Int. J. Dev. Biol. 38: 429-437 (1994)

Original Article

Developmental expression of neurofilament and glial filament proteins in rat cerebellum

LEENA A. SAWANT, NALINI N. HASGEKAR and LALITHA S. VYASARAYANI*

Neurooncology Division, Cancer Research Institute, Tata Memorial Centre, Parel, Bombay, India

ABSTRACT Neurofilament protein (NFP) consists of three subunits: NF200, NF150 and NF68. Several studies on expression of NFP in developing brain have shown that NF200 appears later than NF150 and NF68. However, there are some reports on simultaneous appearance of these subunits in development. The present study is an attempt to resolve this controversy. Rat cerebellum was chosen as most of its development takes place during the first three weeks of postnatal period. Cytoskeletal and NFP preparations from newborn (P0), postnatal day 8 (P8), P15, P21, P30 and adult (3 months) rat cerebella were subjected to electrophoresis on 7.5% SDS-PAGE. All the NFP subunits were present from P0 onwards and there was an increase in NFP content and glial fibrillary acidic protein (GFAP) with age as revealed by the densitometric scanning. Immunoblots of NFP preparations confirmed the presence of NF200 in the early postnatal cerebellum. *In vivo* phosphorylation studies indicated the presence of phosphorylated NF subunits from P8 onwards, which was confirmed by staining in immunoblots by SMI31. Immunohistochemical studies on Bouin's fixed tissues revealed that in P0 cerebella, the deeper neurones (soma and processes) expressed all the NFP subunits while from P8 onwards they were negative for NF200. Similarly, Purkinje cells (soma) expressed transiently NF200 subunits on P8 and ceased to express them from P15 onwards. The white matter was immunopositive for NF200 and NF150 on P0 and the intensity of staining increased progressively. Astrocytes expressing GFAP were seen in cerebellar white matter from P8 onwards and the staining in radial glia could be detected from P15 onwards. The polyclonal antibodies used in this study, recognize predominantly the phosphorylated forms of NFP.

KEY WORDS: *NFP, GFAP, developing cerebellum, phosphorylation, Purkinje cells*

Introduction

Glial filaments and neurofilaments, which belong to the family of intermediate filaments are known to be developmentally regulated and their expression is specific to astrocytes and neurones respectively (Bignami and Dahl, 1977; Shelanski and Liem, 1979). Neurofilament protein (NFP) is composed of 3 polypeptide subunits with apparent molecular masses, as determined by SDS-PAGE, of 68-73 kDa (NF-L/NF68), 140-160 kDa (NF-M/NF150) and 195-200 kDa (NF-H/NF200) (Liem *et al.*, 1978; Lazarides, 1982; Schlaepfer, 1987). Each NFP subunit is immunochemically distinct (Lee *et al.*, 1982). Phosphorylation of NFP is a well established post-translational modification and it occurs mainly on the peripheral carboxy-terminal domain (Jones and Williams, 1982; Julien and Mushynski, 1983; Carden *et al.*, 1985). NF200 and NF150 are extensively phosphorylated while NF68 has very few phosphate groups. Monoclonal antibodies against NF200 and NF150, whose specificities are dependent on the state of phosphorylation, have been generated (Sternberger and Sternberger, 1983; Lee *et al.*, 1986; Carden *et al.*, 1987). Biochemical and histochemical analyses have shown that NF200 and NF150 are less phosphorylated in the cell body and the dendrites, while they are highly phosphorylated in the axons. Thus, the phosphorylation state of NFP reflects the regional specialization of the cytoskeleton in neurones (Sternberger and Sternberger, 1983; Bennett and Dilullo, 1985; Peng *et al.*, 1986; Trojanowski *et al.*, 1986; Dahl *et al.*, 1987; Nixon *et al.*, 1987; Oblinger, 1987).

According to one of the initial reports using an antiserum against the mixture of all 3 subunits, NFP appeared very early in embryonic rat brain by E12 (Raju *et al.*, 1981; Bignami *et al.*, 1982). Subsequent investigations have employed antisera against individual NFP subunits and one such study in the developing rat brain

Abbreviations used in this paper: GFAP, glial fibrillary acidic protein; Mab, monoclonal antibody; NF, neurofilament; NFP, neurofilament protein; DAB, diaminobenzidine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate buffered saline.

***Address for reprints:** Neurooncology Division, Cancer Research Institute, Tata Memorial Centre, Parel, Bombay 400012, India. FAX: 91-22-4146089.

0214-6282/94/$03.00

Printed in Spain

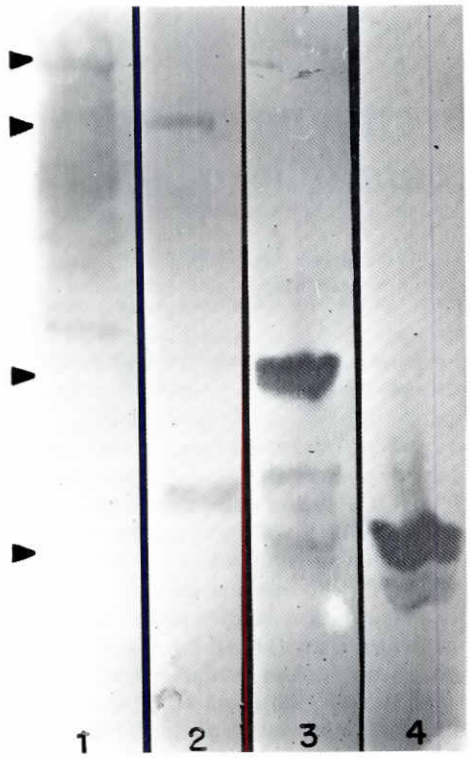

Fig. 1. Immunoblot of cytoskeletal preparations from adult rat brain *stained with antiserum against NF200* **(lane 1)**, *NF150* **(lane 2)**, *NF68* **(lane 3)** *and GFAP* **(lane 4)**.

describes NF68 and NF150 as appearing first followed by NF200 (Shaw and Weber, 1982). In the mouse brain all the NFP subunits could be detected simultaneously by 9-10 days of gestation (Cochard and Paulin, 1984). With the availability of antibodies against NFP and GFAP there are a number of reports on developmental

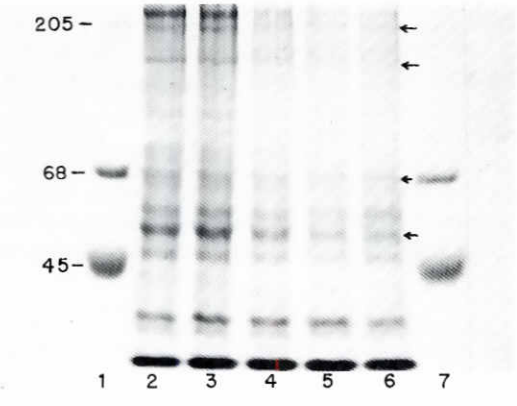

Fig. 2. Coomassie blue stained cytoskeletal preparations of developing rat cerebellum separated using 7.5% SDS-PAGE. (Lanes 2 to 6) *P30, P21, P15, P8 and P0 respectively.* **(Lanes 1 and 7)** *Molecular weight standards. The gel shows the presence of NFP subunits and GFAP (arrows) even on P0. There is a progressive increase of all the 4 proteins with advancing age.*

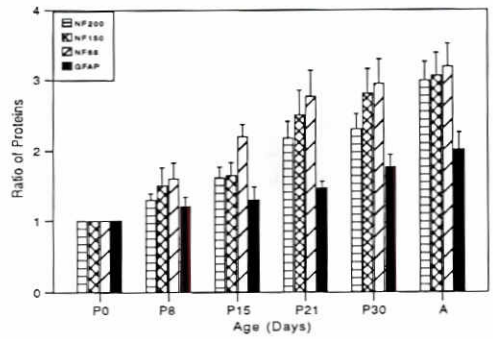

Fig. 3. Histogram depicting the ratio of mean values±SEM (n= 5), obtained on densitometric scanning of the gel. *The ratio was calculated considering the value on P0 as 1. A differential increase of NFP subunits and GFAP is seen.*

expression of these cell-type specific intermediate filament proteins, in different regions of the brain including brain stem and cerebellum (Willard and Simon, 1983; Pachter and Liem, 1984; Harry *et al.*, 1985; Marc *et al.*, 1986). Moreover there are very few comprehensive reports on appearance of all the 3 NFP subunits along with GFAP in the developing cerebellum. The present investigation aims at defining the sequential expression of NFP subunits, their phosphorylation and GFAP in the postnatal rat cerebellum.

Results

Specificity of the antiserum used

The immunoblots of cytoskeletal proteins extracted from adult rat cerebellum probed with the antisera obtained in our laboratory showed that they recognized their respective antigens, and additional bands which could be their degradation products as shown in Fig. 1. In immunohistochemical preparations the antiserum against NF68 did not stain the white matter though it recognized

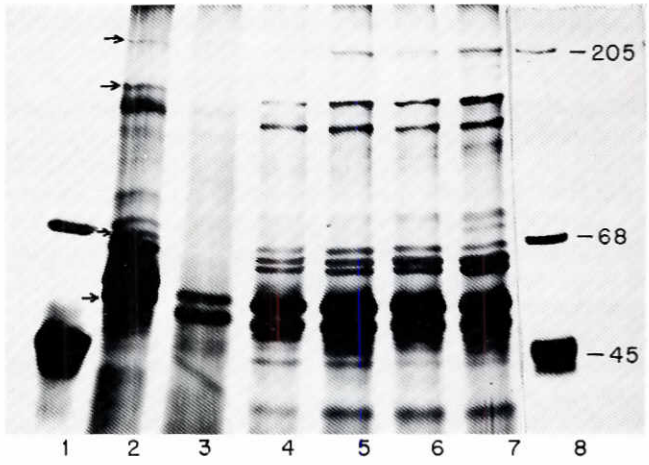

Fig. 4. Silver stained gel of NFP preparation of developing rat cerebellum, separated using 7.5% SDS-PAGE. (Lanes 2 to 7) *P0, P8, P15, P21, P30 and adult (A) respectively.* **(Lanes 1 and 8)** *Molecular weight standards. Progressive increase with age of NFP subunits and GFAP is evident. NF200 and NF150 are seen distinctly even on day 0 (indicated by arrows).*

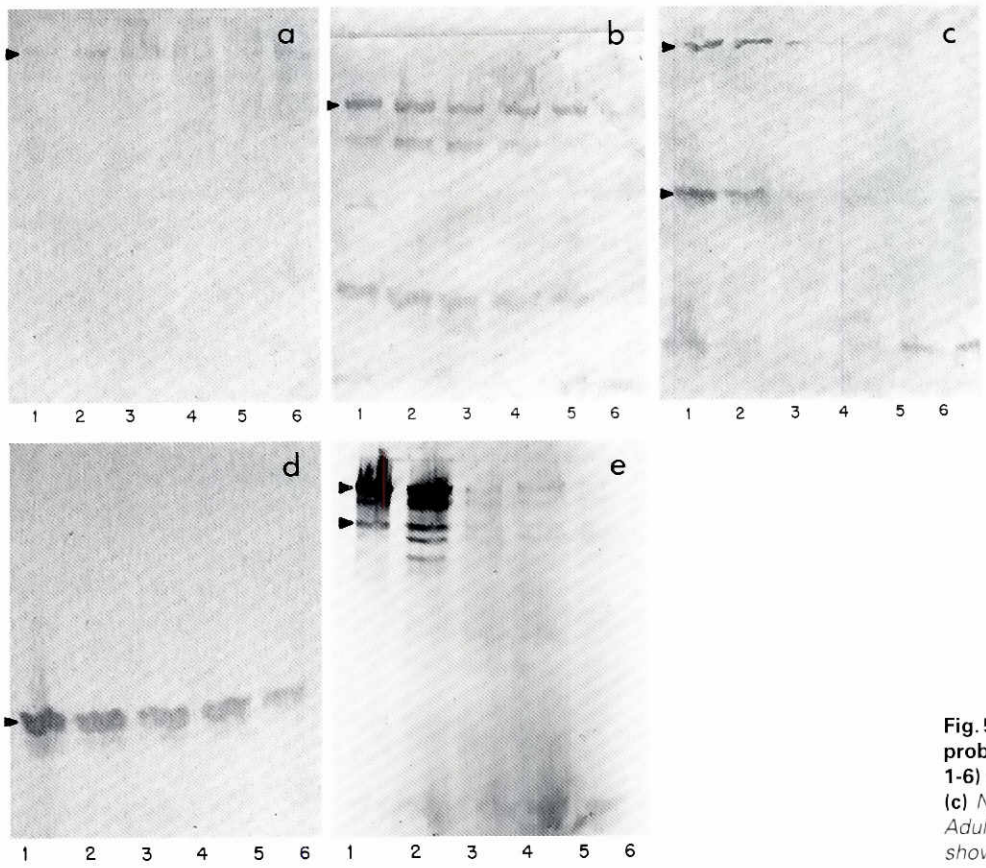

Fig. 5. Immunoblot of cytoskeletal preparation probed with antiserum against (a) *NF200*; **(lanes 1-6)** *P0, P8, P15, P21, P30 and adult.* **(b)** *NF150,* **(c)** *NF68,* **(d)** *GFAP, and* **(e)** *SMI31.* **(Lanes 1-6)** *Adult, P30, P21, P15, P8 and P0. Arrow heads show the positions of NF subunits and GFAP.*

NF200 on immunoblots. The pattern of staining exhibited by antiserum against NF200 and NF150 on rat cerebella sections was similar to that shown by SMI31 which recognizes phosphorylated NF200 and NF150.

Electrophoresis

Cytoskeletal preparations

The developmental time course of NFP and GFAP expression in the cytoskeletal preparations of cerebella from P0, P8, P15, P21, P30 and adult is shown in Fig. 2. The protein profile is similar for all the time points studied. All NFP subunits are present on P0 and there is an increase in their content with increasing age. A similar change is observed on comparison of mean values for all four proteins (Fig. 3). There is a steady increase in NF68, from P0 for the first 3 weeks without much change later. Increase in NF200 is more obvious in the third week and after day 30, whereas the increase in NF150 is more obvious during the first and third weeks. GFAP also increases progressively with age.

NFP preparation

Fig. 4 shows silver stained gel of NFP preparation from developing rat cerebellum. The protein pattern is similar for all time points studied. As seen in cytoskeletal preparations, all 3 NFP subunits are present at P0 and there is a gradual increase thereafter.

Immunoblotting

Fig. 5a-e shows the immunoblot of NFP preparations from P0, P8, P15, P21 P30 and adult stained with antiserum against NF200, NF150, NF68, GFAP and SMI31 respectively. The intensity of bands increased steadily from P0 onwards. The increase was differential for both NFP subunits and GFAP and was more obvious with respect to NF68 and GFAP (Fig. 6). The monoclonal antibody SMI31 strongly reacted with NF200 and NF150 in the cytoskeletal preparations from P30 and adult, while it weakly stained both these subunits on P8, P15 and P21. It also stained bands below NF200 and NF150 which may be the proteolytic products of the NF subunits (Fig. 5e)

Autoradiography

The autoradiographs of cytoskeletal preparations for P0 and P6 do not show a clear signal among the phosphorylated proteins at any of the positions of the NFP subunits, while the autoradiograph of P8, P15, P21 and P30 shows labelled bands at the position of NF200 and NF 150 (Fig. 7).

Immunohistochemistry

(i) On the NBF fixed cerebella, the immunoreactivity for NFP first appears on P15, while that for GFAP appears only from P21 onwards in astrocytes of white matter. On treating the sections with trypsin, the staining of radial glia with GFAP antibodies and the reactivity for NF68 in the neurones are more intense than in the

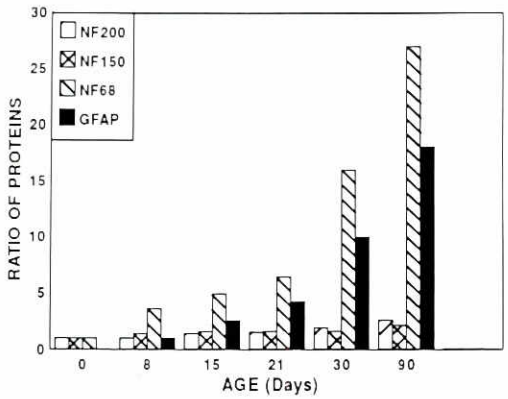

Fig. 6. Histogram showing the ratio of proteins obtained on densitometric scanning of the immunoblots from Fig. 5. *The ratio was calculated considering the values for P0 as 1.*

untrypsinized sections (data not shown). (ii) In Bouin's fixed cerebellum, the staining for both NFP and GFAP is brighter than with formalin fixation. Immunoreactivity is observed even in cerebella of P0 for NFP and P8 for GFAP. The distribution of NFP and GFAP at various time intervals in the postnatally developing cerebellum is shown in the Tables 1 and 2. On P0, immature Purkinje cells were distributed irregularly in several rows between the molecular layer and the cerebellar white matter. As shown in Table 1, none of the antisera against NFP subunits stains the Purkinje cell cytoplasm and dendrites. The white matter is stained by antisera against NF200 and NF150. The staining of deeper neurones in cerebellar nucleus is moderate for NF200 and NF68 (Fig. 8a and c) whereas it is brighter for NF150 (Fig. 8b). The brain

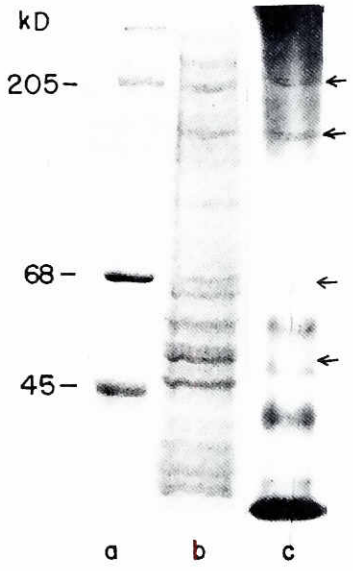

Fig. 7. Cytoskeletal preparations from P8 rat cerebellum on 7.5% SDS-PAGE. (Lane a) *Molecular weight standards,* **(lane b)** *Coomassie stained gel and* **(lane c)** *autoradiograph showing phosphorylation of NF200 and NF150. Arrows indicate the positions* of NF200, NF150, NF68 and GFAP.

TABLE 1

DISTRIBUTION OF NFP SUBUNITS IN DEVELOPING RAT CEREBELLUM

		P0	P8	P15	P21	P30	A
NF200	Molecular layer:						
	Transverse fibers	-	-	++	+++	++	++
	Purkinje cytoplasm	-	+	-	-	-	-
	Basket axons	-	-	++	+++	++	++
	White matter	+	++	++	+++	++	++
	Deeper neurones in cerebellum	++	-	-	-	-	-
	Brain stem neurones	++	-	-	-	-	-
NF150	Molecular layer:						
	Transverse fibers	-	-	+	+++	++	++
	Purkinje cytoplasm	-	++	+	++	+	+
	Basket axons	-	-	+	+++	++	++
	White matter	+	+++	+++	++	+++	++
	Deeper neurones in cerebellum	+++	++	++	+++	++	++
	Brain stem neurones	+++	++	++	+++	++	++
NF68	Molecular layer:						
	Transverse fibers	-	-	-	-	-	-
	Purkinje cytoplasm	-	+	+	+	+	+
	Basket axons	-	-	-	-	-	-
	White matter	-	-	-	-	-	-
	Deeper neurones in cerebellum	++	+++	+	+++	+++	++
	Brain stem neurones	++	++	+	+++	++	++

stem neurones which are at a more advanced stage of differentiation are stained intensely for NFP subunits (Fig. 9a,b,c).

On P8, Purkinje cells are aligned in a single row between the molecular and internal granular layer. The cytoplasm of Purkinje cells was highly immunopositive for NF150 (Fig. 10b) while the staining is moderate for NF200 and NF68 (Fig. 10a). NF200 and NF150 stained fibers of the white matter while NF68 failed to stain them. The neurones of the cerebellar deep nuclei are immunopositive for NF150 and NF68, but negative for NF200 at this developmental stage (Table 1). The astrocytes in the white matter expressing GFAP are first discernible at this stage (Fig. 10c, Table 2).

On P15, the Purkinje cells show clear signs of dendritic growth with many branches. The immunoreactivity of Purkinje cell cytoplasm for NF200 disappears completely at this stage, while that for NF150 and NF68 persists, though faint. Its dendrites exhibit weak to moderate immunoreactivity for both NF150 and NF200. The nerve fibers in the white matter are moderately positive for NF200 and NF150 and negative for NF68. The basket axons around

TABLE 2

EXPRESSION OF GFAP IN DEVELOPING RAT CEREBELLUM

	P0	P8	P15	P21	P30	A
Molecular layer: Radial glia	-	-	+	++	++	++
White matter: Astrocytes	-	+	++	++	+++	++

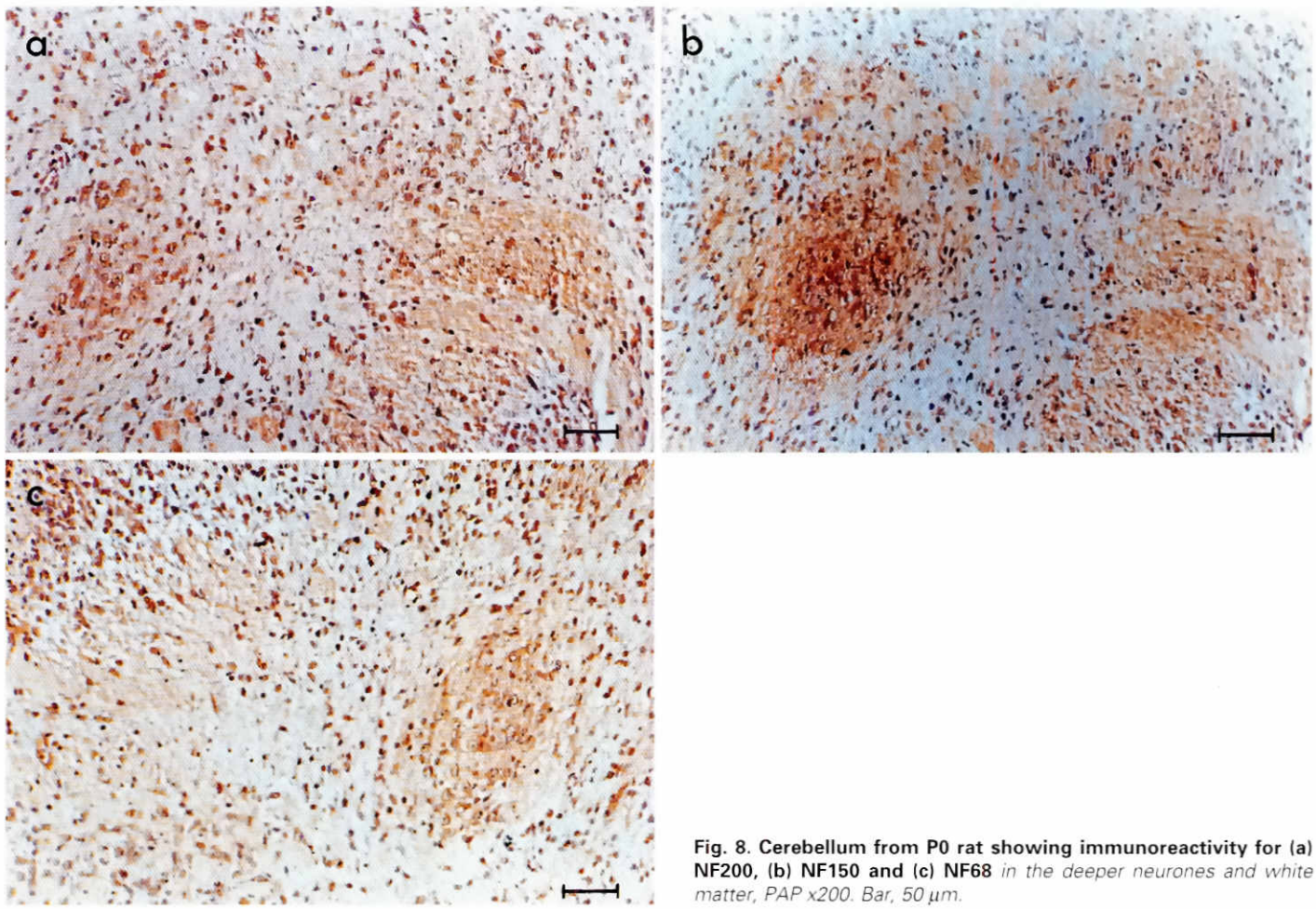

Fig. 8. Cerebellum from P0 rat showing immunoreactivity for (a) NF200, (b) NF150 and (c) NF68 *in the deeper neurones and white matter, PAP x200. Bar, 50 μm.*

Purkinje cells are stained more intensely for NF200 than for NF150. In addition, transverse fibers immunoreactive for NF200 and NF150 are found in the molecular layer, most of them running parallel to the surface of the cerebellum. The neurones of the cerebellar deep nuclei are intensely stained for NF150 and NF68, (Table 1). The radial glia in the molecular layer, immunopositive for GFAP are first seen at this stage. The astrocytes in the white matter continue to express GFAP immunoreactivity (Table 2).

On P21, the staining for all the 3 NFP subunits is brighter than on P15 though the pattern is similar (Fig. 11a,b,c). The Purkinje cells have an adult orientation and their dendritic arborization extends towards the surface of the cerebellum. The basket axons are positive for NF200 and NF150. The radial glia and astrocytes in the white matter show GFAP immunoreactivity. On P30 and in the adult, the intensity and pattern of staining for all the 3 NFP subunits as well as GFAP is very similar to that on P21.

Discussion

The findings in the present study indicate that all 3 NFP subunits are present in the cerebellum since birth. According to some reports, NF200 subunit is absent in brains of newborn rat and rabbit (Shaw and Weber, 1982; Willard and Simon, 1983). On the other hand, in a few studies the presence of NF200 has been recorded in newborn rat brain (Calvert and Anderton, 1982; Hofstein *et al.*, 1985) and in embryonic brains of mice and rat (Cochard and Paulin 1984; Carden *et al.*, 1985). The developmental profile of NF200 and its mRNA have been described in various regions of the brain (Leiberburg *et al.*, 1989; Roussel *et al.*, 1991) and they could be detected prior to birth. Although Shaw and Weber (1982) could not observe NF200 in postnatal rat brain on P0, using the same extraction procedure, we were able to detect in newborn rat cerebellum NF200 on gel, immunoblots and sections immunostained by specific antisera. The only difference between the study of Shaw and Weber (1982) and our study was that the whole brain was used for NFP extraction in the former, while only the cerebellum was chosen in the latter. Also, it is known that brain regions giving rise to neurones with long-axons (spinal cord, cerebellum, pons and medulla) contain high levels of the NF200 message as well as NF200 polypeptide (Leiberburg *et al.*, 1989). The differential increase in NFP subunits in the developing rat cerebellum observed by us (Fig. 3) is similar to that reported by Harry *et al.* (1985), in the rat brain stem. The cross-reactivity exhibited by our antisera in immunoblots is possibly due to antigenic determinants shared by

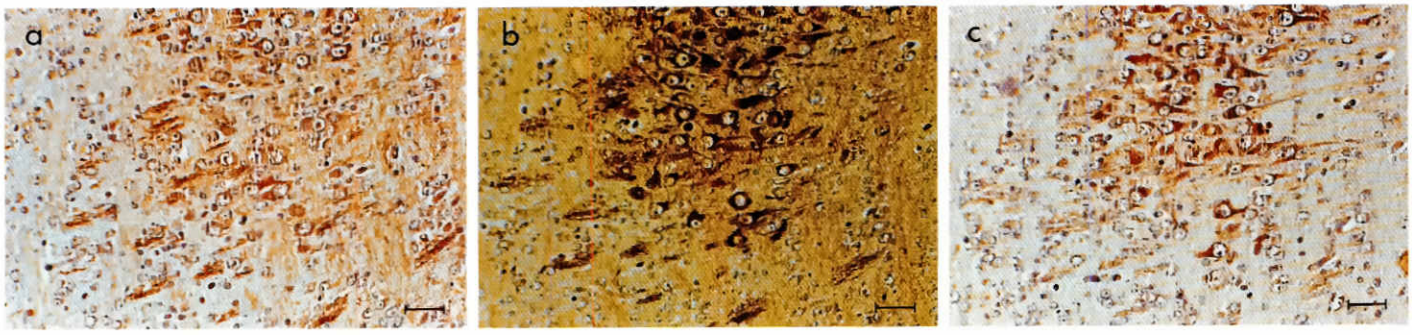

Fig. 9. Brain stem neurones and white matter stained for (a) NF200, (b) NF150 and (c) NF68, *PAP x 300. Bar, 33 μm.*

the subunits as suggested by Calvert and Anderton (1982), Willard and Simon (1983), Dahl (1987) and Dahl *et al.* (1987).

In the present study, phosphorylated NFP could be detected from P8 onwards, while in P0 and P6 it was not seen. However, Noetzel *et al.* (1986) recorded the presence of the phosphorylated form of all 3 NFP subunits in newborn rat brain, spinal cord and sciatic nerves. Failure to detect phosphorylated forms of NFP in autoradiographs of P0 and P6 cerebella in the present study is probably due to fewer phosphate groups which defy detection. The presence of phosphorylated NF200 and NF150 in P8 is confirmed by the reactivity of SMI31 on immunoblots of cytoskeletal preparation (Fig. 5e). Also there are reports stating that NF200 first appears in the non-phosphorylated form and then gradually becomes phosphorylated. In the rat optic nerve, NF200 is present on day 10 in the non-phosphorylated form and by day 18 the phosphorylated NF200 could be detected (Dahl *et al.*, 1986). The appearance of mature NF200 in dorsal root ganglion (DRG) neurones (Oblinger, 1987) was a late phenomenon. Studies on phosphorylation of NFP (Komiya *et al.*, 1986) in the sciatic nerve also revealed that the existing NFP could be phosphorylated by phosphates. Extensive phosphorylation of NFP (NF200 and NF150) at the carboxy-terminal domains, which are believed to be projected outward from the filament core, has been considered one of the means by which neurofilaments cross link and stabilize the axonal cytoskeleton (Leterrier *et al.*, 1982; Sharp *et al.*, 1982; Julien and Mushynski, 1983; Hirokawa *et al.*, 1984; Carden *et al.*, 1985; Nixon and Sihag, 1991; Nixon and Shea, 1992).

Bouin's fixative has been recommended for NFP immunohistology by Sternberger and Sternberger (1983) and Trojanowski *et al.* (1985). Our findings of immunopositivity for NF200 and NF150 in Purkinje cells on Bouin's fixed P8 rat cerebella are very similar to the observations reported earlier. Bignami *et al.* (1982) have shown that NF200 is expressed transiently in immature Purkinje cells of chick cerebellum till day 20, when it starts appearing in basket axons. According to a recent study (Kondo *et al.*, 1991) faint immunoreactivity for NF triplets appear in the somata and dendrites of Purkinje cells of the postnatal rat cerebellum from P8 and P10, fixed in formalin. A similar phenomenon has been described (Watanabe *et al.*, 1990) with reference to the progressive elevation of neuron specific enolase (NSE) immunoreactivity in the Purkinje cell somata and their axons until P9 in the developing cerebellum. Yachnis *et al.* (1993) have also reported the transient expression of highly phosphorylated NF-M and H in the Purkinje cell of the human cerebellum at gestational ages 20-40 weeks after which it disappeared. According to them this may be due to the differences in the location or activity of NF kinases or phosphatases during development and in the adult. An extensive study on the expression of developmentally regulated neuronal and glial polypeptides in the human cerebellar cortex and dentate nucleus (Yachnis *et al.*, 1993) and spinal cord (Tohyama *et al.*, 1991) has been carried out. Interestingly, the neurones of the deep nuclei of cerebellum also show transient expression of NF200 on P0 and it disappears by P8, while the other two subunits persist. It is believed that NF polypeptides are synthesized in the neuronal

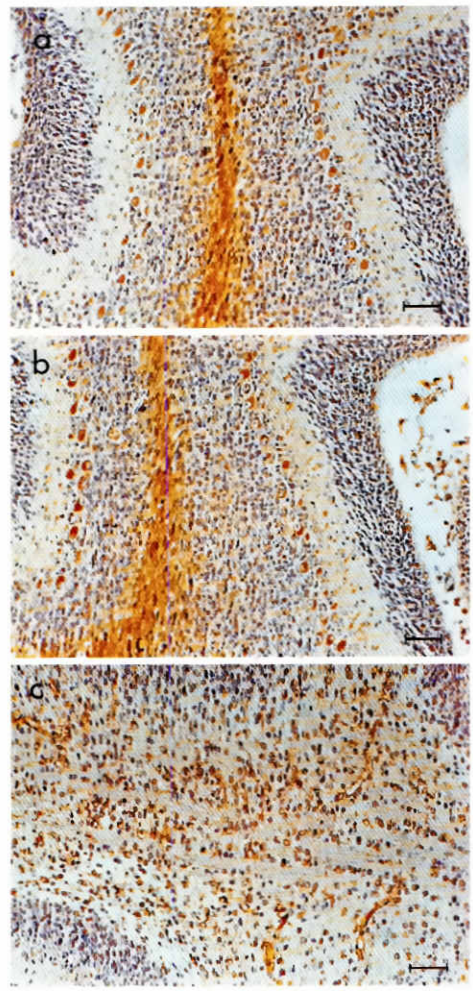

Fig. 10. Cerebellar folia of P8 rat stained with (a) *NF200 antiserum* **(b)** *showing immunopositivity in the Purkinje cytoplasm and white matter, PAP x200.* **(c)** *White matter astrocytes showing immunopositivity for GFAP, PAP x200. Bar, 50 μm.*

somata and move down the axons by slow axoplasmic transport (Hoffman and Lasek, 1975). It is well known that NFP can undergo post-translational modification during the axoplasmic transport and it is restricted to certain parts of neurones (Nixon *et al.*, 1982; Goldstein *et al.*, 1983; Sternberger and Sternberger, 1983; Bennett and Dilullo, 1985; Trojanowski *et al.*, 1985; Nixon and Sihag, 1991). There appear to be at least two possible mechanisms for the differential distribution of NFP immunoreactivity in different domains of the neurones during development: a) axoplasmic transport and b) post-translational modification. Accordingly the accumulation of NFP in the Purkinje cell cytoplasm during the period of rapid growth i.e., P8 (Altman, 1972) is influenced by the onset of slow axoplasmic transport which may not be very effective in that particular developmental stage. Alternatively the phosphorylation of NFP subunits in some neurones occurs even in the soma in the embryonic brain so that they could be easily discerned on reacting with antisera recognizing predominantly the phosphorylated epitopes. It is generally believed that the cell body contains less phosphorylated NF while these are more concentrated in the axons. The studies by Lee *et al.* (1986) described for the first time a group of Mab (dP series) that recognize exclusively the non-phosphorylated or enzymatically dephosphorylated forms of NF200 in the Purkinje cell cytoplasm. That the partially phosphorylated forms of NF-M was present in perikarya and neurites of E12 neurones and extremely phosphorylated isoforms of NF-M appeared in E13 axons has been reported by Carden *et al.* (1987) .

It is possible that all NF subunits are rapidly transported to neuronal processes following synthesis and that only a small amount of NF remains in the perikarya of some neurones which escapes detection by the antibodies used. It could also be that the transient and distinct appearance of the subunits of NFP at the neonatal stages in the present study represents premature occurrence of the phosphorylation in the deeper neurones and Purkinje cell somata and that the phosphorylation may become restricted to the Purkinje axons at later postnatal stages. Both NF200 and NF150 exist in multiple phosphorylated forms within the same neurones and this phosphorylation of NF200 is region dependent (Lee *et al.*, 1986, 1987; Black and Lee, 1988). The important observation by Black and Lee (1988) was that NF-M and NF-L can be phosphorylated in neuronal cell bodies and distal as well as proximal locations within axons. The presence of NF-H and NF-M in Purkinje cell cytoplasm on P8 is probably due to the fact that our antisera recognize the phosphorylated epitopes of NF-H and NF-M. The antiserum used in the current report is against NFP from adult brain which contains more of phosphorylated forms of NFP and the staining pattern is very similar to SMI31 that recognizes phosphorylated NF200 and NF150.

Our findings of GFAP in cytoskeletal preparations of P0 rat cerebellum supports the report of GFAP gene expression in mouse brain which commences from P3 onwards (Riol *et al.*, 1992). GFAP positive cells have been observed in the brain and spinal cord on embryonic E18 *(in vivo)* in cultures derived from 10 day old embryos (Raju *et al.*, 1981). Our observation on the first appearance of GFAP positive radial glia on P15 and white matter astrocytes on P8 is similar to those of earlier investigators (Schnitzer *et al.*, 1981; Edwards *et al.*, 1990). The GFAP immunoreactivity in both the types of astrocytes increased progressively with glial maturation. In contrast, in the later fetal period Mab RC1 stained radial glia of the mouse cerebellum have been found to disappear progressively followed by appearance of immunoreactive scattered immature astrocytes bearing multiple processes (Edwards *et al.*, 1990).

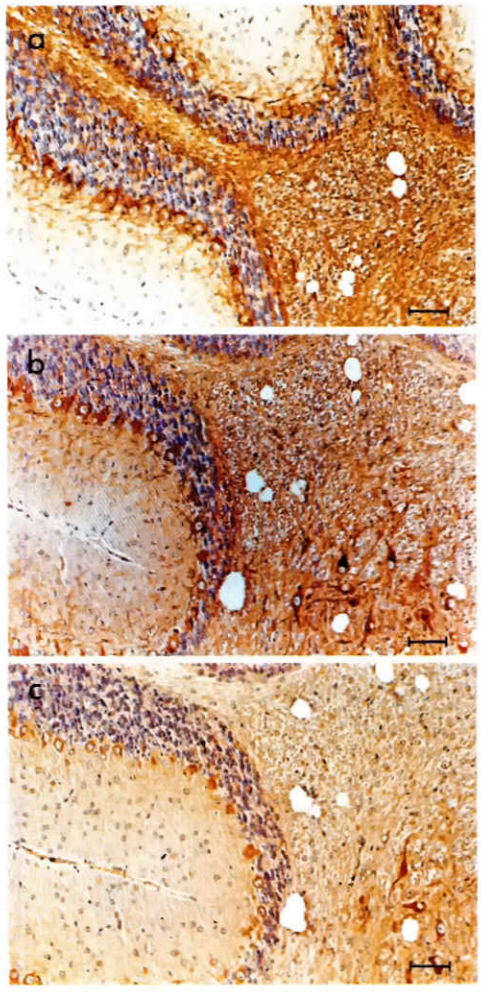

Fig. 11. Cerebellar folia of P21 rat stained with antisera against (a) NF200 (b) NF 150 and (c) NF68. *Note that deeper neurones are devoid of NF200 immunoreactivity and Purkinje cell cytoplasm expresses NF150 and NF68. PAP x200. Bar, 50 μm.*

To conclude, neurofilament subunit expression and its maturation are complex phenomena in cerebral cortex. The NFP appears early in differentiating neurones, forms filaments which are stabilized by phosphorylation occurring mostly in axons. The post-translational modification rather than genetic transition is an important mechanism that brings about the stabilization of neurofilaments, which is a gradual process. *In situ* hybridization analysis of developing cerebellum using cDNA to NFP along with the knowledge of status of their phosphorylation will enhance our understanding of the mechanism of the present developmental phenomenon. Expression of an incomplete set of NF proteins (i.e. NF150 and NF68, but not NF200) in certain neurones poses an interesting question about the control of NFP gene expression in developing neurones. NF68 and NF150 genes seem to be tightly linked, since both were always present in the same cells viz. deeper nuclei of cerebellum. The concurrent expression of NF150 and NF68 genes would also suggest that the same signal induces them in newly formed neurones. The significance of transient expression of NFP in some neurones (Purkinje cells) during development is an interesting feature which awaits elucidation.

Materials and Methods

Newborn (P0), P8, P15, P21, P30 and adult (A-3 months) Sprague-Dawley (SD) rats were anesthetized with ether and sacrificed by decapitation. Cerebella were quickly removed and stored at -70°C until all tissue samples were obtained for the isolation of NFP and GFAP. Sagittal sections of the cerebella were fixed either in Bouin's fixative or in neutral buffered formalin (NBF) for histochemical studies.

Cytoskeletal proteins

Cytoskeletal proteins were prepared from all the tissues at one time using the method described by Chiu *et al.* (1981). About 50 mg of the tissue (cerebellum) were disrupted in 1 ml of buffer T (50 mM-Tris-HCl, pH 6.8, 2 mM-PMSF [phenylmethyl sulphonyl fluoride] and 0.5% Triton X100) using a Teflon homogenizer. The homogenate was centrifuged at 17,000 rpm for 10 min at 4°C. The pellet containing cytoskeletal material was homogenized in 1 ml of buffer T containing 30%, sucrose and similarly centrifuged. The pellet was dissolved in sample solvent containing 2% SDS, 2 mM-EDTA, 2 mM-PMSF and 10% glycerol and stored at -20°C.

NFP preparation

Neurofilament proteins were isolated from the cerebellum from P0, P8, P15, P21, P30 and adult (3 months) using the method described by Shaw and Weber (1982). About 1.5 g of starting material was dounce homogenized in 20 vols of Dahl buffer pH 7.1. After centrifugation for 10 min at 12,000 rpm at 4°C in an SS34 Sorvall rotor, the supernatant (S_1) was saved, and the pellet was again homogenized with the same volume of Dahl's medium. After further centrifugation, the supernatant (S_2) was again saved. The pellet was resuspended in 8 ml of Dahl's medium and sonicated using a sonifier at a setting of 3W for 10 s. The material was spun in the Sorvall centrifuge as before. The supernatant was saved (S_3) and the pellet was again resuspended in 8 ml of Dahl's medium and sonicated for 10 s at 4W. The supernatant (S_4) was collected after a further centrifugation. All supernatants were centrifuged in the Beckman type 40T1 rotor at 4°C for 30 min at 35,000 rpm. The pellets produced were dissolved in 1% SDS and stored at -20°C. Protein was estimated using the method of Lowry *et al.* (1951).

Phosphorylation of NFP

Sprague-Dawley rats aged postnatal P0, P6, P8, P15, P21 and P30 were injected intracerebrally with 20 μCi of carrier free [^{32}P]-*ortho*phosphate (BRIT-Board of Radiation and Isotope Technology, India). The animals were sacrificed 20 hours later and the cytoskeletal proteins were isolated from the cerebella using the method of Chiu *et al.* (1981). The gels were stained with Coomassie, destained. dried, exposed to X-ray films and developed.

Electrophoresis

The cytoskeletal or NFP preparations were dissolved by boiling in the solution containing 2% SDS, 2 mM-EDTA, 2 mM-PMSF and 10% glycerol and then separated on 7.5% SDS-PAGE. The gels were stained with Coomassie or silver stained, destained and dried on a gel dryer. The bands corresponding to the Molecular Weight (Mr) of NFP and GFAP were quantitated as the area under the peaks generated by densitometric scanning using the UVP program. The ratios of the proteins at P8, P15, P21, P30 and A were calculated considering the value for P0 as one and are represented as mean±SEM (n= 5).

Antisera against NFP and GFAP

NFP from rat brain was extracted using the method described earlier. GFAP was isolated from human spinal cord obtained at autopsy by hydroxyapatite chromatography as described by Bignami *et al.* (1980). Antisera to different subunits of NFP and GFAP were raised in rabbits using proteins eluted from gels, about 600 μg of gel-purified proteins were emulsified with complete Freund's adjuvant and injected into the foot pad of each rabbit. A second injection was given a week later in the same manner except that incomplete Freund's adjuvant was used. Blood was drawn from the vein a week after the second injection. The antiserum specificity was checked by Western blotting and immunohistochemistry on sections of adult rat cerebellum using the appropriate dilutions of the antiserum.

Immunoblotting

Cytoskeletal proteins were separated by 7.5% SDS-PAGE as described by Laemmli (1970). Electrophoretic transfer of polypeptides from gels to nitrocellulose paper was performed according to Towbin *et al.* (1979). Nitrocellulose paper was then stained with Ponceau S to check for the transfer of proteins, destained by washing with D/W and probed with antiserum against NFP subunits. GFAP and monoclonal antibody SMI31 using Sternberger's PAP method. Densitometric analysis of the blot was done using the UVP program. The ratio of proteins was calculated as mentioned earlier for different developmental time points studied.

Immunohistochemistry

Immunohistochemical staining was performed on (i) Neutral buffered formalin (NBF) fixed and (ii) Bouin's fixed cerebella from P0, P8, P15, P21, P30 and adult (A) rat using polyclonal antisera against individual NFP subunits and against GFAP (Sternberger *et al.*, 1970). Sections were deparaffinized in xylene, rehydrated, washed in phosphate buffered saline (PBS) pH 7.5. Endogenous peroxidase was inactivated in methanol and H_2O_2 and covered with 3% normal goat serum (NGS) for 30 min. After blotting, they were flooded with primary antisera against NF200, NF150, NF68 and GFAP. Sections were washed with buffer and flooded with goat anti-rabbit IgG for 30 min. After washing, sections were covered for 30 min with PAP complex. Slides were washed and incubated for 10-15 min with 0.05% DAB and 0.01% H_2O_2 in 0.05 M Tris buffered saline (TBS) pH 7.5, washed several times, counter stained with hematoxylin and mounted.

Acknowledgments

The authors are grateful to Mrs. Premlata M. Kotenkar for the preparation of the manuscript. They are also thankful to Dr. Rajeswari Seshadri and Dr. Jyoti Kayal for valuable suggestions during the preparation of the manuscript.

References

ALTMAN, J. (1972). Postnatal development of the cerebellar cortex in the rat. II Phases in the maturation of Purkinje cells and of the molecular layer. *J. Comp. Neurol. 145:* 399-464.

BENNETT, G.S. and DILULLO, C. (1985). Slow posttranslational modification of a neurofilament protein. *J. Cell Biol. 100:* 1799-1804.

BIGNAMI, A. and DAHL, D. (1977). Specificity of glial fibrillary acidic protein for astroglia. *J. Histochem. Cytochem. 25:* 466-469.

BIGNAMI, A., DAHL, D. and RUEGER, D.C. (1980). Glial fibrillary acidic protein (GFAP) in normal neural cells and in pathological conditions. *Adv. Cell. Neurobiol. 1:* 285-310.

BIGNAMI, A., RAJU, T. and DAHL, D. (1982). Localization of vimentin, the nonspecific intermediate filament protein, in embryonal glia and in early differentiating neurons. *In vivo* and *in vitro* immunofluorescence study of the rat embryo with vimentin and neurofilament antisera. *Dev. Biol. 91:* 286-295.

BLACK, M.M. and LEE, V.M-Y. (1988). Phosphorylation of neurofilament proteins in intact neurons: demonstration of phosphorylation in cell bodies and axons. *J. Neurosci. 8:* 3296-3305.

CALVERT, R. and ANDERTON, B.H. (1982). *In vivo* metabolism of mammalian neurofilament polypeptides in developing and adult rat brain. *FEBS Lett. 145:* 171-175.

CARDEN, M.J., SCHLAEPFER, W.W. and LEE, V.M-Y. (1985). The structure, biochemical properties and immunogenicity of neurofilament peripheral regions are determined by phosphorylation state. *J. Biol. Chem. 260:* 9805-9817.

CARDEN, M.J., TROJANOWSKI, J.Q., SCHLAEPFER, W.W. and LEE, V.M-Y. (1987). Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. *J. Neurosci. 7:* 3489-3504.

CHIU, F.C., NORTON, W.T. and FIELDS, K.L. (1981). The cytoskeleton of primary astrocytes in culture contains actin, glial fibrillary acidic protein, and the fibroblast type filament protein vimentin. *J. Neurochem. 37:* 147-155.

COCHARD, P. and PAULIN, D. (1984). Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo *in vivo*. *J. Neurosci. 4:* 2080-2094.

DAHL, D. (1987). Maturation of a large neurofilament protein (NF150K) in rat postnatal development. *J. Neurosci. Res. 17:* 367-374.

DAHL, D., CROSBY, C.J., GARDNER, E.E. and BIGNAMI, A. (1986). Delayed phosphorylation of the largest neurofilament protein in rat optic nerve development. *J. Neurosci. Res. 15:* 513-519.

DAHL, D., GARDNER, E.E. and CROSBY, C.J. (1987). Axonal maturation in development. I. Characterization of monoclonal antibodies reacting with axon-specific neurofilament epitopes. *Int. J. Dev. Neurosci. 5:* 17-27.

EDWARDS, M.A., YAMAMOTO, M. and CAVINESS, V.S., Jr. (1990). Organisation of radial glia and related cells in the developing murine CNS. An analysis based upon a new monoclonal antibody marker. *Neuroscience 36:* 121-144.

GOLDSTEIN, M.E., STERNBERGER, L.A. and STERNBERGER, N.H. (1983). Microheterogeneity ("neurotypy") of neurofilament proteins. *Proc. Natl. Acad. Sci. USA 80:* 3101-3105.

HARRY, G.J., GOODRUM, J.F. and MORELL, P. (1985). The postnatal development of glial fibrillary acidic protein and neurofilament triplet proteins in rat brain stem. *J. Neurosci 3:* 349-352.

HIROKAWA, N., GLICKSMAN, M.A. and WILLARD, M.B. (1984). Organisation of mammalian neurofilament polypeptides within the neuronal cytoskeleton. *J. Cell Biol. 98:* 1523-1536.

HOFFMAN, P.N. and LASEK, R.J. (1975). The slow component of axonal transport: identification of major structural polypeptide of the axon and their generality among mammalian neurons. *J. Cell Biol. 66:* 351-366.

HOFSTEIN, R., MAJOCHA, R.E., BARNSTABLE, C.J. and MAROTTA, C.A. (1985). Non-uniform distribution of neurofilament epitopes in the CNS during development. *Ann. NY Acad. Sci. 455:* 787-789.

JONES, S.M. and WILLIAMS, R.C., Jr. (1982). Phosphate content of mammalian neurofilaments. *J. Biol. Chem. 257:* 9902-9905.

JULIEN, J.P. and MUSHYNSKI, W.E. (1983). The distribution of phosphorylation sites among identified proteolytic fragments of mammalian neurofilaments. *J. Biol. Chem. 258:* 4019-4025.

KOMIYA, Y., TASHIRO, T. and KUROKAWA, M. (1986). Phosphorylation of neurofilament proteins during their axonal transport. *Biomedical Res. 7:* 345-348.

KONDO, H., IWANAGA, H.T., ABE, H., WATANABE, M. and TAKAHASHI, Y. (1991). Differential changes in expression of the neurofilament triplet protein-immunoreactivity in Purkinje cells of the cerebellum during the postnatal development of rats. *Arch. Histol. Cytol. 54:* 437-445.

LAEMMLI, U.K. (1970). Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature 227:* 680-685.

LAZARIDES, E. (1982). Intermediate filaments: a chemically heterogenous developmentally regulated class of proteins. *Annu. Rev. Biochem. 51:* 219-250.

LEE, V.M-Y., CARDEN, M.J. and TROJANOWSKI, J.Q. (1986). Novel monoclonal antibodies provide evidence for the *in situ* existence of a non phosphorylated form of the largest neurofilament subunit. *J. Neurosci. 6:* 850-858.

LEE, V.M-Y., CARDEN, M.J., SCHLAEPFER, W.W. and TROJANOWSKI, J.Q. (1987). Monoclonal antibodies distinguish several differentially phosphorylated states of the two largest rat neurofilament subunits (NF-H and NF-M) and demonstrate their existence in the normal nervous system of adult rats. *J. Neurosci. 7:* 3474-3488.

LEE, V.M-Y., WU, H.L., SCHLAEPFER, W.W. (1982). Monoclonal antibodies recognise individual neurofilament triplet proteins. *Proc. Natl. Acad. Sci. USA 79:* 6089-6092.

LETERRIER, J.F., LIEM, R.K.H. and SHELANSKI, M.L. (1982). Interactions between neurofilaments and microtubule associated proteins. A possible mechanism for intraorganellar bridging. *J. Cell Biol. 95:* 982-986.

LIEBERBURG, I., SPINNER, N., SNYDER, S., ANDERSON, J., GOLDGABER, D., SMULOWITZ, M., CARROLL, Z., EMANUEL, B., BREITNER, J. and RUBIN, L. (1989). Cloning of a cDNA encoding the rat high molecular weight neurofilament peptide (NF-H): developmental and tissue expression in the rat and mapping of its human homologue to chromosomes 1 and 22. *Proc. Natl. Acad. Sci. USA 86:* 2463-2467.

LIEM, R.K.H., YEN, S.H., SALOMON, G.D. and SHELANSKI, M.L. (1978). Intermediate filaments in nervous tissue. *J. Cell Biol. 79:* 637-645.

LOWRY, O.H., ROSEBROUGH, N., FARR, A.L. and RANDALL, R.J. (1951). Protein measurement with the Folin-Phenol reagent. *J. Biol. Chem. 193:* 265-275.

MARC, C., CLAVEL, M.C. and RABIE, A. (1986). Non phosphorylated and phosphorylated neurofilaments in the cerebellum of the rat: an immunohistochemical study using monoclonal antibodies. Development in normal and thyroid-deficient animals. *Dev. Brain Res. 26:* 249-260.

NIXON, R.A. and SHEA, T.B. (1992). Dynamics of neuronal intermediate filaments: a developmental perspective. *Cell Motil. Cytoskel. 22:* 81-91.

NIXON, R.A. and SIHAG, R.K. (1991). Neurofilament phosphorylation: a new look at regulation and function. *Trends Neurosci. 14:* 501-506.

NIXON, R.A., BROWN, B.A. and MAROTTA, C.A. (1982). Post translational modification of a neurofilament protein during axoplasmic transport: implication for regional specialization of CNS axons. *J. Cell Biol. 94:* 150-158.

NIXON, R.A., LEWIS, S.E. and MAROTTA, C.A. (1987). Posttranslational modification of neurofilament proteins by phosphate during axoplasmic transport in retinal ganglion cell neurons. *J. Neurosci. 7:* 1145-1158.

NOETZEL, M.J., ROOTS, B.I. and AGARWAL, H.C. (1986). Appearance and phosphorylation of the 210 kDa neurofilament protein in newborn rat brain, spinal cord and sciatic nerve. *Neurochem. Res. 11:* 363-374.

OBLINGER, M.M. (1987). Characterization of posttranslational processing of the mammalian high-molecular-weight neurofilament protein *in vivo*. *J. Neurosci. 7:* 2510-2521.

PACHTER, J.S. and LIEM, R.K.H. (1984). The differential appearance of neurofilament triplet polypeptides in the developing rat optic nerve. *Dev. Biol. 103:* 200-210.

PENG, I., BINDER, L.I. and BLACK, M.M. (1986). Biochemical and immunological analysis of cyloskeletal domains of neurons. *J. Cell Biol. 102:* 252-262.

RAJU, T., BIGNAMI, A. and DAHL, D. (1981). *In vivo* and *in vitro* differentiation of neurons and astrocytes in the rat embryo. *Dev. Biol. 85:* 344-357.

RIOL, H., FAGES, C. and TARDY, M. (1992). Transcriptional regulation of glial fibrillary acidic protein (GFAP)-mRNA expression during postnatal development of mouse brain. *J. Neurosci. Res. 32:* 79-85.

ROUSSEL, G., FELIX, J.M., DAUTIGNY, A., PHAM-DINH, D., HINDELANG, C., JOLLES, P. and NUSSHAUM, J.L. (1991). *In situ* localization of NF-H neurofilament subunit mRNAs in rat brain. *Dev. Neurosci. 13:* 98-103.

SCHLAEPFER, W.W. (1987). Neurofilaments: structure, metabolism and implications in disease. *J. Neuropathol. Exp. Neurol. 46:* 117-129.

SCHNITZER, J., FRANKE, W.W. and SCHACHNER, M. (1981). Immunocytochemical demonstration of vimentin in astroglia and ependymal cells of developing and adult mouse nervous system. *J. Cell Biol. 90:* 435-447.

SHARP, G.A., SHAW, G. and WEBER, K. (1982). Immunoelectron microscopical localization of the three neurofilament triplet proteins along neurofilaments of cultured dorsal root ganglion cells. *Exp. Cell Res. 137:* 403-413.

SHAW, G., and WEBER, K. (1982). Differential expression of neurofilament triplet proteins in brain development. *Nature 298:* 277-279.

SHELANSKI, M.L. and LIEM, R.K.H. (1979). Neurofilaments. *J. Neurochem. 33:* 5-13.

STERNBERGER, L.A. and STERNBERGER, N.H. (1983). Monoclonal antibodies distinguish phosphorylated and nonphosphorylated forms of neurofilaments *in situ*. *Proc. Natl. Acad. Sci. USA 80:* 6126-6130.

STERNBERGER, L.A., HARDY, P.N., CUCULIS, J.J. and MEYER, H.G. (1970). The unlabelled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-anti-horseradish peroxidase) and its use in the identification of spirochaetes. *J. Histochem Cytochem. 18:* 315-333.

TOHYAMA, T., LEE, V.M-Y., RORKE, L.B. and TROJANOWSKI, J.Q. (1991). Molecular milestones that signal axonal maturation and the commitment of human spinal cord precursor cells to the neuronal or glial phenotype in development. *J. Comp. Neurol. 310:* 285-299.

TOWBIN, H., STAEHELIN, T. and GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA 76:* 4350-4354.

TROJANOWSKI, J.Q., OBROCKA, M.A. and LEE, V.M-Y. (1985). Distribution of neurofilament subunits in neurons and neuronal processes: immunohistochemical studies of bovine cerebellum with subunit specific monoclonal antibodies. *J. Histochem. Cytochem. 33:* 557-563.

TROJANOWSKI, J.Q., WALKENSTEIN, N. and LEE, V.M-Y. (1986). Expression of neurofilament subunits in neurons of the central and peripheral nervous system: an immunohistochemical study with monoclonal antibodies *J. Neurosci. 6:* 650-660.

WATANABE, M.K., SAKIMURA, K., TAKAHASHI, V. and KONDO, H. (1990). Ontogenic changes in expression of neuron-specific enolase (NSE) and its mRNA in the Purkinje cells of the rat cerebellum: immunohistochemical and *in situ* hybridisation study. *Dev. Brain Res. 53:* 89-96.

WILLARD, W. and SIMON, C. (1983). Modulations of neurofilament axonal transport during the development of rabbit retinal ganglion cells. *Cell 35:* 551-553.

YACHNIS, A.T., RORKE, L.B., LEE, V.M-Y. and TROJANOWSKI, J.Q. (1993). Expression of neuronal and glial polypeptides during histogenesis of the human cerebellar cortex including observations on the dentate nucleus. *J. Comp. Neurol. 334:* 356-369.

Accepted for publication: June 1994