Temporal and Topographic Relationships Between the Phosphorylated and Nonphosphorylated Epitopes of the 200 kDa Neurofilament Protein During Development *in vitro*

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The ontogeny of the triplet of neurofilament proteins (NF), and the phosphorylated and nonphosphorylated derivatives of the 200 kDa neurofilament subunit (NF200P, NF200D) have been investigated in dissociated cultures prepared from gestational day 13 mouse spinal cord and dorsal root ganglia (DRG), using immunocytochemical methods.

Neurofilament-like immunoreactivity (NF-LI), as detected with antiserum, occurred in the somata and processes of all neurons from day 1 in culture, and reached a maximum density and intensity at days 16–20. The first labeling of neurons by NF200D antibodies occurred at day 3, and was confined to DRG cells. Only a small, proximal portion of the axons from these cells exhibited NF200D-LI. At later stages, however, this immunoreactive region extended to include progressively more distal parts. Spinal cord neurons first became NF200D-positive at day 9; however, many NF200D-negative neurons still remained in mature cultures. Also at these later stages, some axons were stained for less than their full length with the NF200D antibody. NF200P-LI was first apparent at day 17, in smooth and varicose axons and only where NF-LI was also present. In contrast, NF200P- and NF200D-LI were usually localized in mutually exclusive populations of axons and other fibers. In some, predominantly thick axons, however, the proximal segment was NF200D-positive, whereas the distal part exhibited solely NF200P-LI.

In contrast to NF70 and NF150, the 200 kDa neurofilament is dilatory in its appearance in most neurons in culture. The development of the nonphosphoderivative precedes that of the phosphoderivative, and the respective ontogenies are specific for different neuronal types. Posttranslational phosphorylation of NF200 seems therefore to occur at a later stage of development than the induction of NF200 itself, while there is a wide variation in its rates of phosphorylation during passage down different axons.

The family of intermediate filaments found in neurons has been shown to comprise at least 3 proteins, of 70, 150, and 200 kDa (Liem et al., 1978; Schlaepfer and Freeman, 1978). The last protein, NF200, exists in various degrees of phosphorylation (Julien and Mushynski, 1982), and these phosphoproteins (NF200P) may be selectively discriminated with monoclonal antibodies (Sternberger and Sternberger, 1983; Carden et al., 1985; Dahl and Bignami, 1985). Since the phosphorylated epitopes of NF200 were found only in axons of mature neurons, but were absent from immature axons *in vivo* (Dahl and Bignami, 1985), it has been suggested that a posttranslational modification of nascent NF200, e.g., phosphorylation, renders this subunit recognizable to the NF200P antibodies. This hypothesis could explain the discordance between several groups concerning the time of development of NF200 (Tapscott et al., 1981; Shaw and Weber, 1982; Willard and Simon, 1983; Cochard and Paulin, 1984; Pachter and Liem, 1984), the variation being due to the ability of the antibody to recognize either the phosphorylated or nonphosphorylated forms. However, there is no evidence as yet that the posttranslational phenomenon does indeed follow NF200 expression.

We have used antibodies directed against the phospho- and nonphosphoderivatives of NF200 and compared their evolution in dissociated cultures of mouse spinal cord and dorsal root ganglia (DRG). In this way it has been possible to compare the spatial and temporal relationship between NF200P and NF200D, and thus to shed light on the mechanisms of their respective ontogenies.

Materials and Methods

Tissue culture

Dissociated cultures of mouse spinal cord with DRG were produced as previously described (Ransom et al., 1977). Briefly, the spinal cords, complete with the attached DRG, were dissected from embryonic C57BL/6J mice at day 13 of gestation and treated with 0.1% trypsin at 37°C for 30 min. The tissue was dissociated by trituration through a flame-polished pasteur pipette, and the cell density adjusted to 2.5×10^5 cells/ml, then plated onto 35 mm dishes previously coated with collagen. Five days after plating, cultures were treated with FuDR for 4 d in order to halt the division of glial cells. Cultures were fed twice a week with minimum essential medium with Earle's salts (MEM) and supplemented with 5% horse serum, glutamine, and N_3 (Romijn et al., 1982).

Immunocytochemistry

Polyclonal antiserum against neurofilament was raised in the rabbit against a chick brain antigen (Dahl and Bignami, 1977). Immunoaffinity

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Figure 1. Immunoperoxidase labeling of NF200P- (*A*), NF- (*B*) and NF200D-LI (*C,D*) in dissociated cultures of mouse spinal cord and dorsal root ganglia. At day 1 after plating (*A–C*), there is moderate to intense staining of all neurons with NF antibodies (*B*). Antibodies directed against NF200P or *D*, however, gave no specific staining at day 1 (*A* and *C*, respectively). The weak, artifactual labeling of neuronal somata with the NF200P antibody was unaffected by prior phosphatase treatment. At day 3, the first detectable NF200-LI in neurons was apparent, but could only

chromatography and immunostaining of gels have previously shown that the antiserum reacted specifically with all 3 NFs (Dahl et al., 1984). The same antigen was used to raise monoclonal antibodies in mice. The monoclonal antibody to NF200P used in the present study was shown to react specifically with the 200 kDa NF by immunoblotting (Bignami et al., 1985). In addition, since prior incubation of tissue sections with phosphatase (in the presence of a protease inhibitor) abolished staining with the antibody, the antibody was considered to recognize only the phosphorylated form of the NF200 protein (Dahl and Bignami, 1985).

Monoclonal antibodies against NF200D were prepared by immunization of mice with phosphatase-treated rat NF200 kDa excised from gels, and subsequent fusion of the spleen cells with the mouse myeloma cell line (SP2/0-Ag14), as described earlier (Lee et al., 1982; Schlaepfer et al., 1984). Analysis by immunoblotting using rat spinal cord extracts demonstrated that the antibody recognized only NF200, and only in the nonphosphorylated form (Lee et al., 1987).

At various times after plating, cultures were rinsed with MEM at 37°C for 2 min, and then fixed for 30 min with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at room temperature. The dishes were washed 3 times with 0.01 M PBS (pH 7.4) containing 0.9% NaCl over a period of 15 min. PBS containing 20% normal goat serum (for subsequent NF staining) or 20% normal horse serum (for NF200P or NF200D staining), and 0.1% Triton was applied for 1 hr at room temperature. Following a brief rinse in PBS, the cultures were incubated overnight at 4°C with primary antibodies against either NF (diluted 1:1000 in PBS containing 0.1% Triton and 0.1% sodium azide), NF200P (diluted 1:10), NF200D (diluted 1:100), or with a mixture of either NF + NF200D (final dilutions, 1:100 and 1:10, respectively), of NF + NF200P (final dilutions, 1:100 and 1:2, respectively). Cultures were also stained sequentially for NF200P (1:10), followed by NF200D (1:100 or 1:10). After rinsing 3 times in PBS, the cultures were processed either for peroxidase staining, using the avidin-biotin complex (ABC) technique, or for immunofluorescence, using the methods of Coons and collaborators (Coons, 1958).

ABC peroxidase. Biotinylated goat anti-rabbit immunoglobulins (1:200 in PBS) or biotinylated horse anti-mouse immunoglobulins (1:200) were applied for 2 hr at room temperature to cultures previously incubated, respectively, with antibodies against NF, against NF200D, or against NF200P. After being rinsed in PBS, the dishes were incubated with avidin linked to biotinylated peroxidase (1:100; Vector Laboratories) for 2 hr at room temperature, rinsed again, and then the diaminobenzidine (0.75 mg/ml)-hydrogen peroxide (0.03%) mixture was added for 5 min. Following 3 rinses in Tris-buffered saline (TBS) (pH 7.4), the cultures were osmicated (0.1% OsO₄ in 0.1 M cacodylate buffer), dehydrated through an ascending series of ethanol, and embedded in Epon. The stained cultures were viewed in a Zeiss light microscope, and micrographs taken on Panatomic-X film.

Immunofluorescent simultaneous double-labeling. Dual-labeled cultures were rinsed in PBS and then incubated at 37°C for 30 min with a mixture of tetramethyl rhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit immunoglobulins (1:300; Boehringer, W. Germany) and Fab' fragments of anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (FITC) (1:10, Cappel, USA) in PBS containing 0.1% Triton. After being rinsed 3 times in PBS, the cultures were immersed in glycerol/phosphate (5:1) containing 0.2% *p*-phenylenediamine to prevent fading of the fluorescence.

Immunofluorescence sequential double-labeling. If one wishes to study the localization of 2 antibodies raised in the same species within the same tissue section or culture dish, coincubations with the 2 antibodies are obviously unsuccessful. A sequential staining protocol was therefore used. Antibody against NF200P (diluted 1:10) was applied, followed by two 1 hr incubations with biotinylated horse anti-mouse antibodies (1:200 in PBS). Avidin linked to FITC (1:200) was then added for 2 hr at room temperature, rinsed, and, after blocking of the unoccupied avidin binding sites with excess biotin, cultures were incubated with either biotinylated horse anti-mouse antibodies followed by rhodamine 600 conjugated to avidin (1:200; Vector, USA), or rhodamine-labeled goat anti-mouse antibodies (1:300; Boehringer). The staining was examined under illumination for both fluorescein and rhodamine to ensure that

no structures were as yet labeled by the latter fluorophore. Antibodies against NF200D (1:100 or 1:10) were added to the dishes for 18 hr at 4°C and, after rinsing in PBS, either biotinylated horse anti-mouse antibodies and then rhodamine 600 conjugated to avidin or rhodamine-linked goat anti-mouse was applied as before. The dishes were finally rinsed in PBS, and immersed in *p*-phenylenediamine antifading solution. The cultures were viewed in a Zeiss fluorescence microscope fitted with epi-illumination and equipped with filter sets BP546/12 and LP590 for rhodamine, and BP450-490 and LP520 for fluorescein. Micrographs were taken on Agfapan film.

Controls

Control experiments included incubation of cultures with normal rabbit serum in place of the neurofilament antibody, or with PBS instead of the NF200P or NF200D monoclonal antibodies. No immunostaining was seen under these conditions (Fig. 8D). At all stages in culture, when either NF200D- or NF200P-LI was not displayed, more mature cultures (approximately 4 weeks old) were also stained in the same experiment and used as positive controls. In experiments involving simultaneous double-labeling, lack of cross-reactivity between the secondary antibodies was confirmed by the disappearance of rhodamine labeling when no NF antibodies were applied, and of fluorescein labeling after the omission of NF200P or NF200D antibodies.

In order to test whether the NF200P antibody was directed against a phosphorylated epitope, cultures were fixed as before, rinsed in PBS with 0.1% Triton for 15 min, and then incubated for 3 hr with 1.5 U/ml of phosphatase from phosphatase from *E. coli*, type III-N (Sigma) in Tris (pH 8.0)-buffered saline (TBS) containing 1 mM ZnSO₄, at 37°C. Following rinsing in TBS + 1 mM EDTA, the cultures were processed for immunocytochemistry as before.

Results

Days 1-4 in culture

At day 1 in culture, all multipolar, phase-bright, neuronlike cells also contained neurofilament-like immunoreactivity (NF-LI) (Fig. 1B). In addition, many cells displayed smooth NF-immunoreactive processes that extended several hundred microns.

At the same time, but in sister cultures, no immunostaining against the nonphosphorylated epitope of the 200 kDa NF (NF200D) was detectable (Fig. 1C). By day 3, however, the first NF200D-containing structures had appeared (Fig. 1D). Intense staining was found in large cells (20-30 μ m in diameter) that had an eccentric nucleus and were either bipolar or multipolar. These morphological characteristics are typical only of DRG neurons at this stage in culture. The narrow diameter of the processes containing NF200D-LI was consistent with this identification. Despite the observation that the DRG neurons displayed processes stained for NF-LI that were several millimeters long, the labeling of NF200D-LI extended only 10-20 μ m from the perikaryon. By day 4, though, the segment of the process containing NF200D-LI had increased in length to approximately 300-400 μ m. Phosphatase pretreatment of the cultures did not affect the profile of NF200D-LI.

No specifically stained NF200P-immunoreactive structures were visible in cultures at days 1 (Fig. 1A) and 4.

Days 5-9 in culture

The proximal part of the DRG fibers continued to contain both NF-LI and NF200D-LI, whereas in the more distal regions, only NF-LI was apparent (Fig. 2, A, B, D). However, the proportion

be observed with antibodies against the nonphosphorylated form (D): no NF200P-LI could be visualized in sister cultures at the same age. NF200D-LI at day 3 was contained only in presumed DRG neuronal somata (arrows), but not their processes (arrowheads). Scale bar, 50 μ m. Magnification was the same for all micrographs.

Figure 2. Immunoperoxidase labeling of NF200D- (*A–C*) and NF-LI (*D*) in dissociated cultures of mouse spinal cord and DRG. At day 5 after plating, DRG neuronal somata are the most intensely stained cells using NF200D antibodies (*A*; higher magnification in *B*). In addition, the proximal portion of the processes emanating from these cells is now NF200D-positive, although the distal part remains unstained. Some spinal cord cells, most notably, but not exclusively, those near labeled DRG neurons, are also NF200D-immunoreactive (arrows). At day 9 (*C*), NF200D-

Figure 3. Immunoperoxidase labeling of NF200P- (*A*), NF200D- (*B*), and NF-LI (*C*) in dissociated cultures of spinal cord and DRG at day 17 after plating. NF200P-LI was seen for the first time at day 17 (*A*) in fibers in and around neuronal clusters. No specifically immunoreactive somata were visible, however. In sister cultures at the same age, NF200D-LI was much more extensive (*B*). Dense networks of fibers and intensely stained large and medium-sized spinal cord cells were now visible. Comparison with sister cultures labeled with NF antibodies revealed that despite their containing NF-LI, most small neurons were still NF200D-negative. Scale bar, 20 μ m in *A*, 50 μ m in *B* and *C*.

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LI is now apparent in many neurons other than those derived from the DRG (*arrows*). Note the pseudo-unipolar cell (*large arrow*), typical of the mature DRG neurons *in situ*. Despite the increase in NF200D-LI, sister cultures at the same age (*D*) displayed more extensively distributed NF-LI: all neurons were immunoreactive to NF antibodies at this stage. Scale bar, 50 μ m in *A*, *C*, and *D*, 20 μ m in *B*.

Figure 4. Immunoperoxidase labeling of NF200D- (*A–C*) and NF200P-LI (*D*) in dissociated cultures of mouse spinal cord and DRG at day 28 after plating. Intensely stained NF200D-immunoreactive neurons exist in the same areas of the culture dish as totally unlabeled neuronal somata (arrows) (*A*). At the same age (*B*), axons emanating from, for example, large spinal cord neurons (arrows) contain NF200D-LI for long distances (arrowheads). However, in the same dish, fibers emerging from presumed flattened DRG cells (large arrow) or spinal cord cells (small arrow) exhibit decreased NF200D-LI at their peripheral parts (*C*). A higher-power micrograph of NF200P-LI in sister cultures at the same age shows a rich network of immunoreactive axons, ramifying in all parts of the culture dish (*D*). Note the bifurcating axon (arrowheads). NF200P-LI could not be demonstrated in neuronal dendrites. Scale bar, 50 μ m in *A–C*, 20 μ m in *D*.

in which both antigens were present had increased substantially over earlier stages. These NF200D-immunoreactive processes had proliferated markedly by days 8 and 9. In places, especially near the cells of origin, a moderately dense network of fibers was visualized. In addition, by day 9 (Fig. 2*C*), many smaller cell bodies (10–12 μ m in diameter) were now exhibiting weak NF200D-LI, although their processes were unstained. The neurons were most obvious where they were aggregated in clusters;

no isolated single cells, apart from DRG, displayed NF200D-LI. NF200P-LI was still undetectable at these stages in culture.

Days 10–15 in culture

By days 10–12 in culture, the full length of the DRG axons, as visualized by NF antibodies, was also NF200D-positive. Over the same time period, many more cell bodies became NF200D-immunoreactive and stained progressively more intensely. They

Figure 5. Immunofluorescence photomicrographs of NF200P- and NF-LI in the same field in dissociated cultures of mouse spinal cord and DRG at day 17 after plating. Individual NF200P-LI positive axons were also NF-immunoreactive (arrows). Other NF-positive axons of similar intensity were, however, NF200P-negative (arrowheads). Scale bar, 20 μ m. Magnification was the same for both micrographs.

still comprised, however, a minority of the total number of neurons, as determined not only by simultaneous dual-labeling with NF polyclonal antibodies, but also by their brightness under conditions of phase contrast. Between days 13 and 15, many strongly NF-positive neurons had adopted the morphology of typical large multipolar spinal cord cells. They were 25–35 μ m in diameter and exhibited thick dendritic processes, which divided several times close to the soma. Other new cell morphologies were also found. For instance, a few large, flat, and strongly NF-immunopositive cells appeared at day 15. These were thought to be DRG neurons on the basis of the size and number of the thin processes that they sent out. An example of such a cell, but at a later stage in culture, is shown in Figure 4C. Also over days 13–15, a large increase in the number of perikarya containing NF200D-LI occurred, such that by the latter stage, about 50% of all neuronal somata were immunopositive. Principally, these neurons were lightly stained, bipolar or multipolar, and had a diameter of 10–14 μ m. They occurred both singly and in groups of 5–15 somata. In addition, a number of the large, multipolar spinal cord neurons, having somata 25–30 μ m in diameter and exhibiting multiply branching dendrites, were also observed. Many of these were almost as densely stained as the DRG neurons. Finally, bipolar neurons, with a diameter of 5 μ m, were also apparent, possessing long, undivided processes. No NF200P-immunoreactive structures could be perceived in cultures up to and including day 15.

Days 16–24 in culture

The development of NF-LI in cultures was essentially complete by days 16–20 (Fig. 3C), although the number of neurons displaying the morphology of flattened cells and of the large, multipolar spinal cord type apparently increased. Nonetheless, the total number of NF-positive somata declined by 13% between days 15 and 20 in culture, but the density of fiber networks remained approximately the same. In contrast, the increase in the number of NF200D-positive neurons continued apace. By days 17 (Fig. 3B) and 18, for example, approximately 70% of the neuronal somata were immunoreactive, and the meshwork

of labeled dendrites and axons was now extremely dense. Nevertheless, no varicose fibers containing NF200D-LI were demonstrable.

At day 17 in culture, the first NF200P-immunoreactive structures were observed (Fig. 3A). These were occasional, smooth neuronal processes, coursing either within one of the fascicles between the neuronal clusters or across unoccupied space (Fig. 5A). Their length was greater than several hundred microns, and they branched only rarely, which suggests that they were axons. The cells of their origin, however, could not be discerned. By day 22, it was possible to visualize NF200P-positive varicose axons. Incubation of fixed cultures with phosphatase prior to immunocytochemical processing completely abolished all axonal labeling. Incubation with TBS alone, on the other hand, failed to attenuate NF200P-LI.

Double-labeling at day 17 and at subsequent stages revealed that NF200P- and NF200D-LI were found only in processes that were also strongly immunoreactive to antibodies against the neurofilament triplet (Fig. 5, A, B). Conversely, there were many NF-positive axonal and dendritic processes that failed to display either one or both epitopes of NF200.

Days 25–56 in culture

At day 25 in culture, there remained a conspicuous absence of NF200D-positive varicose fibers—only smooth processes could be visualized. Moreover, many NF-positive processes were still NF200D-negative (Fig. 6, A, B). Strikingly, in contrast to earlier stages, where the full length of axons appeared to contain NF200D-LI, from day 25 onwards the more distal extent of some was either much more weakly NF200D-positive or entirely negative. Thick axons, mainly from large spinal cord cells, displayed this phenomenon best, although a similar declining gradient of NF200D-LI could also be visualized in medium axons, such as from DRG, and in a few thin axons. The distance from the large spinal cord cell body to the point at which a reduction in staining could be substantiated varied from about 100 μ m to over 1000 μ m (Fig. 4, B, C). This range of distances was similar at all stages after day 25. However, the number of

Figure 6. Immunofluorescent labeling of NF200D- (*A*, *C*, *E*), NF200P- (*D*, *F*), and NF-LI (*B*) in dissociated cultures of mouse spinal cord and dorsal root ganglia at day 37 after plating. *A* and *B*, *C* and *D*, and *E* and *F* are, respectively, of identical fields in the same cultures, stained for 2 different antigens. Even at the late stage in culture, many NF-positive cells and fibers (*B*) still fail to display NF200D-LI (*A*). *C* and *D* show the same field from a culture labeled for NF200D and NF200P-LI, respectively, and demonstrate the lack of cross-reactivity between the 2 antibodies, despite their being raised in the same species. Many fibers are NF200P-positive/NF200D-negative (arrowheads in *C*) and vice versa (arrowheads in *D*). The proximal axon of the cell arrowed in *E* is NF200D-immunoreactive (arrowheads), but NF200P-negative (*F*). More distally, however, NF200D-LI decreases, whereas NF200P-LI becomes clearly visible (arrowheads in *F*). Scale bar, 50 μ m in *A*–*D*, 42.5 μ m in *E* and *F*.

Figure 7. Immunofluorescence photomicrographs of NF200P-LI (*A*) and NF200D-LI (*B*) of identical fields in a dissociated culture of mouse spinal cord and DRG at day 37 in culture after plating. *P* indicates the end of an axon proximal to the cell body, which is NF200D-immunoreactive but NF200P-negative (arrowheads in *B* only). More distally is an extensive region of the axon in which both NF200P-LI and NF200D-LI are visible (arrowheads in *A* and *B*). More distally still, NF200D-LI can no longer be detected, whereas the axon remains NF200P-immunoreactive (arrowheads in *A* only). Scale bar, 50 μ m. Magnification was the same for both micrographs.

Figure 8. Effects of prior phosphatase treatment on NF200P- (*A*, *B*) and NF200D-LI (*C*) in dissociated cultures of mouse spinal cord and DRG at day 37 after plating. Labeling of NF200P-LI is seen in axons in untreated cultures (*A*). In addition, weak staining of cell bodies is apparent. However, pretreatment with phosphatase abolished the axonal staining in sister cultures, but not in the somata (*B*). The latter was therefore considered to be artifactual. Prior phosphatase had no effect on NF200D-LI (*C*). Cultures processed for immunocytochemistry with PBS in place of the primary antibody exhibit no labeling of any structures (*D*). Scale bar, 50 μ m. Magnification was the same for all micrographs.

axons exhibiting this distal decrement in NF200D-LI increased with time in culture, such that at days 48 and 56 it was observed in most of the thick axons for which the proximal and distal regions could be unequivocally delineated. In the DRG neurons, however, staining was occasionally restricted to the cell body and initial segment, whereas in many others it extended several hundred microns (Fig. 4*C*), even at day 56. Tracing the fine fibers in order to determine their distal and proximal parts was impossible in the extremely dense networks of other NF200D-immunoreactive processes.

The number and staining intensity of NF200P-immunoreactive processes continued to increase (Fig. 4*D*). The labeling was confined to axons alone, the somata and dendrites remaining unstained. In some cases it was possible to locate the negative cell body and initial segment of a NF200P-positive axon.

In view of the reciprocity of NF200P and NF200D staining in some processes, cultures were labeled with both antibodies. It was then apparent that some fibers were NF200P-positive, NF200D-negative for their whole length (Fig. 6, *C*, *D*); others were NF200P-negative, NF200D-positive in their entirety (Fig.

6, C, D); while yet others displayed solely NF200D-LI in their proximal segment, only NF200P-LI in their distal extreme, and colocalized the 2 antigens in an intermediate region of about 500 μm in length (Figs. 6, E, F; 7, A, B). This alteration in the phosphorylation state of NF200 was most prominent in the thick axons emerging from spinal cord cells.

At the latest stage in culture investigated (day 56), NF200D-LI was still found in many axons, but only of the nonvaricose type. On the other hand, NF200P-LI occasionally existed in beaded axons, as well as, more commonly, in smooth fibers.

Also, at day 56, no neuronal somata containing NF200P-LI were detectable. There was, however, a weak staining of cell bodies, as at all ages from day 1 in culture, but since this was unaffected by prior phosphatase treatment, it was considered not to be due to NF200P (Fig. 8, A, B). Conversely, the staining in axons obtained with NF200P antibody was completely abolished by pretreatment with phosphatase (Fig. 8, A, B).

The number of somata containing NF200-LI continued to increase with time; by day 56, approximately 85–90% of all neuronal cell bodies were NF200D-immunoreactive. Both small (5 μm in diameter) and medium-sized (10–14 μm) phase-bright somata were included among the unstained cell bodies.

Discussion

The development of the neurofilament protein triplet and of the phosphorylated and nonphosphorylated derivatives of NF200 have been analyzed immunocytochemically in dissociated cultures of mouse spinal cord and dorsal root ganglia, using polyclonal and monoclonal antibodies against various neurofilament epitopes.

Using antisera to the triplet of NF, immunostaining was apparent after 1 d in culture. The NF-LI was thought to be due to the 70,000 and/or 150,000 *M*_r proteins, since results from the present experiments have demonstrated that neither NF200P nor NF200D exhibits detectable immunostaining at days 1 and 2 in culture, a period when all neuronal somata are moderately to strongly NF-immunoreactive.

Of the many epitopes of NF200, the nonphosphorylated form was the first to appear in culture, at day 3. It is possible that other, phosphorylated, epitopes were also present at this stage, but were undetected by the NF200P monoclonal antibody used in the present study. However, it is unlikely that the same NF200D antibody can react with both the phosphorylated and dephosphorylated epitopes (Carden et al., 1985). Furthermore, phosphatase digestion failed to enhance the intensity or extent of NF200D-immunoreactive neurons at any age, indicating that no phosphorylated epitopes of NF200 were being exposed by digestion. Extremely intense staining was observed in presumptive DRG neuronal somata over days 3–12, whereas no other neurons were stained. At subsequent stages, large spinal cord neurons became NF200D-positive, and later, many medium and small-sized neurons also developed NF200D-LI. In the last stage in culture examined, approximately 10–15% of the neurons remained unlabeled by NF200D antibody. It was apparent, therefore, that the ontogeny of NF200D-LI differed from one neuronal type to another. This variation was not dependent on size alone; DRG and large spinal cord neurons, for example, display markedly different dates of evolution of NF200D-LI within their perikarya, despite the similarity in their somatic diameter. The possibility that the relative detectability of NF200D staining results from an artifact of the size of neurons can, therefore, probably be excluded. Presumably, then, some of the factors that normally determine the induction of NF200D

in vivo are still functioning *in vitro*; it is not clear at present, however, whether these factors are controls inherent to the neuron itself or trophic influences present in the culture medium.

Some diversity in the ontogeny of NF200 has been previously reported (Tapscott et al., 1981; Shaw and Weber, 1982; Willard and Simon, 1983; Cochard and Paulin, 1984; Pachter and Liem, 1984). One explanation for this inconsistency is that some antibodies were directed against a derivative of NF200, rather than the native molecule itself (Dahl and Bignami, 1985). Our results indicate that, although this may also have been the case, considerable variation is found in the development of the non-phosphorylated epitope of NF200 even when the same antibody is used. In other words, the date of development of NF200D is dependent on neuronal type.

Although the processes of the NF200D-positive neurons were also stained, the extent and intensity of immunoreactivity varied with time. Despite the extremely strong NF200D-LI visualized in DRG cell bodies at day 3, the greater part of each axon was immunonegative—only the most proximal 10–20 μm was labeled—whereas the NF-LI could be traced in the same fibers for at least 300–400 μm . The proportionate length of the DRG process containing NF200D-LI increased until about days 10–12, by which time its full extent was immunoreactive. These observations indicate that the induction of NFs is not unitarily controlled, and that the NF200, for example, can appear independently of either NF70 or NF150, even within the same neuron. At later stages in culture, there was in some axons a reversal of this distally directed propagation of NF200D-LI, such that by day 48, the NF200D-positive region comprised merely the dendrites, soma, and the initial 500 μm segment of axons, most notably those arising from large spinal cord cells. However, many of the same cell type still displayed NF200-LI for the full length of their axons, i.e., 2–3 mm. In addition, very dense plexuses of NF200D-positive axons were still found throughout the culture, even at the last stage examined, indicating that this apparent regression of NF200D-LI was not a ubiquitous phenomenon. Since a similar attenuation of NF-LI in axons could not be observed over the same time period, it was concluded that the decline in axonal NF200D-LI possibly reflected, not a nonspecific deterioration in neuronal functional integrity, such as axonal transport, but an alteration in the structure of NF200D to a form unrecognizable by the NF200D antibody. In view of the differential exhibition of NF200D-positive distal processes within specific cell populations, it is tempting to suggest that the factors controlling the onset of the modification of the NF200D epitope are epigenetic in nature.

NF200P-LI was also found in the cultures of spinal cord and DRG, and its first appearance was at day 17. Staining was found only in axons, and was abolished by treatment with alkaline phosphatase in TBS, but not after TBS alone. The enzyme has previously been shown to exert no proteolytic activity on NF200 (Carden et al., 1985). Moreover, no alteration in NF200D-LI was observed after phosphatase digestion. Consequently, it is unlikely that disappearance of NF200P immunostaining results from anything other than loss of phosphate moieties at the recognition site for the antibody. In addition, the antibody has been shown to bind only to the NF200 protein (Bignami et al., 1985). It is highly probable, therefore, that it stains a phosphorylated epitope of NF200.

The first manifestation of NF200P-LI was over 2 weeks after that of NF200D-LI, in common with observations of a 10 d delay between the 2 epitopes in the rat optic nerve *in vivo* (Dahl et al., 1986). *In vitro*, however, it was not always obvious which

neuronal cell types gave rise to the NF200P-positive processes, since somata were always negative. As a result, it was difficult to identify the precise time lag between the ontogeny of the phosphorylated and nonphosphorylated analogs within a given cell type. The diameter and length of some of the immunoreactive processes at day 25, however, suggested that they emanated from the large spinal cord cells, and subsequent observations in double-labeled cultures supported this view. These processes were known to have contained NF200D in their entirety at about days 12–14. Double-staining with the 2 NF200 antibodies indicated that at day 27, for example, this was no longer the case: although NF200D-LI was still present in the soma, dendrites and the proximal part of the axon, more distally the staining weakened and eventually disappeared. Over the same stretch of axon, the labeling of NF200P-LI, which was absent from the cell body and initial segment, increased. For a distance of approximately 500 μm , both forms of NF200 were present. The close complementarity of the appearance of NF200P and the disappearance of NF200D within the same axon supported the proposal that the molecular transformation being exerted on the NF200D was indeed one of phosphorylation. The fact that the 2 epitopes can overlap to a considerable degree within the intermediate region of the same axon may be due to the slow rate of phosphorylation compared to that of axonal transport of the neurofilaments.

The proportion of an axon containing only NF200D-LI, or solely NF200P-LI, is presumably governed by both the rate of phosphorylation of the NF200D and the speed of axonal transport. The latter parameter, which operates via slow component A (Hoffman and Lasek, 1975), varies widely between different tissues, species, and ages of development (Hoffman and Lasek, 1975; Willard and Simon, 1983; Nixon and Logvinenko, 1986). It seems reasonable to suggest that the rate of transport may also vary significantly between neurons in our cultures. It is conceivable, then, that axons containing slowly moving NF200 proteins display shorter segments containing NF200D-LI than do those axons with more rapidly transported NF200. It is also feasible that the rate of phosphorylation varies between neurons. In the squid axon, the appropriate kinases copurify with the neurofilaments themselves (Pant et al., 1986), so the rate of enzyme synthesis may lead to parallel changes in phosphorylation activity. However, it is more possible that cofactors, or inhibitors, of the kinase exert a more influential regulation of enzyme activity.

Little change in the disposition of NF-LI, NF200D-LI, and NF200P-LI in the cultures was observed over the period 35–56 d, and it was considered that by this time a steady state had been achieved. It was surprising, then, that many axons still stored NF200D-LI, instead of switching to NF200P production, since the latter has been associated principally with mature axons (Dahl and Bignami, 1985). A proportion of the neurons within a given cell type, such as from the DRG, failed to display NF200P-positive axons. However, no morphological differences between the phosphorylated and nonphosphorylated epitope-containing cells was apparent. The finding that NF200D can be detected in axons of the trigeminal ganglion and DRG neurons of the adult rat *in vivo* (Lee et al., 1986, 1987) corroborates our evidence that whereas some mature neurons display NF200P, others in the same culture continue to express NF200D.

In summary, the first neurofilament proteins detectable in dissociated cultures of mouse spinal cord and dorsal root ganglia were the 70,000 and 150,000 M_r units. At subsequent stages NF200D appeared in discrete cell populations, and this was

followed considerably later by NF200P. The delayed appearance of NF200P as compared to NF200D was also observed in rat optic nerve during postnatal development, using the immunoblotting procedure employing antibodies reacting with NF200P, NF200D, and NF150D (Dahl et al., 1986). NF200D was found in progressively more distal axonal regions with time, and then, in some fibers, retracted and replaced by NF200P. Nonetheless, in other axons, NF200D remained, while no NF200P was demonstrable even in apparently mature cultures. The results are consistent with the suggestion that there is a partial or full induction of a phosphorylating enzyme late in the development of some subpopulations of neurons.

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