorescence. Nevertheless, it could never be mistaken for the specific, even weak, green FITC fluorescence. The reverse situation never occurred, i.e., intense FITC fluorescence crossing the N filter system specific for TRITC. In any case, the fact that NF and Vim immunoreactivities were, in most cases, expressed in different structures provides the best internal specificity control and does not allow any doubt to exist regarding the validity of these observations.

Discussion

This study sought to characterize spatially and temporally the early expression of NF proteins in the nervous system of the mouse embryo by indirect immunofluorescence techniques.

Our observations demonstrate that (*1*) the three NF protein subunits appear simultaneously and are, from the onset, strictly related to nervous structures, the only exception being that mitotic cells in all tissues express an antigenic determinant specifically recognized by antibodies directed against the 150,000-dalton NF protein subunit; (*2*) the initial appearance of NF is a very early event during neuronal differentiation, both in the CNS and in the PNS, and is concomitant with the initiation of axon extension; (*3*) the developmental pattern of NF correlates extremely well with the process of neurofibrillar differentiation described in previous studies using silver impregnation techniques; (*4*) NF expression in both central and peripheral neurons takes place after that of the fibroblast type of IF, Vim, although both types can coexist within the same cell for a short period of time; (*5*) in the autonomic nervous system, the expression of NF occurs simultaneously with that of certain neurotransmitter-related properties.

Early expression of the various NF protein subunits. Previous studies have reported that NF-immunoreactive cells and processes could first be consistently found in the brain and spinal cord of 12-day-old rat embryos (Raju et al., 1981; Bignami et al., 1982). These embryos were at a developmental stage comparable to 10.5- to 11-day-old mouse embryos (Rugh, 1968). In the present study, we demonstrate that, in fact, the initial NF expression in the mouse embryo occurs earlier and in more restricted regions of the neural tube. NF-positive cells and processes could first be evidenced at E 9 to E 9.5, in 15- to 18-somite embryos, at the hindbrain level. At later developmental stages (E 10.5 to E 11.5), NF immunoreactivity extended rostrally and caudally in the brain and spinal cord. Our observations at these stages were comparable to those of Raju et al. (1981) and Bignami et al. (1982) in the rat embryo and also to the description of NF immunoreactivity in the chick embryo at 3 days of incubation (Bignami et al., 1980; Tapscott et al., 1981a, b).

However, it must be pointed out that this pattern of early appearance was not always identical for the three NF protein subunits. Although antisera directed against each of the protein subunits recognized nerve cell processes from the onset (E 9), at early stages, cell bodies were intensely stained only with the anti-70,000-dalton and anti-150,000-dalton antisera. At later developmental stages, NF-200,000-dalton immunoreactivity was generally less conspicuous and labeling of cell bodies was much weaker than with the two other antisera.

These observations suggest that the 200,000-dalton subunit does not always develop in a pattern similar to that of the 70,000- and 150,000-dalton proteins. There are some indications that the expression of the three proteins could vary in the different regions of the CNS (D. Paulin, unpublished observations), the 200,000-dalton protein being, in some neurons, synthesized later than the two other proteins.

Nevertheless, our findings contrast with the recent results of Shaw and Weber (1982), who found that the 200,000-dalton protein was expressed in the rat CNS only after birth. Low cross-reactivities between our anti-200,000-dalton antiserum and the 70,000- and/or 150,000-dalton NF subunits would not be sufficient to account for this discrepancy, since (*1*) staining of nerve fibers with the 200,000-dalton antiserum was always very intense and (*2*) the presence of 200,000-dalton immunoreactivity at embryonic stages has been recently confirmed using a monoclonal antibody (P. Cochard and D. Paulin, manuscript in preparation). Thus, we tentatively conclude that the three NF protein subunits are expressed simultaneously at least in some neurons; however, the possibility of sequential expression of the NF subunits remains for certain neuronal types.

Correlation with neurofibrillar differentiation. For almost a century, one of the most widely used staining techniques in neuroanatomical studies has relied upon the selective affinity of neurofibrillar structures for silver salts. Since it has been shown that silver-based reagents stain NF polypeptides (Gambetti et al., 1981), it was of particular interest to confront our results with the previous studies dealing with the appearance and development of neurofibrils.

In young rat and chick embryos, silver-stained cells and processes appear first in the hindbrain around the 14th somite stage and shortly thereafter in the mesencephalon and diencephalon (Tello, 1923; Windle and Baxter, 1936; Windle and Austin, 1936; Lyser, 1966). These early differentiating neurons, which are also those that first express NF, belong to the medial motor column of the rhombencephalon. They represent the initial stage of the formation of visceral motor nuclei of cranial nerves. It is interesting to note that it is at the same precise stage and level that the corresponding sensory afferents from cranial ganglia differentiate in terms of both NF and neurofibrils (Windle and Austin, 1936; Windle and Baxter, 1936). Correspondence between neurofibrillar and NF expression can also be established at the cellular level. As in silver-stained preparations (see Ramón y Cajal, 1909; Tello, 1923; Windle and Austin, 1936; Windle and Baxter, 1936; Lyser, 1964), NF

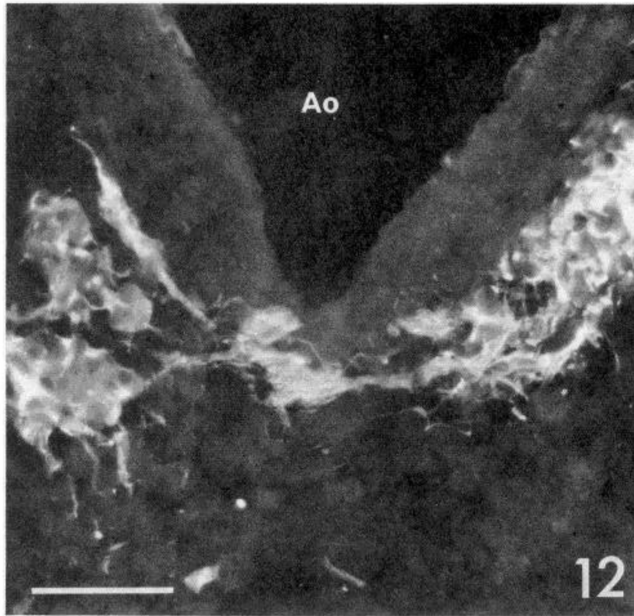
Figure 7. Initial expression of NF-70,000-dalton immunoreactivity in the PNS; transverse section at the rhombencephalic level of a 9.5-day-old embryo. A few cells aggregated close to the neural tube (*NT*) exhibit NF immunofluorescence. They presumably represent the primordium of the trigeminal ganglion. The *bar* represents 50 μm.

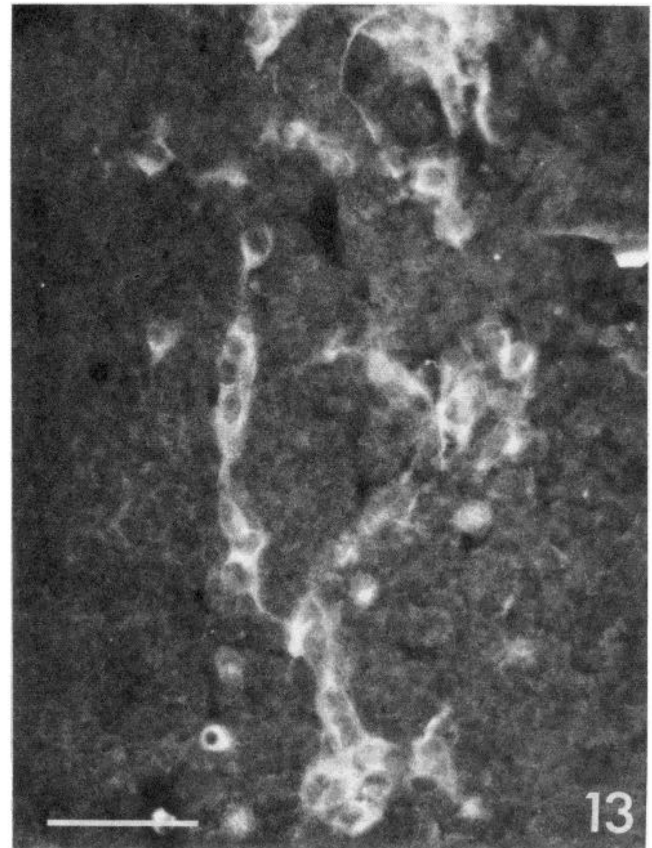
Figure 8. Initial expression of NF-70,000-dalton immunoreactivity in the PNS; transverse section at the trunk level of a 10.5-day-old embryo. Faintly immunoreactive cell processes and more intensely labeled cell bodies are found in an early dorsal root ganglion. The *bar* represents 20 μm.

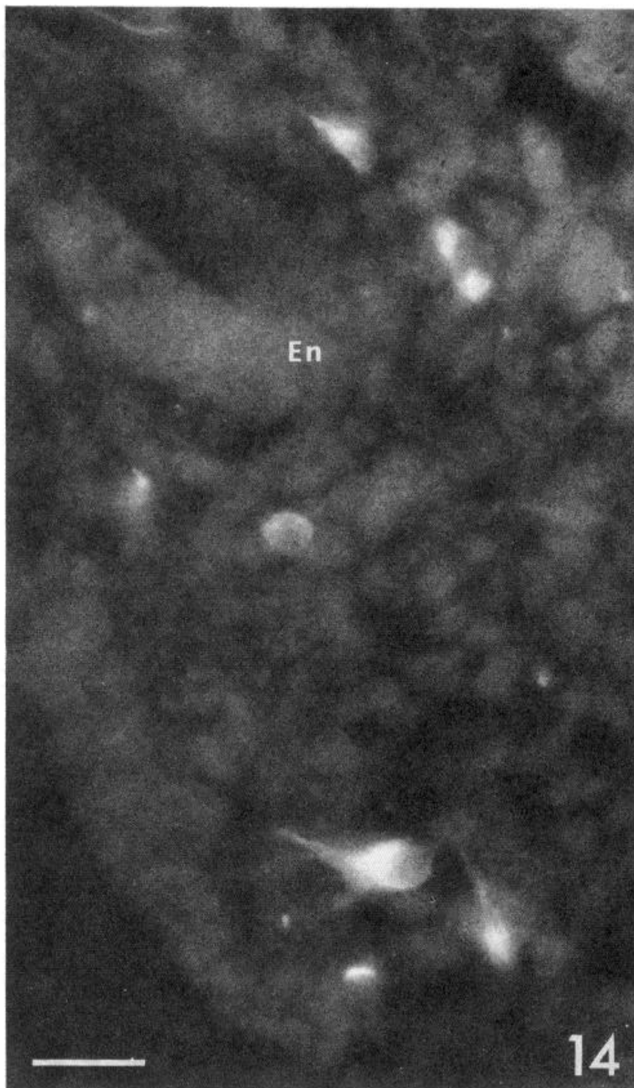
Figure 9. NF-70,000-dalton immunoreactivity; transverse section though a dorsal root ganglion in the trunk region of a 11.5-day-old embryo. Intense immunofluorescence is found in the cytoplasm of neurons extending NF-positive processes in both ventral and dorsal directions. Note at the *top* of the micrograph the intensely labeled dorsal white column. The *bar* represents 50 μm.

Figure 10. Initial expression of NF-70,000-dalton immunoreactivity in sympathetic cells; transverse section at the trunk level in a 10.5-day-old embryo. Cells aggregating at the dorsolateral aspect of the aorta (*Ao*) already express NF immunoreactivity. Note that the fluorescence is mostly confined at one pole of the cell from which emerges an NF-positive cytoplasmic process. The *bar* represents 50 μm.

Figure 11. NF-70,000-dalton immunoreactivity in sympathetic ganglion cells; transverse section at the trunk level in an 11.5-day-old embryo. Immunoreactive cells are grouped in a compact ganglion apposed to the aortic wall. *Ao*, aorta. The *bar* represents 20 μm.







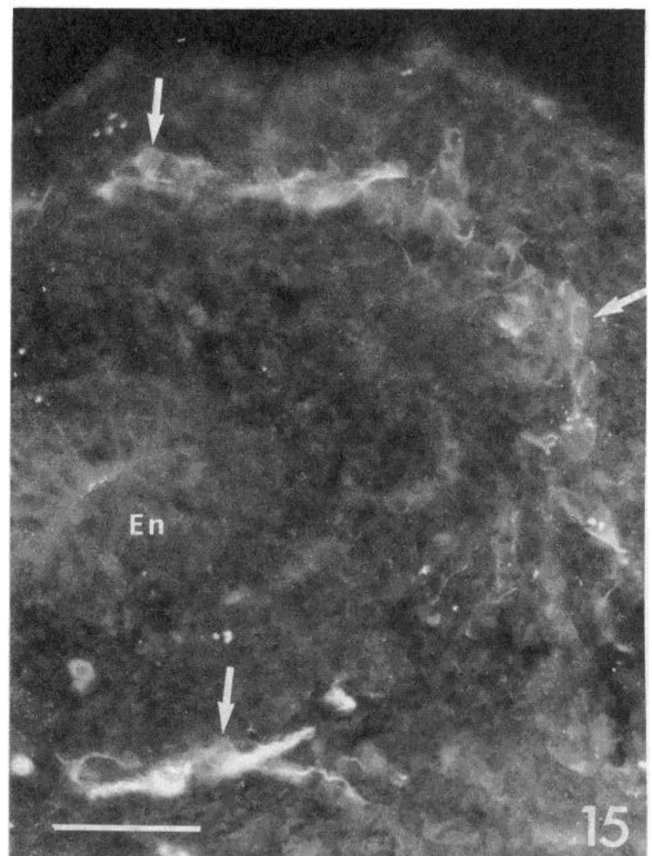
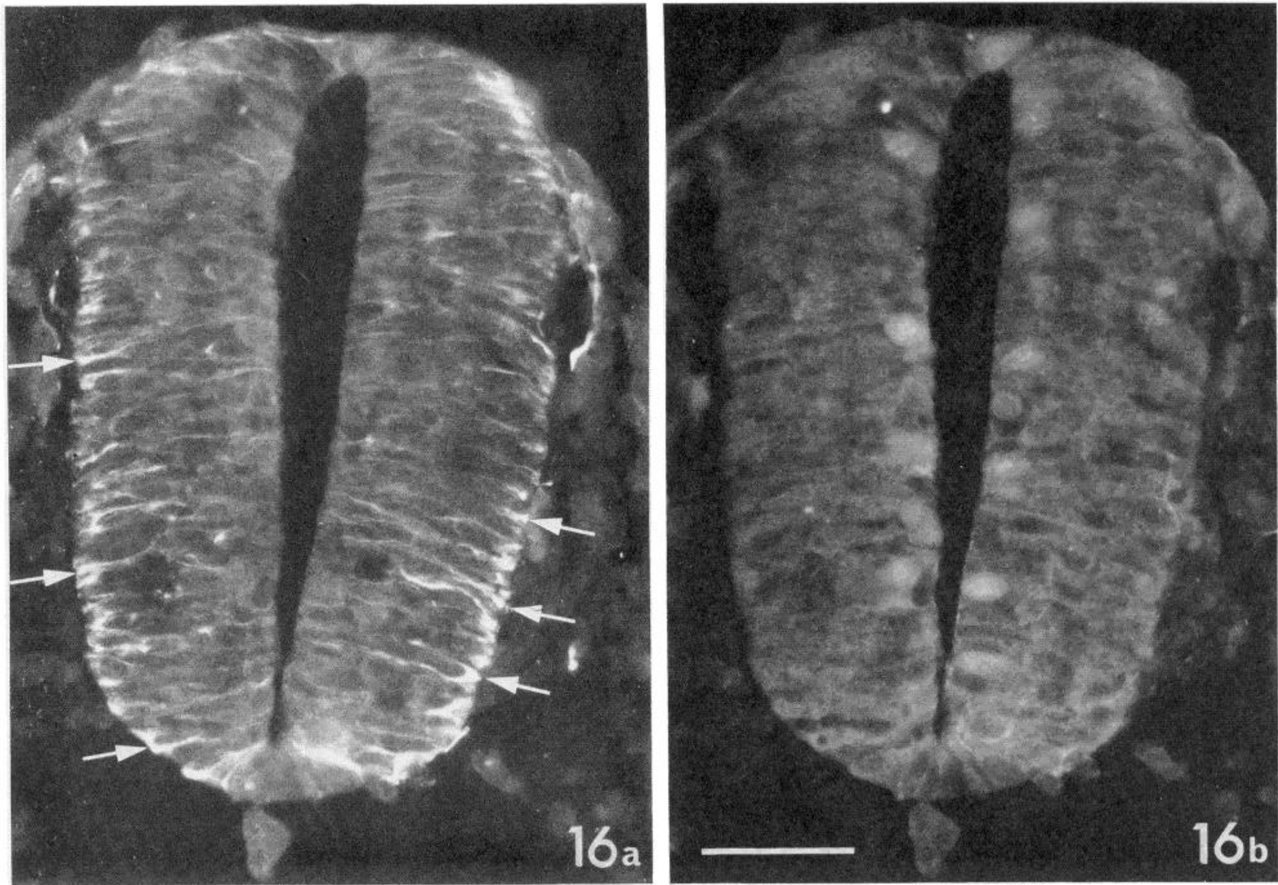


Figure 12. NF-150,000-dalton immunoreactivity; transverse section in the trunk region of a 12.5-day-old embryo. Conspicuous NF staining is found in cells and processes forming the aortic plexus. *Ao*, aorta. The *bar* represents 50 μm.

Figure 13. NF-150,000-dalton immunoreactivity; transverse section in the lumbar region of a 12.5-day-old embryo. NF staining appears in cells constituting the primordium of the celiac plexus. The *bar* represents 50 μm.

Figure 14. Initial expression of NF-70,000-dalton immunoreactivity in the enteric nervous system. Section of the intestine of a 10.5-day-old embryo. Cells scattered in the gut mesenchyme exhibit NF-immunofluorescence. Staining is exclusively cytoplasmic and, as in sympathetic cells, is concentrated at one pole of the cell from which emerges a single process. *En*, endoderm. The *bar* represents 20 μm.

Figure 15. NF-150,000-dalton immunoreactivity; transverse section through the gut of a 12.5-day-old embryo. NF-positive cell bodies (*arrows*) and fibers form a dense network surrounding the developing muscular layer of the gut. *En*, endoderm. The *bar* represents 50 μm.



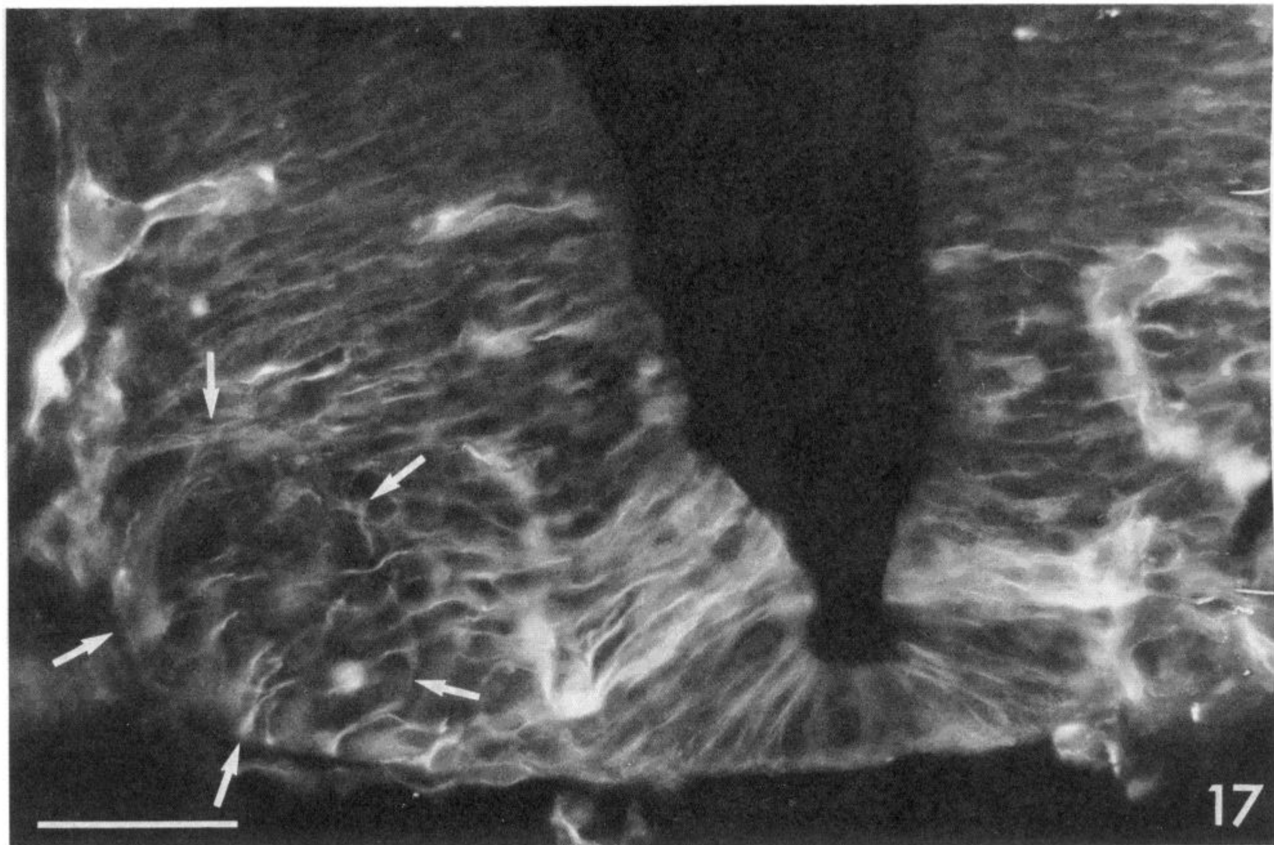


Figure 16. Simultaneous detection of Vim (*a*) and NF (*b*) immunoreactivities in the CNS; transverse section through the trunk neural tube of a 9.5-day-old embryo. *a*, Radially oriented fibers expressing Vim immunofluorescence span the entire neural tube. Note that the fibers end in enlarged boutons at the level of the pial surface (*arrows*). *b*, The same section viewed with the rhodamine filter block demonstrates the total absence of NF immunoreactivity in the neural tube at this stage and level. The *bar* represents 50 μm.

Figure 17. Vim immunoreactivity; transverse section in the trunk region of a 10.5-day-old embryo showing the ventral part of the neural tube. In most of the neuroepithelium, Vim-positive filaments are numerous and oriented radially. In the ventrolateral zone (*arrows*), which corresponds to the main area for NF expression at this level (see Figs. 4 and 6), they are more rarely found and have no specific orientation. The *bar* represents 50 μm.

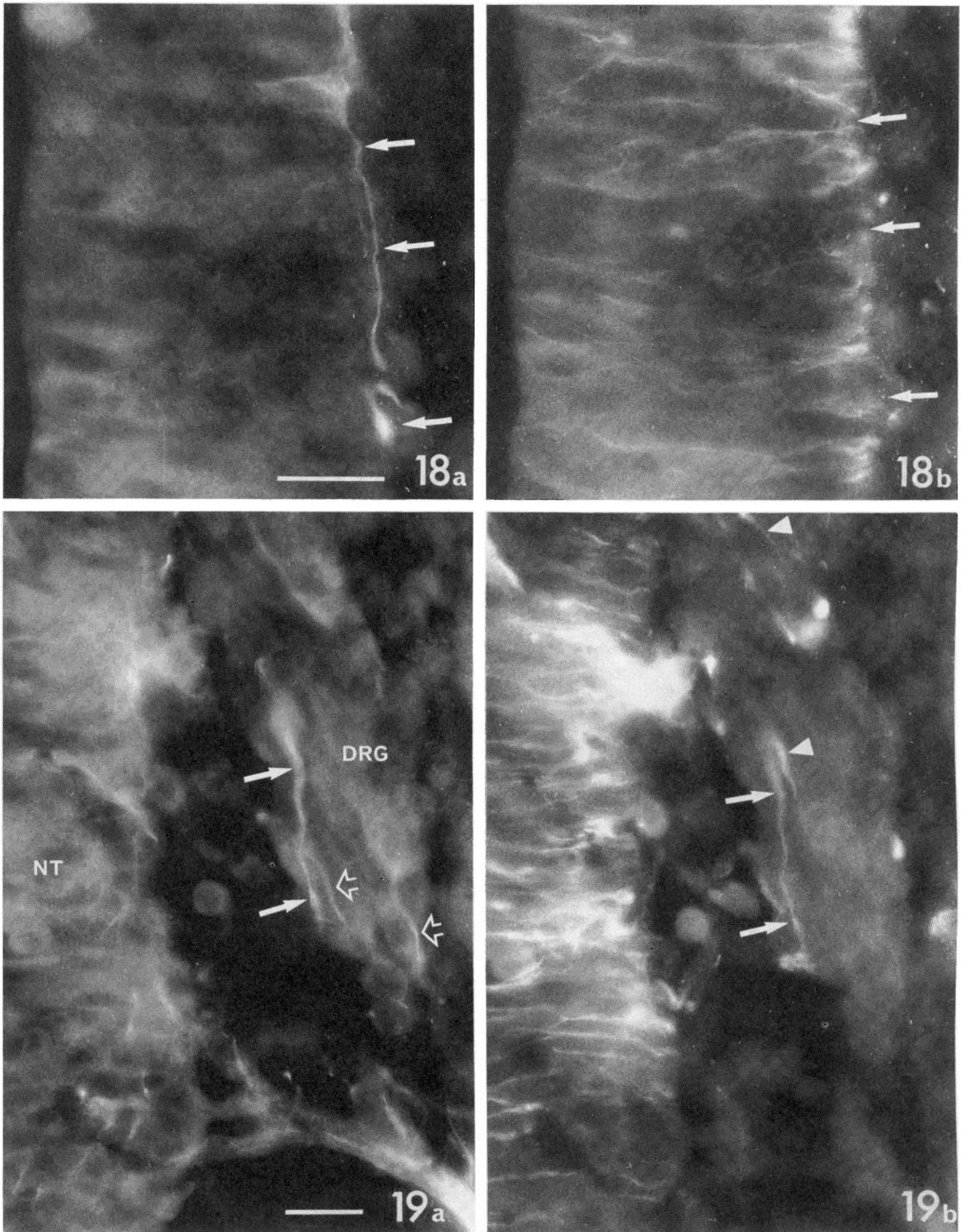


Figure 18. Simultaneous detection of NF (*a*) and Vim (*b*) immunoreactivities; rhombencephalon of a 9.5-day-old embryo. *a*, NF-150,000-dalton immunofluorescence is present in a thin, long process extending ventrally near the external surface of the neural tube (*arrows*). *b*, The same section demonstrates the absence of Vim immunoreactivity in this cell process. However, Vim-positive filaments are present and extend radially. The *bar* represents 20 μm.

Figure 19. Simultaneous detection of NF (*a*) and Vim (*b*) immunoreactivities; trunk neural tube (*NT*) and dorsal root ganglion (*DRG*) of a 10.5-day-old embryo. In the neural tube, Vim and NF staining are mutually exclusive. In the DRG, double immunolabeling is found in a cell process extending parallel to the ganglion long axis (*white arrows*), whereas other processes exhibit only NF (*open arrows* in *a*) or Vim (*arrowheads* in *b*). The *bar* represents 20 μm.

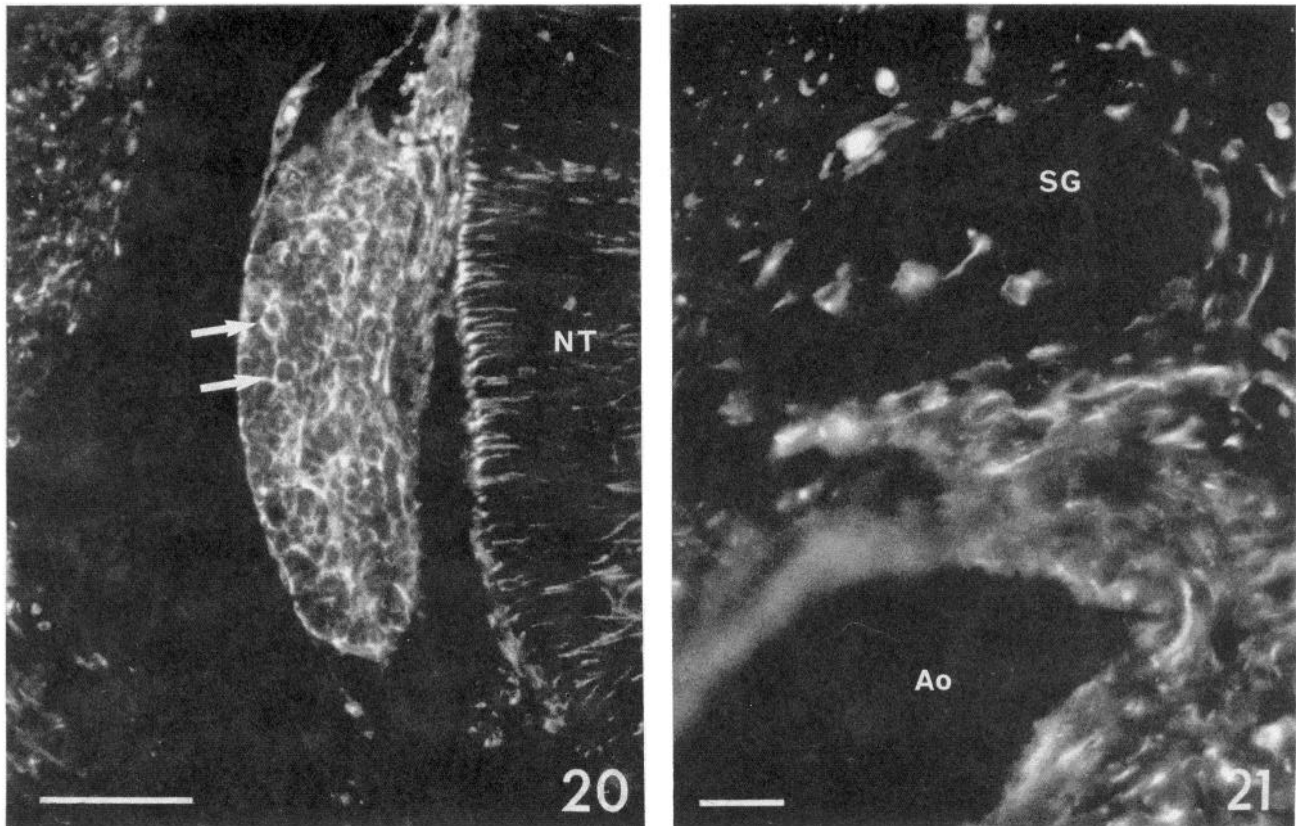


Figure 20. Vim immunoreactivity in a dorsal root ganglion of a 14.5-day-old embryo. Numerous Vim-positive filaments are found throughout the ganglion. They are mostly confined to small satellite cells which surround the ganglion neurons (*arrows*). *NT*, neural tube. The *bar* represents 100 μm.

Figure 21. Vim immunoreactivity in a sympathetic ganglion of a 14.5-day-old embryo. Vim-positive filaments are scarce in the ganglion (*SG*), although the surrounding mesenchyme is intensely labeled. *Ao*, aorta. The *bar* represents 20 μm.

staining was found first in neural tube cells, which still assumed a neuroepithelial morphology, i.e., with processes extending radially between the external and internal limiting membranes. NF immunoreactivity was also prominent in monopolar neurons which had retracted, or lost, the central process while extending a short axon. All of these cells are believed to be postmitotic neurons (see Jacobson, 1978, for references). At subsequent stages, in agreement with the findings of Bignami et al. (1980) in the chick embryo, the pattern of NF staining throughout the brain and spinal cord could be superimposed on the classical pictures of silver-stained structures (see Ramón y Cajal, 1909).

Similarities between neurofibrillar differentiation and NF expression were also evident in the PNS. As stated above, NF-positive cells and processes appeared at E 9 to E 9.5 in cranial sensory ganglia and at E 10 to E 10.5 in spinal and sympathetic anlagen and within the gut mesenchyme. Similarly to what has been described for neurofibrillar differentiation (Paterson, 1890; Ramón y Cajal, 1909; Tello, 1925), NF antibodies reacted initially with bipolar cells in spinal ganglia and in unipolar cells in both sympathetic and gut anlagen, before these cells were aggregated into compact clusters.

Thus, in both CNS and PNS neurons, the onset of NF immunoreactivity coincides with neurofibrillar differentiation and takes place apparently at the time of the initial axon extension. However, a recent report suggests that in the chick neural tube, NFs appear slightly earlier, i.e., during the terminal cell cycle of the neuronal precursors. Using demecolcine, a mitosis-blocking agent, together with NF immunofluorescence, Tapscott et al. (1981a) have shown that 70,000- and 180,000-dalton NF proteins were present in a small percentage of replicating neuroepithelial cells located in the ependymal layer. We did not observe specific NF staining in this region of the mouse neural tube. Nevertheless, our techniques might not be sensitive enough to detect very low amounts of NF, which are more easily identified in cells arrested in metaphase (Tapscott et al., 1981a, b). It is not known at the present time whether this is also true for peripheral neurons. Tapscott et al. (1981a) have suggested that certain peripheral neurons, in the vicinity of the otic vesicle, might synthesize NF for several generations. On the other hand, it has been shown that sympathetic neurons and cells located in the gut mesenchyme can divide while expressing adrenergic neurotransmitter properties (Rothman et al., 1978, 1980; Teitelman et al., 1981). Since there is a good synchrony in the appearance of NF and of adrenergic characteristics in these two cell populations, as discussed below, it is possible that NF expression could occur in replicating neuronal precursors of the autonomic nervous system. We are presently testing this hypothesis.

Relationships between NF and Vim expression. Since Vim occurs in a great variety of cells and tissues and often simultaneously with other types of IF (see Lazarides, 1980; Franke et al., 1982b), its early expression was investigated and compared with that of NF proteins.

Our findings confirm earlier work demonstrating the expression of Vim immunoreactivity by neuroepithelial cells before the onset of NF differentiation (Tapscott et al., 1981a, b; Bignami et al., 1982) and are in excellent agreement with the recent data of Houle and Fedoroff (1983) concerning initial Vim expression which takes place at E 9 in the mouse neural

tube. According to Tapscott et al. (1981a, b), Vim is initially expressed in virtually all neuroepithelial cells of the chick embryo neural tube and is replaced by the neuron-specific IF a short time before neurons withdraw from the cell cycle. Since this replacement is progressive, NF and Vim coexist for some time in immature CNS neurons, as demonstrated in chick (Tapscott et al., 1981a) and rat embryos (Bignami et al., 1982). We also confirm this to be the case in the mouse CNS. From our observations, it appears that coexistence occurs in the cell body and its radial process that still connects the newly born neuron to the luminal surface. By the time the neuron starts extending a neurite in other directions, Vim synthesis must have ceased since this process could only be stained by anti-NF antibodies. However, residual Vim immunoreactivity can sometimes be found, together with NF, in the radial process before it is lost or retracted.

Subsequently, Vim staining was restricted to certain glial cell types, including radial glia, as already reported (Dahl et al., 1981; Tapscott et al., 1981b; Bignami et al., 1982). It is interesting to note that the only case of coexistence of NF and Vim in adult neurons *in situ* is that of the axonless horizontal cells of the retina, which have unusual features and could be intermediate between neuronal and glial cells (Dräger, 1983).

As far as the PNS is concerned, we demonstrate that Vim is present early in neural crest derivatives and is coexpressed with NF at early stages of neuronal differentiation. In fact, using the same antibody, Vim has been demonstrated in virtually all mesencephalic neural crest cells freshly explanted from quail embryos (Ziller et al., 1983). Moreover, in culture conditions promoting the rapid neuronal differentiation of a subpopulation of crest cells, Vim and NF were coexpressed in neurites and perikarya (Ziller et al., 1983). Jacobs et al. (1982) also reported the coexistence of Vim and NF in sensory and sympathetic neurons from 7- to 17-day-old chick embryos and the progressive loss of Vim immunoreactivity during the maturation of sensory neurons. Thus, the expression of IF in the PNS follows a sequence very similar to that found in the CNS. Neural crest cells, as neuroepithelial cells, first express Vim. NFs then appear in Vim-positive neurons and will progressively replace the mesenchymal type of IF.

Interestingly, the time course for this replacement seems to vary among species and, within the same species, among the various types of neurons: coexistence of NF and Vim was found only for a short period of time in dorsal root ganglion neurons of the mouse embryo, whereas in the chick species, it was observed during at least the first 10 days of the development of the sensory ganglia and for even longer periods of time in sympathetic ganglia (Jacobs et al., 1982).

In our study in dorsal root ganglia of E 14.5 embryos, Vim was only found in the numerous satellite cells surrounding each ganglion neuron. Strikingly, however, very few Vim-positive filaments were detected in sympathetic ganglia. This could mean that supporting cells are absent at this stage, possibly remaining outside the ganglionic rudiment which they will later invade. A more likely explanation is simply that sympathetic satellite cells totally lack Vim. If this interpretation is correct, it would be interesting to know to what extent these cells differ from their sensory counterparts.

NF and adrenergic differentiation in the autonomic nervous system. Thanks to the availability of sensitive histochemical and immunocytochemical techniques, the expression of the adrenergic phenotype has been thoroughly studied in the autonomic nervous system of the chick (Enemar et al., 1965; Allan and Newgreen, 1977) and rat embryos (Cochard et al., 1978; Teitelman et al., 1979). Neural crest cells, the progenitors of all autonomic neurons, do not express adrenergic characters while migrating. Catecholamines and their synthesizing enzymes only appear after crest cells have coalesced near the aorta to form the primitive sympathetic chain, and also, in the rat embryo, in cells located in the gut mesenchyme. In the mouse embryo, the onset of catecholaminergic differentiation takes place at 10 days of gestation in both sympathetic and gut cells (Teitelman et al., 1981; P. Cochard, unpublished observations). This is precisely the stage at which we have first observed NFs in cells with a similar localization. In fact, there is a very close spatial and temporal correspondence in the expression of both neuronal and neurotransmitter-related phenotypes. This finding immediately raises the following question: Is the expression of these two phenotypes concomitant in the same cell? Several lines of evidence suggest that it might not always be the case. For example, "extraneuronal" neurotransmitter metabolisms have been described (e.g., see Sastry and Sadavongvivad, 1979). Furthermore, cholinergic traits are present in otherwise apparently undifferentiated migrating neural crest cells (Smith et al., 1979; Fauquet et al., 1981; Cochard and Coltey, 1983), and adrenergic neurotransmitter properties can be expressed by cultivated neural crest cells which have not achieved typical neuronal differentiation (Fauquet et al., 1981). Thus, are the acquisition of neurotransmitter properties and the expression of a neuronal status independent during normal *in vivo* development? Experiments involving double staining of cells with anti-NF antibodies and antibodies directed against the catecholamine-synthesizing enzymes are in progress and might help to clarify this question.

References

Allan, I. J., and D. F. Newgreen (1977) Catecholamine accumulation in neural crest cells and the primary sympathetic chain. Am. J. Anat. *149:* 413–421.

Bennett, G. S., S. J. Tapscott, and H. Holtzer (1982) Switches in intermediate filament subunit types during neurogenesis. In *Changing Concepts of the Nervous System*, A. R. Morrison and P. L. Strick, eds., pp. 131–149, Academic Press, Inc., New York.

Bignami, A., L. P. Kozar, and D. Dahl (1980) Molecular markers for the analysis of neural differentiation in culture. In *Tissue Culture in Neurobiology*, E. Giacobini, A. Vernadakis, and A. Shahar, eds., pp. 63–74, Raven Press, New York.

Bignami, A., T. Raju, and D. Dahl (1982) Localization of vimentin, the nonspecific intermediate filament protein, in embryonal glia and in early differentiating neurons. Dev. Biol. *91:* 286–295.

Cochard, P., and P. Coltey (1983) Cholinergic traits in the neural crest: Acetylcholinesterase in crest cells of the chick embryo. Dev. Biol. *98:* 221–238.

Cochard, P., M. Goldstein, and I. B. Black (1978) Ontogenetic appearance and disappearance of tyrosine hydroxylase and catecholamines in the rat embryo. Proc. Natl. Acad. Sci. U. S. A. *75:* 2986–2990.

Cochard, P., M. Goldstein, and I. B. Black (1979) Initial development of the noradrenergic phenotype in autonomic neuroblasts of the rat embryo *in vivo*. Dev. Biol. *71:* 100–114.

Coons, A. M. (1968) Fluorescent antibody methods. In *General Cytochemical Methods*, J. F. Danielli, ed., pp. 399–422, Academic Press, Inc., New York.

Dahl, D., D. C. Rueger, A. Bignami, K. Weber, and M. Osborn (1981) Vimentin, the 57,000 dalton protein of fibroblast filaments, is the major cytoskeletal component in immature glia. Eur. J. Cell Biol. *24:* 191–196.

Delacourte, A., G. Filliatreau, F. Boutteau, G. Biserte, and J. Schreud (1980) Study of the 10-nm filament fraction isolated during the standard microtubule preparation. Biochem. J. *191:* 543–546.

Dellagi, K., J. C. Brouet, J. Perreau, and D. Paulin (1982) Human monoclonal IgM with autoantibody activity against intermediate filaments. Proc. Natl. Acad. Sci. U. S. A. *79:* 446–450.

Dräger, U. C. (1983) Coexistence of neurofilaments and vimentin in a neurone of adult mouse retina. Nature *303:* 169–172.

Drews, U. (1975) Cholinesterase in embryonic development. In Prog. Histochem. Cytochem. *7:* 1–52.

Duband, J. L., and J. P. Thiery (1982) Distribution of fibronectin in

the early phase of avian cephalic neural crest cell migration. Dev. Biol. *93:* 308–323.

Enemar, A., B. Falck, and R. Hakanson (1965) Observations on the appearance of norepinephrine in the sympathetic nervous system of the chick embryo. Dev. Biol. *11:* 268–283.

Eng, L. F., and J. W. Bigbee (1978) Immunohistochemistry of nervous system specific antigens. In *Advances in Neurochemistry*, B. W. Agranoff, and M. H. Aprison, eds., Vol. 3, pp. 43–98, Plenum Press, New York.

Fauquet, M., J. Smith, C. Ziller, and N. M. Le Douarin (1981) Differentiation of autonomic neuron precursors *in vitro*: Cholinergic and adrenergic traits in cultured neural crest cells. J. Neurosci. *1:* 478–492.

Franke, W. W., E. Schmid, S. Winter, M. Osborn, and K. Weber (1979) Widespread occurrence of intermediate filaments of the vimentin-type in cultured cells from diverse vertebrates. Exp. Cell Res. *123:* 25–46.

Franke, W. W., C. Grund, C. Kuhn, B. W. Jackson, and K. Illmensee (1982a) Formation of cytoskeletal elements during mouse embryogenesis. III. Primary mesenchymal cells and the first appearance of vimentin filaments. Differentiation *23:* 43–59.

Franke, W. W., E. Schmid, D. L. Schiller, S. Winter, E. D. Jarasch, R. Moll, H. Denk, B. W. Jackson, and K. Illmensee (1982b) Differentiation-related patterns of expression of proteins of intermediate-size filaments in tissues and cultured cells. Cold Spring Harbor Symp. Quant. Biol. *46:* 431–454.

Gambetti, P., L. Autilio-Gambetti, and S. C. Papasozomenos (1981) Bodian's silver method stains neurofilament polypeptides. Science *213:* 1521–1522.

Goldstein, M. E., L. A. Sternberger, and N. H. Sternberger (1983) Microheterogeneity ("neurotypy") of neurofilament proteins. Proc. Natl. Acad. Sci. U. S. A. *80:* 3101–3105.

Gown, A. M., and A. M. Vogel (1982) Monoclonal antibodies to intermediate filament proteins of human cells. Unique and cross-reacting antibodies. J. Cell Biol. *95:* 414–424.

Granger, B. L., and E. Lazarides (1983) Expression of the major neurofilament subunit in chicken erythrocytes. Science *217:* 553–556.

Hoffman, P. N., and R. J. Lasek (1975) The slow component of axonal transport. Identification of major structural polypeptides of the axon and their generality among mammalian neurons. J. Cell Biol. *66:* 351–366.

Holtzer, H., G. S. Bennett, S. J. Tapscott, J. M. Croop, and Y. Toyama (1982) Intermediate-size filaments: Changes in synthesis and distribution in cells of the myogenic and neurogenic lineages. Cold Spring Harbor Symp. Quant. Biol. *46:* 317–330.

Houle, J., and S. Fedoroff (1983) Temporal relationships between the appearance of vimentin and neural tube development. Dev. Brain Res. *9:* 189–196.

Jacobs, M., Q. L. Choo, and C. Thomas (1982) Vimentin and 70 K neurofilament protein co-exist in embryonic neurones from spinal ganglia. J. Neurochem. *38:* 969–977.

Jacobson, M. (1978) *Developmental Neurobiology*, Ed. 2, Plenum Press, New York.

Lane, E. B., B. L. M. Hogan, M. Kurkinen, and J. I. Garrels (1983) Co-expression of vimentin and cytokeratins in parietal endoderm cells of early mouse embryo. Nature *303:* 701–704.

Lazarides, E. (1980) Intermediate filaments as mechanical integrators of cellular space. Nature *283:* 249–256.

Lee, V., H. L. Wu, and W. W. Schlaepfer (1982) Monoclonal antibodies recognize individual neurofilament triplet proteins. Proc. Natl. Acad. Sci. U. S. A. *79:* 6089–6092.

Lehtonen, E., V.-P. Lehto, R. Paasivuo, and I. Virtanen (1983) Parietal and visceral endoderm differ in their expression of intermediate filaments. EMBO J. *2:* 1023–1028.

Liem, R. K. H., S. Yen, G. D. Salomon, and M. L. Shelanski (1978) Intermediate filaments in nervous tissue. J. Cell Biol. *79:* 637–645.

Lyser, K. M. (1964) Early differentiation of motor neuroblasts in the chick embryo as studied by electron microscopy. I. General aspects. Dev. Biol. *10:* 433–466.

Lyser, K. M. (1966) The development of the chick embryo diencephalon and mesencephalon during the initial phases of neuroblast differentiation. J. Embryol. Exp. Morphol. *16:* 497–517.

Mazurkiewicz, J. E., and P. K. Nakane (1972) Light and electron microscopic localization of antigens in tissues embedded in polyetheylene glycol with a peroxidase-labeled antibody method. J. Histochem. Cytochem. *20:* 969–974.

Paterson, A. M. (1890) Development of the sympathetic nervous system of mammals. Philos. Trans. *181:* 159–186.

Paulin, D., C. Babinet, K. Weber, and M. Osborn (1980) Antibodies as probes of cellular differentiation and cytoskeletal organization in the mouse blastocyst. Exp. Cell. Res. *130:* 297–304.

Paulin, D., H. Jakob, F. Jacob, K. Weber, and M. Osborn (1982) *In vitro* differentiation of mouse teratocarcinoma cells monitored by intermediate filament expression. Differentiation *22:* 90–99.

Prochiantz, A., A. Delacourte, M. C. Daguet, and D. Paulin (1982) Intermediate filament proteins in mouse brain cells cultured in the presence or absence of fetal calf serum. Exp. Cell. Res. *139:* 404–409.

Pruss, R. M., R. Mirsky, M. C. Raff, R. Thorpe, A. J. Dowding, and B. H. Anderton (1981) All classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody. Cell *27:* 419–428.

Raju, T., A. Bignami, and D. Dahl (1981) *In vivo* and *in vitro* differentiation of neurons and astrocytes in the rat embryo. Immunofluorescence study with neurofilament and glial filament antisera. Dev. Biol. *85:* 344–357.

Ramón y Cajal, S. (1909) *Histologie du Système Nerveux de l'Homme et des Vertébrés*, Tome I. Consejo Superior de Investigationes Cientificas, Madrid, 1972.

Rothman, T. P., M. D. Gershon, and H. Holtzer (1978) The relationship of cell division to the acquisition of adrenergic characteristics by developing sympathetic ganglion cell precursors. Dev. Biol. *65:* 322–341.

Rothman, T. P., L. A. Specht, M. D. Gershon, T. H. Joh, G. Teitelman, V. M. Pickel, and D. J. Reis (1980) Catecholamine biosynthetic enzymes are expressed in replicating cells of the peripheral but not the central nervous system. Proc. Natl. Acad. Sci. U. S. A. *77:* 6221–6225.

Rugh, R. (1968) *The Mouse—Its Reproduction and Development*, Burgess, Minneapolis.

Sastry, B. V. R., and C. Sadavongvivad (1979) Cholinergic systems in non-nervous tissues. Pharmacol. Rev. *30:* 65–132.

Schlaepfer, W. W., and L. A. Freeman (1978) Neurofilament proteins of rat peripheral nerve and spinal cord. J. Cell Biol. *78:* 653–662.

Schnitzer, J., W. W. Franke, and M. Schachner (1981). Immunocytochemical demonstration of vimentin in astrocytes and ependymal cells of developing and adult mouse nervous system. J. Cell Biol. *90:* 435–447.

Shaw, G., and Weber, K. (1982) Differential expression of neurofilament triplet proteins in brain development. Nature *298:* 277–279.

Smith, J., M. Fauquet, C. Ziller, and N. M. Le Douarin (1979) Acetylcholine synthesis by mesencephalic neural crest cells in the process of migration *in vivo.* Nature *282:* 853–855.

Tapscott, S. J., G. S. Bennett, and H. Holtzer (1981a) Neuronal precursor cells in the chick neural tube express neurofilament proteins. Nature *292:* 836–838.

Tapscott, S. J., G. S. Bennett, Y. Toyama, F. Kleinbart, and H. Holtzer (1981b) Intermediate filament proteins in the developing chick spinal cord. Dev. Biol. *86:* 40–54.

Teitelman, G., H. Baker, T. H. Joh, and D. J. Reis (1979) Appearance of catecholamine-synthesizing enzymes during development of rat sympathetic nervous system. Possible role of tissue environment. Proc. Natl. Acad. Sci. U. S. A. *76:* 509–513.

Teitelman, G., M. D. Gershon, T. P. Rothman, T. H. Joh, and D. J. Reis (1981) Proliferation and distribution of cells that transiently express a catecholaminergic phenotype during development in mice and rats. Dev. Biol. *86:* 348–355.

Tello, J. F. (1923) Les differenciations neuronales dans l'embryon du poulet pendant les premiers jours de l'incubation. Trav. Lab. Rech. Biol. Univ. Madrid *21:* 1–93.

Tello, J. F. (1925) Sur la formation des chaînes primaire et secondaire du grand sympathique dans l'embryon de Poulet. Trav. Lab. Rech. Biol. Univ. Madrid *23:* 1–28.

Thiéry, J. P., J.-L. Duband, and A. Delouvée (1982) Pathways and mechanisms of avian trunk crest cell migration and localization. Dev. Biol. *93:* 324–343.

Towbin, H., T. Staehelin, and J. Gordon (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets:

Procedure and some applications. Proc. Natl. Acad. Sci. U. S. A. *76:* 4350–4354.

Willard, M., and C. Simon (1981) Antibody decoration of neurofilaments. J. Cell Biol. *89:* 198–205.

Windle, W. F., and M. F. Austin (1936) Neurofibrillar development in the central nervous system of chick embryos up to 5 days' incubation. J. Comp. Neurol. *63:* 431–463.

Windle, W. F., and R. E. Baxter (1936) The first neurofibrillar development in albino rat embryos. J. Comp. Neurol. *63:* 173–187.

Ziller, C., E. Dupin, P. Brazeau, D. Paulin, and N. M. Le Douarin (1983) Early segregation of a neuronal precursor cell line in the neural crest as revealed by culture in a chemically defined medium. Cell *32:* 627–638.