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Expression of vimentin and glial fibrillary acidic protein in the developing rat spinal cord: an immunocytochemical study of the spinal cord glial system

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INTRODUCTION

Vimentin and glial fibrillary acidic protein (GFAP) belong to a group of proteins, designated as intermediate filaments (IF, for review see Lazarides, 1980). The phosphoprotein vimentin is present in the cytoskeleton of eukaryotic cells of mesenchymal origin (Cabral & Gottesman, 1979; Gard & Lazarides, 1982). Vimentin (M_r 57 kDa) has been characterised extensively and recently the nucleotide sequence of the vimentin gene and its amino acid sequence were elucidated (Geisler, Plessman & Weber, 1982, 1983; Ferrari *et al.* 1986; Xehner, Li, Roe, Patterson & Sax, 1987). Next to its function in the cytoskeleton (Lazarides, 1980; Traub, 1985), other functions have been suggested for vimentin. It is supposed to play a role in neurogenesis because of its coexpression with neurofilaments in neuroblasts (Houle & Fedoroff, 1983; Cochard & Paulin, 1984; Lee & Page, 1984). More recently, evidence has been published on the role of vimentin in DNA replication and recombination, DNA repair and in gene expression (Geiger, 1987; Georgatos & Blodel, 1987*a*, *b*). In spite of all these indications or results on 'new' functions of vimentin, its precise role has yet to be elucidated.

GFAP (M_r 51 kDa) is present in the cytoskeleton of protoplasmic and fibrous astroglial cells (Ludwin, Kosek & Eng, 1976; Bignami & Dahl, 1977; Bignami, Raju & Dahl, 1983; Bovalenta, Liem & Mason, 1987). Its molecular structure has been characterised and is largely similar throughout the vertebrates (Onteniente, Kimura & Maeda, 1983). GFAP functions as a structural component of the astroglial cytoskeleton. On the other hand, GFAP has been demonstrated in various neural and nonneural cells, which could indicate other functions for it which are yet to be discovered (Hansen, Stagaard & Møllgård, 1989).

From a developmental point of view, vimentin and GFAP are of especial interest. Both proteins are particularly useful as markers of glial differentiation (Steinert & Roop, 1988; Tardy *et al.* 1989). With regard to the development of the central nervous system (CNS), their expression in glial cells has been well documented. Glial cells play an important pivotal role in the developing nervous system (Giulian, Vaca & Johnson, 1988; Kalderon, 1988). Neuronal differentiation and the general structural organisation in the brain depend on the temporal and spatial association with neighbouring astrocytes (Aguayo, David & Bray, 1981; Silver, Lorenz, Wahlsten & Coughlin, 1982). In this respect, diverse actions of glial cells can be considered in the

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CNS such as controlling cellular movement by providing a substrate for migration of maturing neurons (Rakic, 1972; Sidman & Rakic, 1973), modelling fibre pathways by formatting a structural matrix and guiding growing axons (Silver *et al.* 1982; Innocenti, Clarke & Koppel, 1983). Oligodendroglial cells are associated with the production of myelin sheaths (Bunge, 1968) and with the metabolism of neurons (Schmechel & Rakic, 1979). The role of glial cells, and therefore the role of vimentin and GFAP in CNS differentiation processes, both seem of vital importance. On the other hand, in the chick cerebellum a relation between radial glia and the formation of longitudinal cytoarchitectonic patterns could not be demonstrated (Roeling & Feirabend, 1988).

During morphogenesis of the rat spinal cord, 3 different layers can be observed: the ventricular ependymal or matrix layer (ML), the adjacent mantle layer (grey matter) and the peripheral marginal layer (white matter) (Altman & Bayer, 1984). Between embryonal day 11 (E11) and E16, cells of the lateral ML proliferate along a ventral-to-dorsal gradient and form the mantle layer. Cells of the ventral and dorsal part of the matrix layer, designated as the floor plate (FP) and roof plate (RP), respectively, are considered to be the source of the neuroglial cell types of the spinal cord (Altman & Bayer, 1984). Between E12 and E17, axonogenesis and dendrogenesis of the ventral motor neurons take place (Altman & Bayer, 1984). At E13, the first contralaterally and ipsilaterally projecting fibres can be discerned in the ventral funiculus (VF) and the lateral funiculus (LF), respectively (Oudega, 1990). On the same day, the first dorsal root fibres penetrate the spinal cord at the dorsal root entry zone (DREZ; Altman & Bayer, 1984; Oudega, 1990). The peripheral marginal layer contains the various ascending and descending fibre systems of the spinal cord.

The knowledge of the expression of vimentin within the developing mammalian CNS is still far from complete. Vimentin was found throughout the closed murine neural tube (Bovolenta, Liem & Mason, 1984; Cochard & Paulin, 1984). Dupouey, Benjelloun & Gomes (1985) showed an intriguing architecture of vimentin-positive radial glia throughout the embryonic mouse CNS. A similar glial pattern has been found in the rat spinal cord (Bittner, Benjelloun-Touimi & Dupouey, 1987; Hirano & Goldman, 1988). This palisading organisation is thought to serve a role in the structural organisation of the CNS. The expression of GFAP in the CNS has been examined more thoroughly (Dahl & Bignami, 1973; Bignami & Dahl, 1974). In the rat, GFAP in astrocytes was detected at E16 (Abney, Bartlett & Raff, 1981). GFAP and vimentin has been studied in the developing rat pyramidal tract (Bignami & Dahl, 1974; Joosten & Gribnau, 1989). An involvement of vimentin-positive glial cells in the guidance of the pioneer corticospinal tract axons has been suggested (Joosten & Gribnau, 1989).

Studies in which the expression of vimentin and/or GFAP is correlated with CNS developmental processes are rare. The main objective of the present paper was to fill this lack of information. Vimentin and GFAP are demonstrated by using a monoclonal antibody (V9) and a polyclonal antibody (anti-GFAP), respectively. Both antibodies were specified and described before (Baumal *et al.* 1980; Tascos, Parr & Gonatas, 1982; Muijen *et al.* 1984; see also Roeling & Feirabend, 1988).

MATERIALS AND METHODS

Animals

Rats were housed under standard conditions (light/dark regime, 4–12 am lights on, relative humidity of 50–60 %). The rats were fed ad libitum with standard rat chow. Rat

embryos were taken from timed-pregnant females. Mating was allowed during a 4 h period, from 8 to 12 am (Zwet, Tilburg & Poelmann, 1986). The fetuses were removed after anaesthesia of the timed-pregnant mother with Hypnorm (0·3 ml/kg s.c.) and Valium (diazepam, 1 ml/kg i.m.). The age of the fetuses was indicated with the day of conception as embryonal day zero (E0). After a natural birth, the newborn animals were anaesthetised with Valium (1 μl/g) and Hypnorm (0·3 μl/g) before further histochemical processing. The day of birth, usually E22, was indicated as postnatal day zero (P0). Postnatal rats were taken from several litters. A total of 26 fetal and postnatal ages were examined. From E9 on, at least 6 embryos of each embryonal age were studied, whereas the postnatal groups comprised a minimum of 4 rats. The postnatal ages examined were P1, 2, 4, 6, 8, 10, 12, 16, 20, 24, 30 and maturity.

Histological procedures

Nonfixed rat embryos were placed in tissue Tek O.C.T. compound (Miles Laboratories, Naperville, USA) on a tissue holder and frozen in isopentane which was chilled with liquid nitrogen (Marani, 1978). The spinal cords of postnatal nonfixed rats were dissected out and frozen as described above. Transverse cryostat sections (12 μm) were mounted on chrome-alum/gelatin-coated slides, briefly fixed in acetone (-20 °C) and air-dried overnight. Subsequently the sections were fixed in acetone at room temperature for 10 min and air-dried for at least 24 h before further immunocytochemical processing.

Immunocytochemistry

The primary antisera used in this study were anti-vimentin (V9, culture fluid, as specified by Muijen *et al.* 1984; Sanbio, Uden, The Netherlands) and anti-GFAP (rabbit-anticow GFAP antibodies, DAKO No. Z334, specified by Baumal *et al.* 1980, and Tascos *et al.* 1981; DAKO, Glostrup, Denmark). The following secondary antisera were used: RAM/PO, rabbit-antimouse antibodies, conjugated to horseradish peroxidase (DAKO) and GAR/PO, goat-antirabbit antibodies, conjugated to horseradish peroxide (Nordic, Tilburg, The Netherlands).

For indirect immunostaining, sections were rinsed 3 times for 10 min in phosphate-buffered saline (PBS, pH 7·4). Afterwards the sections were incubated overnight with the primary antiserum in moist chambers at room temperature. V9 was used undiluted. Anti-GFAP was used diluted 1:500 in PBS (pH 7·4). The sections were rinsed 3 times for 10 min with PBS (pH 7·4). The sections were then incubated for 2 h in moist chambers at room temperature with the peroxidase-conjugated secondary antiserum. RAM/PO was applied in a 1:500 and GAR/PO in a 1:1000 dilution in PBS (pH 7·4). Next, the sections were rinsed 3 times for 10 min with 50 mM Tris-HCl buffer (pH 7·4) and incubated with 80 mM 3,3′-diamino-benzidine-4-HCl (DAB; Serva, Heidelberg, Germany) and 3 mM H_2O_2 dissolved in 50 mM Tris-HCl buffer (pH 7·4). After thorough rinsing with Tris-HCl buffer, the sections were briefly counterstained with haematoxylin, rinsed in tap water for 10 min and dehydrated through graded alcohols to xylene and coverslipped with Entellan.

Control experiments were carried out for the detection of nonspecific immunocytochemical reactions and endogenous peroxidase activity. Sections of several ages were incubated with preimmune normal mouse serum or preimmune normal rabbit serum. Each separate step of the procedure was also controlled for nonspecific reactions.

RESULTS

Specificity of the antisera

All control incubations were negative. Endogenous peroxidase could only be detected in erythrocytes. Vimentin was found in blood vessels and in the meningeal membranes. The blood vessels were not studied. The meningeal tissue, however, will be described because of its apparent participation in the glial system of the rat spinal cord.

The antiserum employed for GFAP also seemed to recognise neurofilament proteins. From P20 on, the motor neurons and the fibre systems of the spinal cord white matter clearly stained positively. This cross-reactivity of the anti-GFAP antiserum has been described before (Hansen *et al.* 1989). Fortunately, the glial fibres demonstrated a unique staining pattern and, therefore, could easily be distinguished from the neuronal structures.

Vimentin and GFAP in the developing rat spinal cord

The ontogeny of vimentin and GFAP in rat spinal cord was studied with immunocytochemistry. Serial sections were examined at the cervical, thoracic and lumbar levels. For reasons of clarity the vimentin and GFAP localisations will be described as found at the thoracic level. The results will be described separately for each layer of the spinal cord. Figure 1 shows schematic drawings of the rat spinal cord development.

Matrix layer

At E11, the first vimentin staining was found in the most ventral aspect of the ependymal layer or matrix layer. It could not be decided whether or not vimentin was located in the cells or in the membrana limitans externa (MLE). One day later, at E12, vimentin was clearly present in the MLE around the ependymal layer (Fig. 2*a*). In the floor plate (FP) and in the intermediate matrix layer, between the basal and alar plate, short protrusions penetrated the ML. At the same age, a scattered pattern of vimentin staining could be found throughout this layer at cervical and higher thoracic levels. Positivity was found in a ventral-to-dorsal gradient abundantly at the ventrally situated basal plate and more faintly in the dorsally situated alar plate. The alar plate demonstrated a few radially oriented fibres that traverse this region. Throughout the matrix layer, tangles of short vimentin-positive fibres were observed (Fig. 2*a*).

At E13, the floor plate demonstrated a marked radial staining pattern. In the ventral part of the ML, vimentin staining was more regular in comparison with the pattern at E12. By this stage, some radial fibres could also be seen in the intermediate part of this layer. Vimentin-positive tangles were present throughout the ML. Fibres emerging from these tangles coursed in a vertical direction. At E14, a radial staining pattern was observed throughout the ML (Fig. 2*b*), but especially in its intermediate region. Scattered short fibres were also present. The floor plate exhibited especially abundant vimentin immunoreactivity (Fig. 2*b*). In the roof plate (RP) relatively few positive fibres were present.

At E15 and E16, radial vimentin staining was clearly present in the intermediate region of the ML, just beneath the sulcus limitans (Fig. 2*c*). The region of the sulcus limitans itself demonstrated less immunoreactivity. During the next 3 days in development, the ventricle was reduced to the small central canal, which was surrounded by a relatively thin matrix layer. Vimentin-positive fibres mostly originated from the intermediate ML (Fig. 2*d*). Those from the ventral part coursed in the

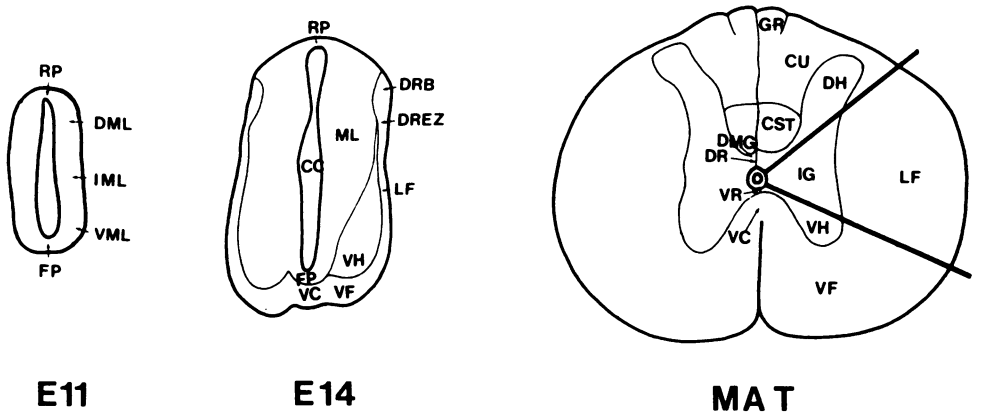


Fig. 1. Schematic representation of stages of the developing rat spinal cord. CC, central canal; CST, corticospinal tract; CU, cuneate fasciculus; DH, dorsal horn; DMG, dorsal medial grey; DML, dorsal matrix layer; DR, dorsal raphe; DRB, dorsal root bifurcation zone; DREZ, dorsal root entry zone; FP, floorplate; GR, gracile fasciculus; IG, intermediate grey; IML, intermediate matrix layer; LF, lateral funiculus; ML, matrix layer; RP, roof plate; VC, ventral commissure; VF, ventral funiculus; VH, ventral horn; VML, ventral matrix layer; VR, ventral raphe.

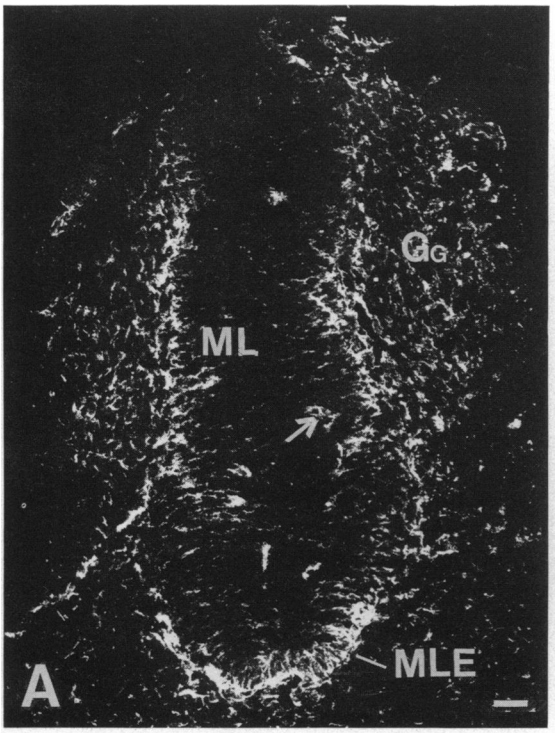
direction of the ventral commissure. This staining pattern persisted until the first postnatal week. The staining intensity, however, decreased during this period. At P4, the ML was almost negative for vimentin (Fig. 2*e*). Surprisingly, after P10 and especially at maturity, short vimentin fibres were found emerging from the matrix layer in all directions (Fig. 2.*f*).

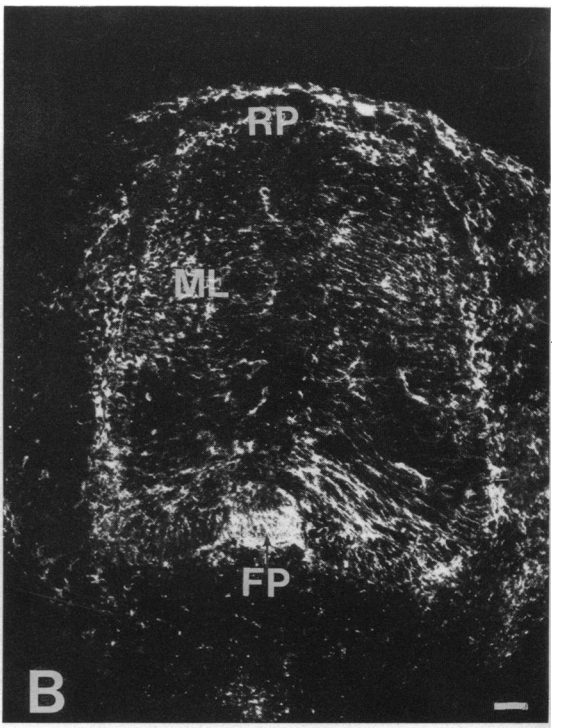
During embryonic life, GFAP immunoreactivity was not detected in the ML, except for sporadic fibres that traverse the layer. During the 1st postnatal week, GFAP-positive fibres were present in the ventral and intermediate regions of the matrix layer (Fig. 2*g*). These thin fibres curved sharply after leaving the layer, forming a small ventral raphe (VR, see below). From P8 on, GFAP-positive fibres emerged from the intermediate aspect of the ML. These fibres curved in vertical direction after penetrating the mantle layer (Fig. 2*h*).

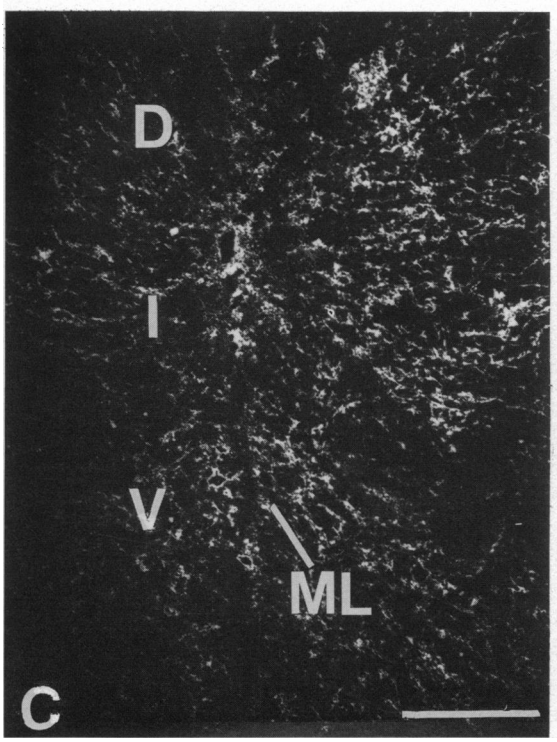
Mantle layer

At E13, the first vimentin immunoreactivity was found in the mantle layer. Its ventral part (ventral horn; VH, see Fig 2*b*) and the incipient intermediate grey (IG) exhibited an intense, radial staining. At E15, the dorsal aspect (dorsal horn; DH) also demonstrated this radial pattern. The fibres originated from the ML (see above). The fibres in the ventral horn followed an oblique course towards the ventrolateral funiculus (Fig. 2*b*). The ventral mantle layer with its obliquely coursing fibres was stained most intensely.

One day later, at E16, the mantle layer still demonstrated an abundant radial fibre pattern (Fig. 3*a*). The superficial layer of the dorsal horn was less intensely stained. At this age, the ventral and dorsal raphe were clearly positive for vimentin. Numerous fibres in the ventral raphe originated in the ventral part of the matrix layer and coursed after a sharp curve in a vertical direction. The dorsal raphe was recognised as a concentration of vimentin-positive fibres, running between the central canal and the dorsal border of the cord. Between E18 and E22, the vimentin fibre pattern did not change but its staining intensity decreased during this period (Fig. 3*b*).







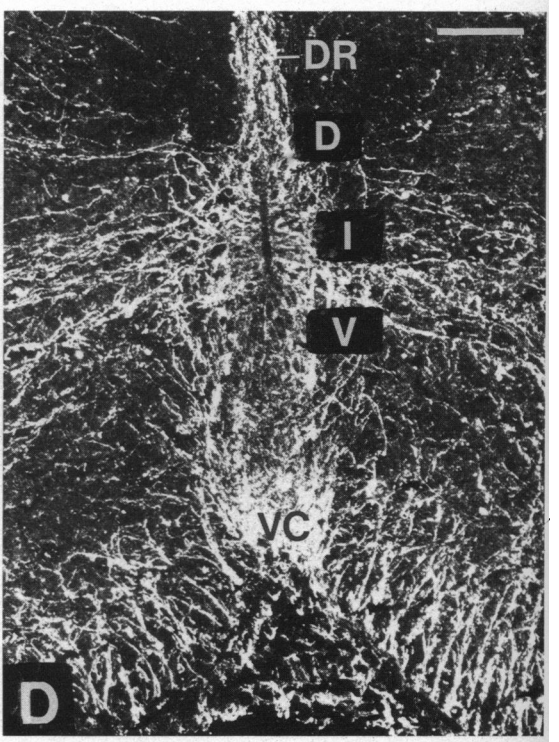
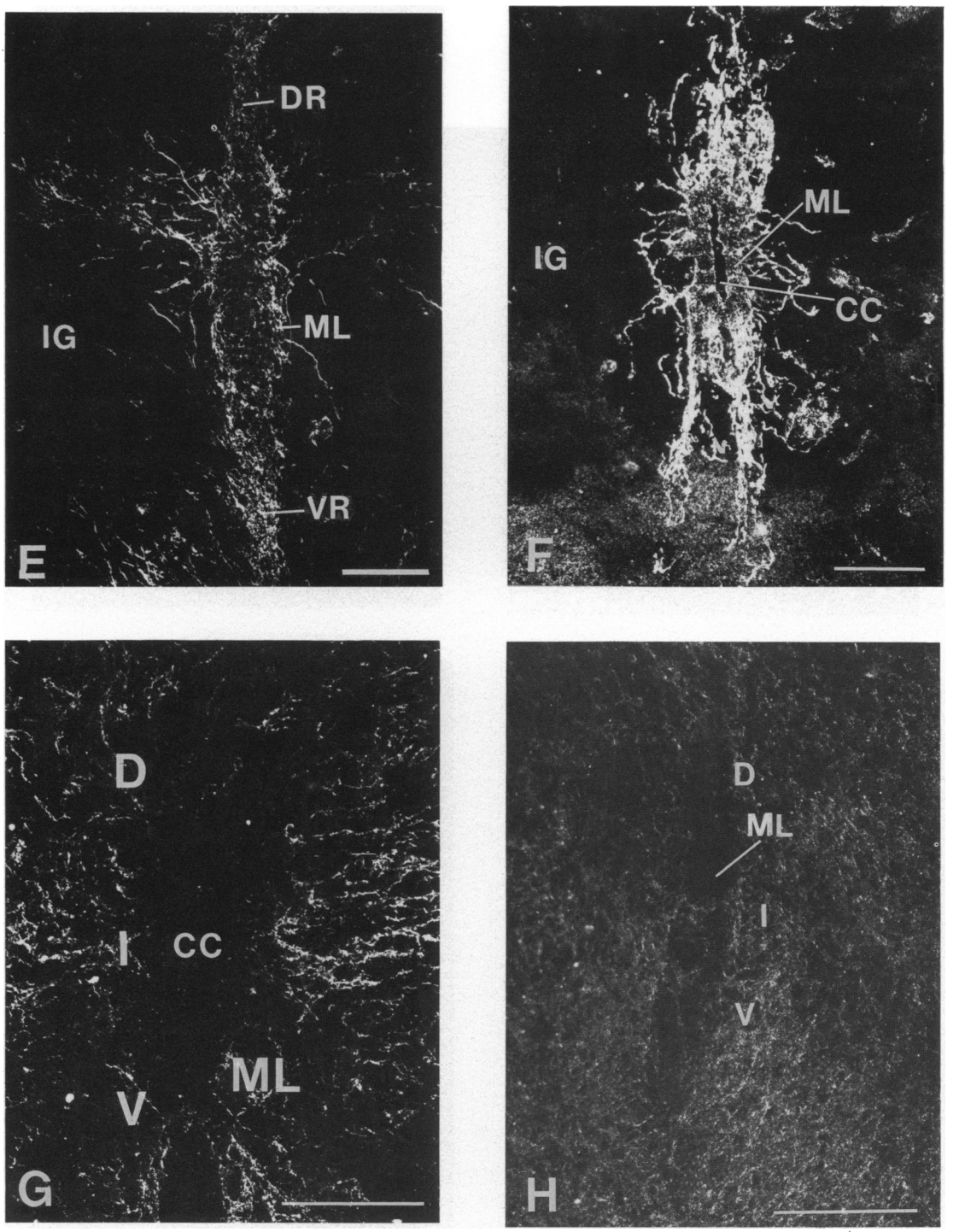


Fig. 2. Expression of vimentin (*a–f*) and GFAP (*g*, *h*) in the matrix layer of the developing rat spinal cord. All figures are dark field photomicrographs. (*a*) E12. Note the abundant expression of vimentin in the dorsal root ganglion (Gg). The arrow points to a tangle of vimentin-positive fibres. (*b*) E14. The intermediate aspect of the matrix layer displays abundant vimentin staining. (*c*) E16. Detail of



the matrix layer. Note the 2 'empty' spots. (*d*) E19. D, I and V represent the dorsal, intermediate and ventral region of the matrix layer. (*e*) P4. Note that the matrix layer is almost negative for vimentin. (*f*) Mature. Vimentin fibres course from the matrix layer in all directions. (*g*) P4. GFAP-positive fibres emerge from the ventral and intermediate matrix layer. (*h*) P20. GFAP-positive fibres from the intermediate matrix layer course in vertical direction. Abbreviations as used in the text; D, I and V represent the dorsal, intermediate and ventral region of the matrix layer. Bars, 10 μm. Other abbreviations as in Figure 1.

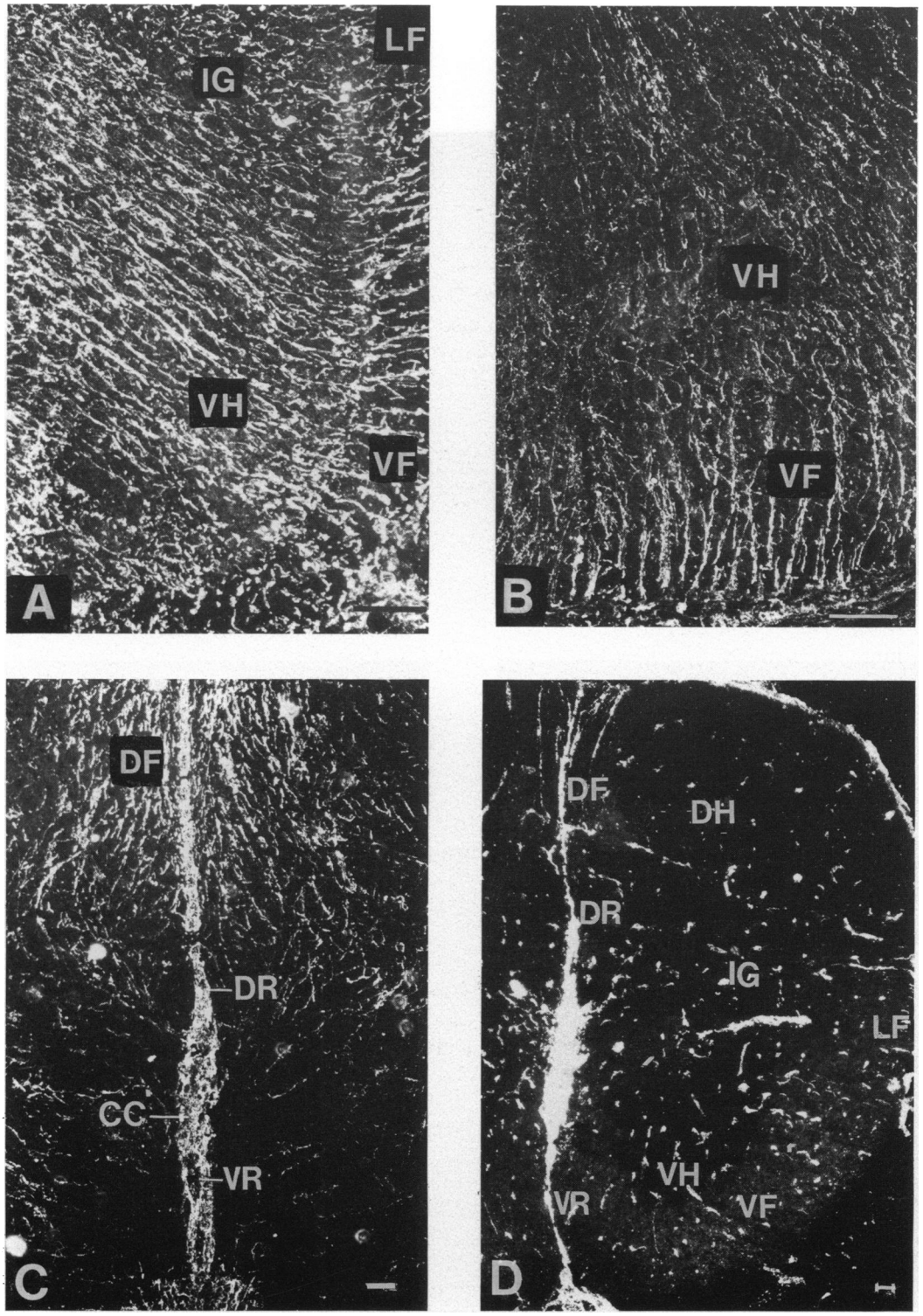


Fig. 3. Vimentin expression in the developing mantle layer of the rat spinal cord. All figures are dark field photomicrographs. (*a*) E16. The ventral mantle layer or ventral horn (VH) contains abundant staining of obliquely coursing fibres. (*b*) E22. Decreased intensity in the ventral horn (VH). (*c*) P6. The ventral and dorsal raphe (VR and DR, respectively) stain abundantly for vimentin. (*d*) P20. Vimentin in the mantle layer has decreased; only some scattered short fibres and blood vessels are present. DF, dorsal funiculus; other abbreviations as in Figure 1. Bars, 10 μm.

During the 1st postnatal week, the overall vimentin-staining intensity gradually decreased in the mantle layer. The raphes, however, stained intensely (Fig. 3*c*). From P10 on, vimentin immunoreactivity could only be found scattered throughout the mantle layer, mostly in cells, but single immunoreactive, short protrusions were also found. By this stage the staining in the ventral and dorsal raphe also decreased in intensity. At P20, the adult configuration was present and only a single vimentin immunoreactive protrusion could be found. In both raphes, a few vimentin-positive fibres were present (Fig. 3*d*).

At E16, some scattered GFAP immunoreactive protrusions were present throughout the mantle layer. Two days later, at E18, GFAP was concentrated in the ventrolateral region of the ventral mantle layer (Fig. 4*a*). The fibres were thin and diffusely scattered over this area. The intermediate region also contained scattered short protrusions. The dorsal region of the mantle layer was almost negative for GFAP.

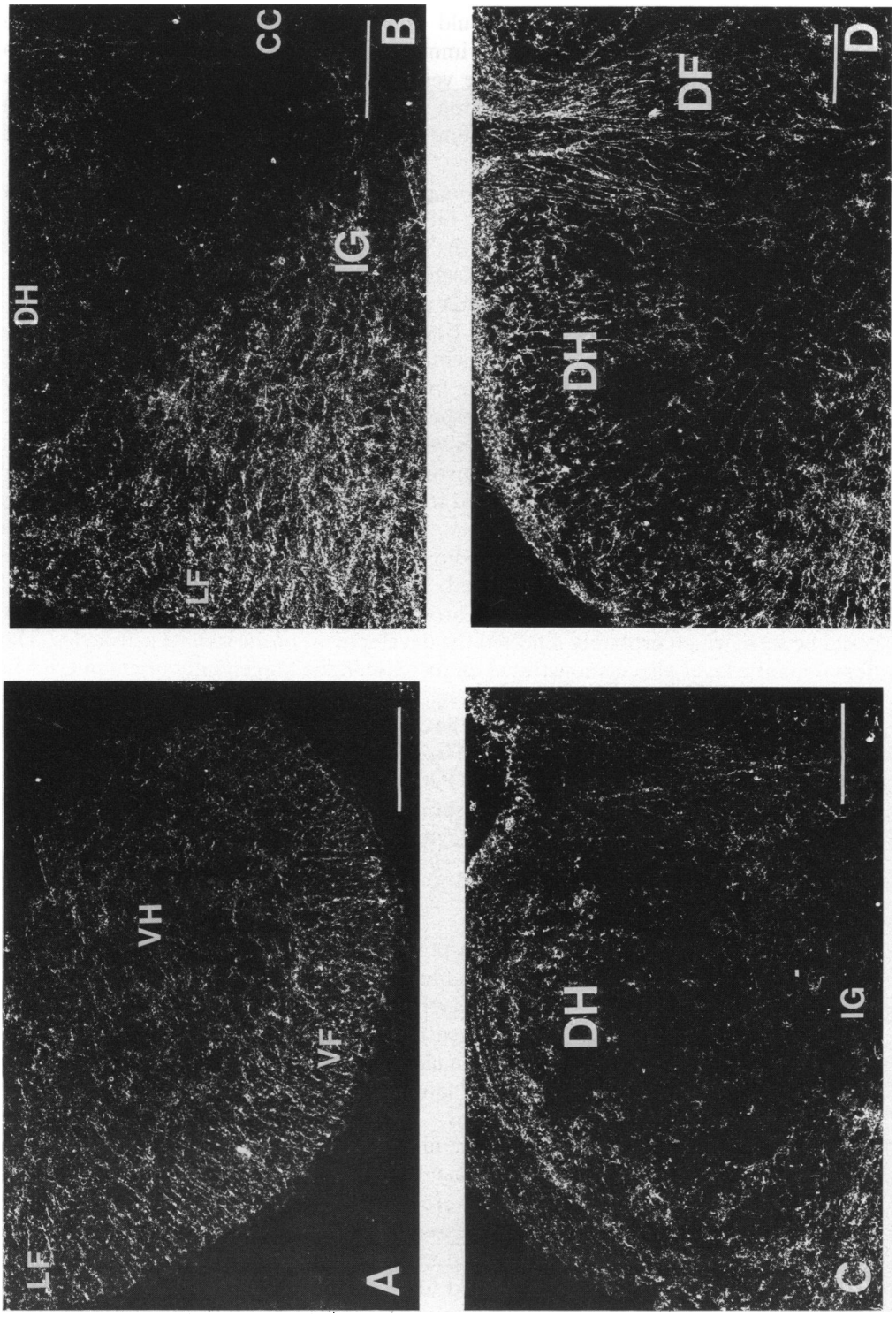
This pattern was also found between E19 and E22, although the general intensity increased. In addition, more radially oriented fibres were present in the intermediate grey (Fig. 4*b*). The dorsal aspect of the mantle layer was almost devoid of GFAP immunoreactivity, apart from a single dispersed small positive tangle (Fig. 4*c*). In the dorsal raphe, a few GFAP-positive fibres were observed. At P2, the ventral horn and especially the intermediate grey demonstrated an abundant staining of radially oriented GFAP fibres. The fibres emerged from the matrix layer and diverged towards the peripheral white matter. At this time, the dorsal horn contained more positive fibres. These fibres coursed in a vertical direction, converging towards the neck of the horn (Rexed's laminae III and IV; Rexed, 1952, 1954). Between P4 and P10, GFAP immunostaining increased explosively. Throughout the mantle layer, short protrusions could be seen, which probably reflected the development of the stellate astrocytes. The dorsal mantle layer also demonstrated an increase in the tangentially oriented GFAP-positive fibres. GFAP-positive tangles were clearly visible in the dorsal horn (Fig. 4*d*). At P6, long, thin fibres were observed in the dorsal raphe. At P8, the dorsal and ventral raphes were markedly GFAP-positive. Their fibres originated from the intermediate region of the matrix layer (Fig. 4*e*). At P20, the adult staining pattern was present. Many GFAP-positive cells, with several short protrusions, were found throughout the mantle layer, which represented the astrocytes of the spinal cord (Fig. 4*f*). Both raphes were markedly positive for GFAP.

Marginal layer

At E13, disarranged short vimentin-positive fibres were found in the ventral funiculus (VF) and in the lateral funiculus (LF). In DREZ and the dorsal root bifurcation zone (DRB; see Altman & Bayer, 1984; Oudega, 1990) only a few positive protrusions were found (Fig. 5*a*). The ventral commissure (VC) contained a distinct vimentin pattern. One day later, at E14, a clear radial vimentin pattern was present in the ventral funiculus, whereas the thin lateral funiculus and the DREZ/DRB still contained the short scattered protrusions.

At E15, a palisade of vimentin-positive fibres was present in the ventral and lateral funiculus. One day later, this could also be observed in the dorsal funiculus. In addition, short vimentin-positive fibres were observed (Fig. 5*b*). Between E18 and P6, the vimentin staining pattern remained essentially the same (Figs 3*c*, 5*c*). In general, the staining intensity gradually decreased after P8.

Around P6, the vimentin fibres seemed to 'withdraw' from the ventral and lateral funiculus. In the dorsal funiculus, the vertically oriented fibres converged towards its ventral apex. At P20, the adult staining pattern was observed. Vimentin fibres by this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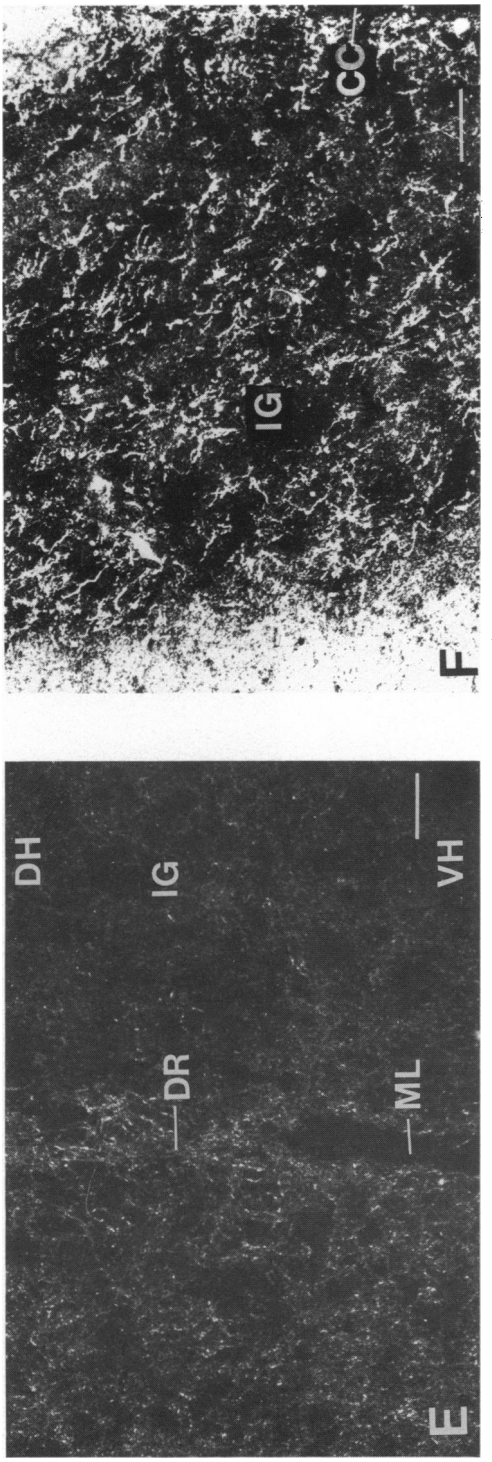

Fig. 4. GFAP immunoreactivity in the developing mantle layer of the rat spinal cord. All figures are dark field photomicrographs. (*a*) E18. Scattered fibres originate from the ventrolateral part of the mantle layer. (*b*) E20. Strong increase of GFAP reactivity in the intermediate grey. (*c*) E20. Dispersed tangles in the dorsal horn (DH). (*d*) P10. Dispersed tangles and some vertically directed fibres in the dorsal horn (DH). (*e*) P8. Fibres in the dorsal and ventral raphe (DR and VR, respectively) that originate from the intermediate region of the matrix layer. (*f*) Mature. GFAP-positive stellate cells are present throughout the mantle layer. Other abbreviations as in Figure 1. Bars, 30 μm

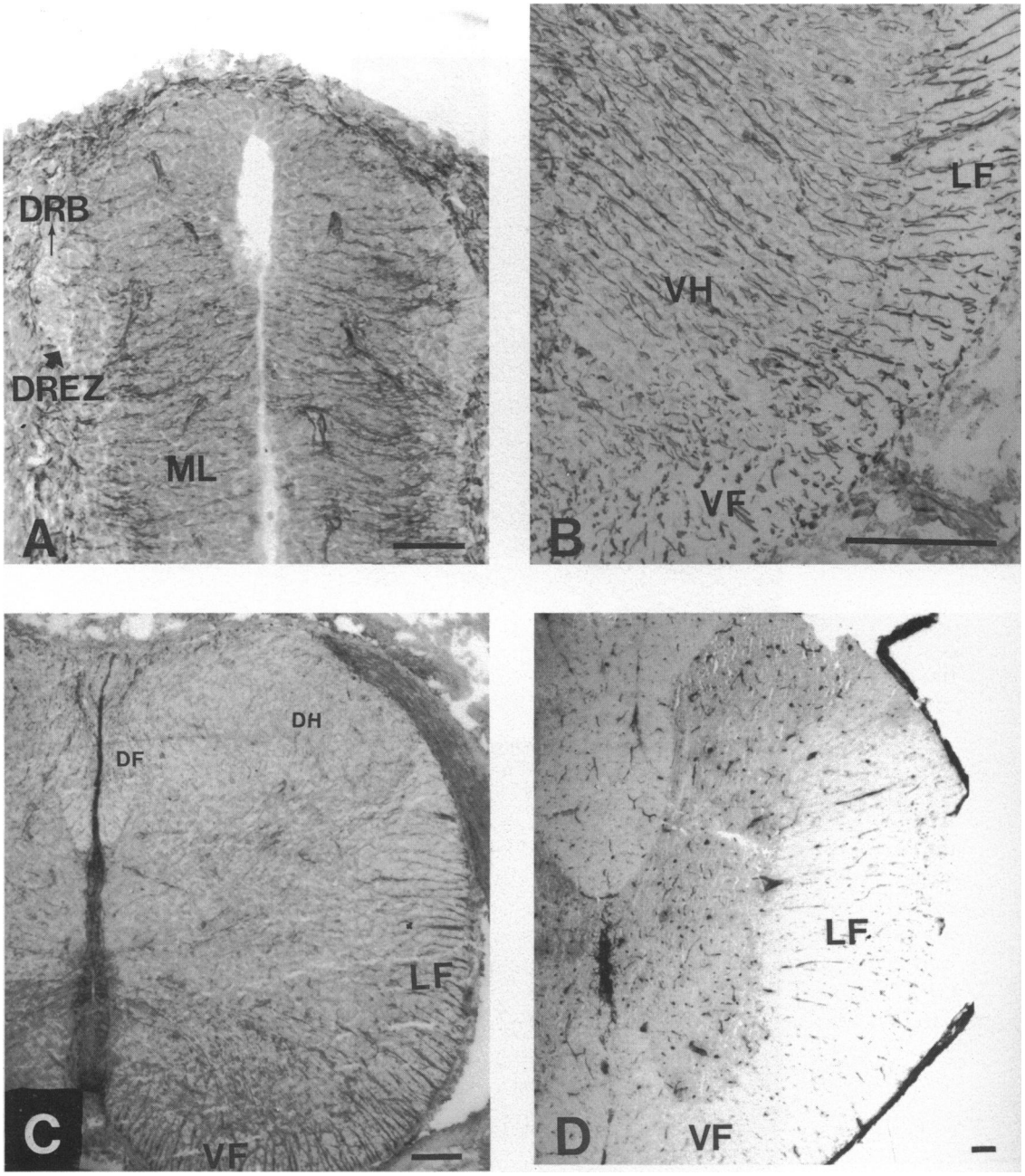

Fig. 5. Immunocytochemical localisation of vimentin and GFAP in the developing white matter of the rat spinal cord. All figures are bright field photomicrographs. (*a*) E13. A few vimentin fibres are seen in the dorsal root bifurcation zone (DRB) and the dorsal root entry zone (DREZ, arrow). Some vimentin tangles are clearly visible. (*b*) E16. Scattered and radially oriented vimentin fibres are present in the ventral and lateral funiculus (VF and LF, respectively). (*c*) P6. Decreased vimentin immunostaining in the white matter. (*d*) P20. The different funiculi are almost devoid of vimentin immunoreactivity. (*e*) P8. General increase of GFAP immunostaining in the white matter. (*f*) Mature. GFAP expression in the adult spinal cord. Other abbreviations as in Figure 1. Bars, 30 μm.

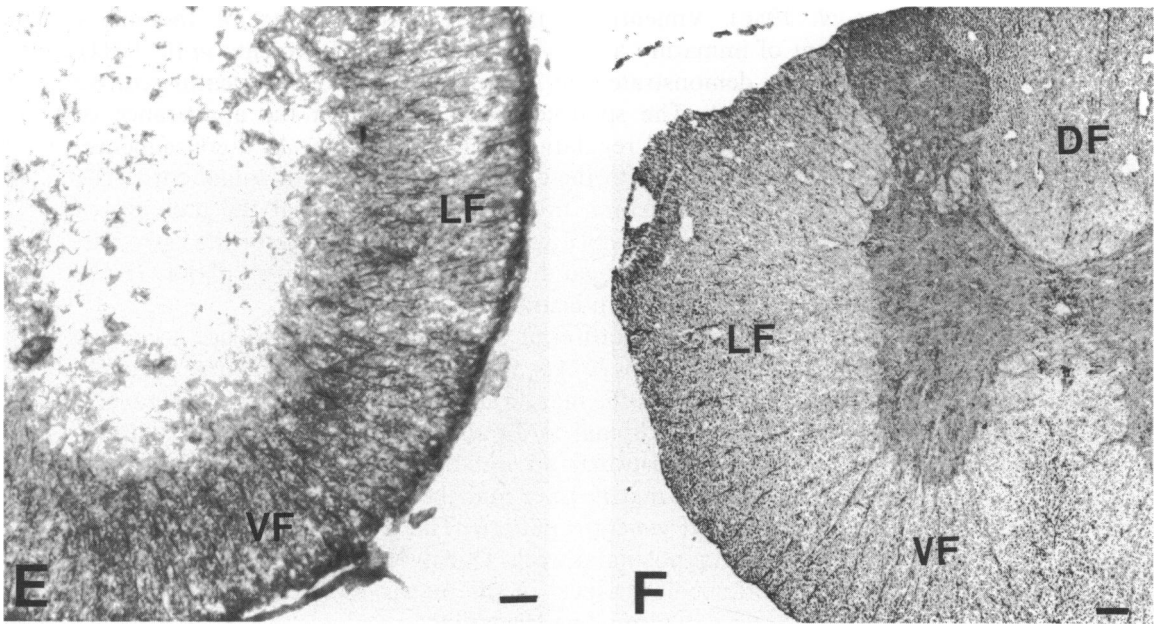

Fig. 5(*e*,*f*). For legend see p. 108.

time had vanished from the ventral and lateral funiculus (Figs 3*d*, 5*d*). A few scattered protrusions were still found in the dorsal funiculus.

GFAP-positive, disarranged short protrusions were first detected in the ventral and lateral funiculus, at E18. These protrusions originated from the ventrolateral aspect of the ventral mantle layer. In addition, radially oriented fibres were found (see Fig. 4*a*). At E19, the first scattered protrusions were present in the dorsal funiculus. In addition, the dorsolateral funiculus contained an abundant GFAP pattern (Fig. 4*c*). At this age, radial GFAP fibres were observed in the ventral funiculus. At E21, this region as well as the lateral funiculus were filled in with a palisade of GFAP-positive fibres. Around P6, GFAP staining in the marginal layer explosively increased (Fig. 5*e*). The fibres were arranged in a palisade pattern. At P20, the mature pattern was attained. The GFAP-positive fibres in the mature marginal layer were distributed in the same pattern (Fig. 5*f*).

DISCUSSION

The development of the glial system in the rat spinal cord was investigated for vimentin and GFAP using immunocytochemistry. The vimentin-GFAP transition period in the rat spinal cord takes up the first 3 postnatal weeks (Joosten & Gribnau, 1989). Moreover, in vitro studies demonstrated a linear increase of GFAP between P3 and P14 (Goldman, Geier & Hirano, 1986). During the transition period, glial cells were found to express GFAP and vimentin simultaneously (Schnitzer, Frank & Schachner, 1981; Pixley & De Vellis, 1984; Joosten & Gribnau, 1989). The present results showed a general transition from vimentin to GFAP in the rat spinal cord during the 2nd and 3rd postnatal weeks, but it could not be decided whether both proteins are expressed simultaneously by the same cell. The replacement of vimentin by GFAP is thought to reflect the differentiation of glioblasts into astrocytes (Dahl,

1981; Herpers *et al.* 1986). Vimentin is therefore considered to be the major cytoskeletal component of immature astrocytes (Dahl, 1981; Bovolenta *et al.* 1984). The present study clearly demonstrated an elaborate network of vimentin-positive fibres in the developing cord. The spatiotemporal pattern in the appearance of vimentin indicated a developmental regulation of its expression. As a consequence, vimentin is likely to play a vital role in the development of the rat spinal cord. The present morphological results, therefore, favour the proposal that the presence of vimentin in the glial cytoskeleton reflects a specific functional stage of the astrocytes (Steinert & Roop, 1988). So far, functional differences between vimentin and GFAP-positive astrocytes have never been demonstrated experimentally.

Within the matrix layer, the proliferation of the neuroblasts takes place according to a ventral-to-dorsal gradient (Nornes & Das, 1974; Altman & Bayer, 1984). Between E11 and E16, these neuroblasts form the mantle layer. In addition, the matrix layer is regarded as the site of origin for the spinal cord radial glial cells (Hirano & Goldman, 1988). The present results demonstrated an initial pattern of scattered vimentin immunoreactive structures in the matrix layer and the mantle layer. Later on, both layers exhibited a distinct radial vimentin pattern. The replacement of vimentin by GFAP took place during the 1st postnatal week. During this period, the GFAP fibres which originated in the intermediate aspect of the matrix layer curved sharply and coursed into the ventral and dorsal midline structures.

The (early) scattered vimentin pattern in the matrix layer suggests the presence of vimentin in neuroblasts rather than in developing glial cells. Earlier a coexpression of vimentin with neurofilament subunits in neuroblasts had been demonstrated, which indicated a role of vimentin in neurogenesis (Bignami *et al.* 1983; Cochard & Paulin, 1984; Lee & Page, 1984). In addition, a temporal relationship between vimentin and neural tube development was suggested by Houle & Federoff (1983).

The radial fibre pattern in the developing and mature mantle layer may indicate the involvement of vimentin and GFAP in the structural organisation of the spinal cord (Aguayo *et al.* 1981; Silver *et al.* 1982). This radial orientation suggested a role of spinal cord glial cells in neuroblast migration, from the matrix layer towards their final location in the mantle layer. Glial cells have been found to serve as an appropriate substrate for migrating neurons (Rakic, 1972; Sidman & Rakic, 1973).

At birth, GFAP fibres in the dorsal horn are oriented tangentially, which is contrary to the general orientation of the spinal cord glial fibres. The fibres of the dorsal horn converged towards its apex, the so-called neck region of the dorsal mantle layer. This pattern may indicate the involvement of GFAP-positive glial fibres with ingrowing primary afferents.

At E11 and E12, vimentin was already present in the MLE. Short protrusions seemed to penetrate into the most ventral part of the matrix layer. The precise function of these vimentin-positive protrusions has yet to be determined. Tangles of vimentin fibres were found in the early development of the matrix layer. Their origin and function also remains to be elucidated.

The motor neurons of the ventral horn, which originate from the ventral matrix layer at E11–E12, form their axons between E12 and E14. Dendrogenesis takes place between E14 and E17 (Nornes & Das, 1974; Altman & Bayer, 1984). The possible involvement of vimentin with these developmental processes was discussed above. At E18, however, GFAP-positive fibres in the ventral horn are clearly associated with the motor neurons in the ventral motor columns. These GFAP-positive glial fibres did not originate from the matrix layer. It is therefore suggested that these glial extrusions develop from a local source.

The floor plate and roof plate are considered to be the sources of glial cells in the rat spinal cord (Altman & Bayer, 1984). The present study clearly demonstrated the intermediate matrix layer as the site of origin of the glial fibres in the ventral and dorsal spinal cord raphe too. From E14 on, vimentin fibres were present in the ventral raphe. During the 2nd postnatal week, vimentin immunoreactivity diminished whereas GFAP immunoreactivity increased. The GFAP-positive fibres originated from the intermediate region of the matrix layer. The development of the dorsal raphe showed large similarities with the ventral raphe. In the dorsal raphe, a major vimentin barrier was also replaced by a thinner GFAP barrier at maturity. The function of these 'glial-barriers' is not completely understood. A physical role in the guidance of growing axons has been suggested (Hankin & Silver, 1986).

The marginal layer of the spinal cord contains the ascending and descending fibre systems. In the different funiculi, a striking radial arrangement could be demonstrated. Already at E14, vimentin was found as palisades in both the ventral and lateral funiculus. Later, the dorsal funiculus also showed the same palisade pattern. The vimentin–GFAP transition in the developing white matter occurred between P4 and P10. At E18, however, the first GFAP fibres in the ventral funiculus were already present. These fibres originated from the ventrolateral region of the ventral horn (see above). Later on, GFAP-positive fibres of the palisades in the ventral funiculus also originated from the matrix layer. A function of glia in the control of fibre growth was proposed earlier. The glial fibres should format the structural matrix for axonal growth (Singer, Norlander & Edgar, 1979; Silver *et al.* 1982; Hankin & Silver, 1986).

As a more speculative approach to the understanding of the function of these changes in expression from vimentin early in development towards GFAP positivity later in development it must be noted that it concerns a large population of the same glia cells that switch from vimentin to GFAP expression in the rat developmental spinal cord (Joosten & Gribnau, 1989).

Vimentin-positive radially oriented glial fibres are involved in the migration of the ventral motor neuroblasts, the neurons of the intermediate grey of the spinal cord and in generation and migration of neurons of the dorsal horn (see Houle & Fedoroff, 1983). Later on (E15–P2) vimentin is present in the developing white matter. Vimentin-positive glial fibres play a role in the formation of the funiculi (Joosten & Gribnau, 1989). During the first 2 postnatal weeks, GFAP-positive radially oriented glial fibres are involved in the development of the ventral horn, intermediate grey and dorsal horn. These GFAP-positive fibres also fulfil a role in the maintenance of the mature funiculi. GFAP positivity in the dorsal horn seems to be related to ingrowing primary afferents.

In conclusion, vimentin-positive radially oriented glial fibres contribute to the migration of neuroblasts in the matrix layer and in the mantle layer. They are also involved in the development of fibre systems in the spinal cord white matter. GFAP only contributes to a limited late period in the migration of neuroblasts. GFAP-positive glial fibres are involved in the postnatal outgrowth of fibre systems and the maintenance of the mature situation.

SUMMARY

The glial system in the developing rat spinal cord was studied using immunocytochemistry. Antibodies to vimentin and glial fibrillary acidic protein (GFAP) were used. At E11, vimentin was first found in the membrana limitans externa. In the matrix layer, short vimentin protrusions were found near the membrana limitans externa at

E12. In addition, vimentin was scattered throughout the matrix layer, where it was also present as vimentin-positive tangles. Later in development, vimentin immunoreactivity was distributed in a distinct radial pattern in the matrix layer. During the first postnatal weeks, vimentin was replaced by GFAP which is therefore expressed in a similar radial pattern. This orderly structural organisation of vimentin and GFAP in the matrix layer could indicate the involvement of both proteins in morphogenetic processes such as neuron migration and cell organisation.

In the mantle layer, a distinct radial vimentin immunoreactivity was replaced by GFAP immunoreactivity during the first 2 postnatal weeks. In addition, GFAP fibres appeared first, at E18, in the ventral mantle layer associated with the motor neuron columns. These glial fibres originated from a local source. In the dorsal mantle layer, GFAP-positive fibres were oriented tangentially, which is different from the overall radial arrangement. This expression pattern may be related to the ingrowth of primary afferents. In the ventral and dorsal raphe, a major vimentin expression was replaced by a minor presence of GFAP.

Within the white matter, a vimentin-positive radial pattern was demonstrated which, after birth, was replaced by GFAP. This palisading pattern suggested an involvement of both proteins in the development and guidance of the ascending and descending spinal cord fibre systems.

The general transition from the expression of vimentin to the expression of GFAP in the rat spinal cord takes place during the first 3 postnatal weeks.

REFERENCES

ABNEY, E. R., BARTLETT, P. P. & RAFF, M. C. (1981). Astrocytes, ependymal cells, and oligodendrocytes develop on schedule in dissociated cell cultures of embryonic rat brain. *Developmental Biology* **83**, 301–310.

ALTMAN, J. & BAYER, S. A. (1984). The development of the rat spinal cord. In *Advances in Anatomy, Embryology and Cell Biology*, vol. 85 (ed. J. F. Beck). Berlin: Springer.

AGUAYO, A. H., DAVID, S. & BRAY, G. M. (1981). Influences of the glial environment on the elongation of axons after injury: transplantation studies in adult rodents. *Journal of Experimental Biology* **95**, 231–240.

BAUMAL, R., KAHN, H. J., BAILEY, B., PHILIPS, M. J. & HANNA, W. (1980). The value of immunohistochemistry in increasing diagnostic precision of undifferentiated tumours by the surgical pathologist. *Histochemical Journal* **16**, 1061–1078.

BIGNAMI, A. & DAHL, D. (1974). Astrocyte specific protein and radial glia in the cerebral cortex of newborn rat. *Nature* **252**, 55–56.

BIGNAMI, A. & DAHL, D. (1977). Specificity of the glial fibrillary acidic protein for astroglia. *Journal of Histochemistry and Cytochemistry* **25**, 466–469.

BIGNAMI, A., RAJU, T. & DAHL, D. (1983). Localization of vimentin, the nonspecific intermediate filament protein, in embryonic glia and in early differentiating neurons. *Developmental Biology* **91**, 286–295.

BITTNER, C., BENJELLOUN-TOUIMI, S. & DUPOUEY, P. (1987). Palisading pattern of subpial astroglial processes in the adult rodent brain: relationship between the glial palisading and the axonal and astroglial organization. *Developmental Brain Research* **37**, 167–178.

BOVOLENTA, P., LIEM, R. K. H. & MASON, C. A. (1984). Development of cerebral astroglia transitions in form and cytoskeletal content. *Developmental Biology* **102**, 248–259.

BOVOLENTA, P., LIEM, R. K. H. & MASON, C. A. (1987). Glial filament protein expression in astroglia in the mouse visual pathway. *Developmental Brain Research* **33**, 113–126.

BUNGE, R. P. (1968). Glial cells and the central myelin sheath. *Physiological Reviews* **48**, 197–251.

CABRAL, F. & GOTTESMAN, M. M. (1979). Phosphorylation of the 10-nm filament protein from Chinese hamster ovary cells. *Journal of Biological Chemistry* **254**, 6203–6206.

COCHARD, P. & PAULIN, D. (1984). Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo in vivo. *Journal of Neuroscience* **4**, 2080–2094.

DAHL, D. (1981). The vimentin–GFA protein transition in rat neuroglia cytoskeleton occurs at the time of myelination. *Journal of Neuroscience Research* **6**, 741–748.

DAHL, D. & BIGNAMI, A. (1973). Immunochemical and immunofluorescence studies of the glial fibrillary acidic protein in vertebrates. *Brain Research* **61**, 279–293.

DUPOUEY, P., BENJELLOUN, S. & GOMES, D. (1985). Immunohistochemical demonstration of an organized cytoarchitecture of the radial glia in the CNS of the embryonic mouse. *Developmental Neuroscience* **7**, 81–93.

FERRARI, S., BATTINI, R., KACZMAREK, L., RITTLING, S., CALABRETTA, B., KIM DE RIEL, J., PHILIPONIS, V., WEI,

J.-F. & BASERGA, R. (1986). Coding sequence and growth regulation of the human vimentin gene. *Molecular and Cellular Biology* **6**, 3614–3620.

GARD, D. L. & LAZARIDES, E. (1982). Cyclic AMP-modulated phosphorylation of intermediate filament proteins in cultured avian myogenic cells. *Molecular and Cellular Biology* **2**, 1104–1114.

GEIGER, B. (1987). Intermediate filaments looking for a function. *Nature* **329**, 392–393.

GEISLER, N., PLESSMAN, U. & WEBER, K. (1982). Related amino acid sequence in neurofilaments and non-neuronal intermediate filaments. *Nature* **296**, 448–450.

GEISLER, N., PLESSMAN, U. & WEBER, K. (1983). Amino acid sequence characterization of mammalian vimentin, the mesenchymal intermediate filament protein. *Federation of European Biochemistry Societies Letters* **163**, 22–24.

GEORGATOS, S. D. & BLODEL, G. (1987*a*). Two distinct attachment sites for vimentin along the plasma membrane and the nuclear envelope in avian erythrocytes: a basis for a vectorial assembly of intermediate filaments. *Journal of Cell Biology* **105**, 105–115.

GEORGATOS, S. D. & BLODEL, G. (1987*b*). Laminin B constitutes an intermediate filament attachment site at the nuclear envelope. *Journal of Cell Biology* **105**, 117–127.

GIULIAN, D., VACA, K. & JOHNSON, B. (1988). Secreted peptides as regulators of neuron–glia and glia–glia interactions in the developing nervous system. *Journal of Neuroscience Research* **21**, 487–500.

GOLDMAN, J. E., GEIER, S. S. & HIRANO, M. (1986). Differentiation of astrocytes and oligodendrocytes from germinal matrix cells in primary culture. *Journal of Neuroscience* **6**, 52–60.

HANKIN, M. H. & SILVER, J. (1986). Mechanisms of axonal guidance. The problem of intersecting fiber systems. In *Developmental Biology* (ed. L. W. Browder), pp. 565–603. New York: Plenum.

HANSEN, S. H., STAGAARD, M. & MØLLGÅRD, K. (1989). Neurofilament-like pattern of reactivity in human foetal PNS and spinal cord following immunostaining with polyclonal anti-glial fibrillary acidic protein antibodies. *Journal of Neurocytology* **18**, 427–436.

HERPERS, M. J. H. M., RAMAEKERS, F. C. S., ALDEWARELDT, I., MOESKER, O. & SLOOFF, J. (1986). Co-expression of glial fibrillary acidic protein and vimentin-type intermediate filaments in human astrocytomas. *Acta Neuropathologica* **70**, 333–339.

HIRANO, M. & GOLDMAN, J. E. (1988). Gliogenesis in rat spinal cord: evidence for origin of astrocytes and oligodendrocytes from radial precursors. *Journal of Neuroscience Research* **21**, 155–167.

HOULE, J. & FEDEROFF, S. (1983). Temporal relationship between appearance of vimentin and neural tube development. *Developmental Brain Research* **9**, 189–195.

INNOCENTI, G. M., CLARKE, S. & KOPPEL, H. (1983). Transitory macrophages in the white matter of developing visual cortex. II. Development and relations with axonal pathways. *Developmental Brain Research* **11**, 55–66.

JOOSTEN, E. A. J. & GRIBNAU, A. A. M. (1989). Astrocytes and guidance of outgrowing corticospinal tract axons in the rat. An immunocytochemical study using anti vimentin and anti glial fibrillary acidic protein. *Neuroscience* **31**, 439–452.

KALDERON, N. (1988). Differentiating astroglia in nervous tissue histogenesis/regeneration: studies in a model system of regenerating peripheral nerve. *Journal of Neurocience Research* **21**, 510–512.

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

LEE, V. M.-Y. & PAGE, C. (1984). The dynamics of nerve growth factor-induced neurofilament and vimentin filament expression and organization in PC12 cells. *Journal of Neuroscience* **4**, 1705–1714.

LUDWIN, S. K., KOSEK, J. C. & ENG, L. F. (1976). The topographical distribution of S-100 and GFA proteins in the adult rat brain: an immunohistochemical study using horseradish peroxidase labeled antibodies. *Journal of Comparative Neurology* **165**, 197–208.

MARANI, E. (1978). A method for orienting cryostat sections for three-dimensional reconstructions. *Stain Technology* **53**, 265–268.

MUIJEN, G. N. P. VAN, RUITER, D. J., LEEUWEN, C. VAN, PRINS, F. A., RIETSEMA, K. & WARNAAR, S. O. (1984). Cytokeratin and neurofilament in lung carcinomas. *American Journal of Pathology* **116**, 363–369.

NORNES, H. O. & DAS, G. D. (1974). Temporal pattern of neurogenesis in the spinal cord of rat. I. An autoradiographic study. Time and sites of origin and migration and settling patterns of neuroblasts. *Brain Research* **73**, 121–138.

ONTENIENTE, B., KIMURA, H. & MAEDA, T. (1983). Comparative study of the glial fibrillary acidic protein in vertebrates by PAP immunohistochemistry. *Journal of Comparative Neurology* **215**, 427–436.

OUDEGA, M. (1990). Development of the rat spinal cord. Histochemistry of some functional and structural parameters. Thesis, University of Leiden.

PIXLEY, S. K. R. & DE VELLIS, J. (1984). Transition between immature radial glia and mature astrocytes studied with a monoclonal antibody to vimentin. *Developmental Brain Research* **15**, 201–209.

RAKIC, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. *Journal of Comparative Neurology* **145**, 61–84.

REXED, B. (1952). The cytoarchitectonic organization of the spinal cord in the cat. *Journal of Comparative Neurology* **96**, 415–495.

REXED, B. (1954). A cytoarchitectonic atlas of the spinal cord in the cat. *Journal of Comparative Neurology* **100**, 297–379.

ROELING, T. A. P. & FEIRABEND, H. K. P. (1988). Glial fiber pattern in the developing chicken cerebellum: vimentin and glial fibrillary acidic protein (GFAP) immunostaining. *Glia* **1**, 398–402.

SCHMECHEL, D. E. & RAKIC, P. (1979). A Golgi study of radial glial cells in developing monkey telencephalon: morphogenesis and transformation into astrocytes. *Anatomy and Embryology* **156**, 115–152.

SCHNITZER, J., FRANKE, W. W. & SCHACHNER, M. (1981). Immunohistochemical demonstration of vimentin in astrocytes and ependymal cells of developing and adult nervous system. *Journal of Cell Biology* **90**, 435–447.

SIDMAN, R. L. & RAKIC, P. (1973). Neuronal migration, with special reference to developing human brain: a review. *Brain Research* **62**, 1–35.

SILVER, J., LORENZ, S. E., WALHSTEN, D. & COUGHLIN, J. (1982). Axonal guidance during development of the great cerebral commissures: descriptive and experimental studies in vivo on the role of performed glial pathways. *Journal of Comparative Neurology* **210**, 10–29.

SINGER, M., NORDLANDER, R. H. & EDGAR, M. (1979). Axonal guidance during neurogenesis and regeneration in the spinal cord of the newt. The blueprint hypothesis of neuronal pathway patterning. *Journal of Comparative Neurology* **185**, 1–22.

STEINERT, P. M. & ROOP, D. R. (1988). Molecular and cellular biology of intermediate filaments. *Annual Review of Biochemistry* **57**, 593–625.

TARDY, M., FAGES, C., RIOL, H., LEPRINCE, G., RATABOUL, P., CHARRIÈRE-BERTRAND, C. & NUÑEZ, J. (1989). Developmental expression of the glial fibrillary acidic protein mRNA in the central nervous system and in cultured astrocytes. *Journal of Neurochemistry* **52**, 162–167.

TASCOS, N. A., PARR, J. & GONATAS, N. K. (1982). Immunocytochemical study of the glial fibrillary acidic protein in human neoplasms of the central nervous system. *Human Pathology* **13**, 454–458.

TRAUB, P. (1985). *Intermediate Filaments: A Review*. Heidelberg: Springer Verlag.

XEHNER, Z. E., LI, Y., ROE, P. R., PATTERSON, B. M. & SAX, C. M. (1987). The chicken vimentin gene. *Journal of Biological Chemistry* **262**, 8112–8120.

ZWET, J. VAN DER, TILBURG, P. A. VAN & POELMANN, R. E. (1986). Oren wapperen, een betrouwbare manier om de bronst bij ratten te bepalen. *Biotechnology* **3**, 48.