The Journal of Neuroscience, September 1987, 7(9): 2711–2720

Light and Electron Microscopic Localization of a Cell Surface Antigen (NG2) in the Rat Cerebellum: Association with Smooth Protoplasmic Astrocytes

Joel M. Levine[1] and J. Patrick Card[2,a]

Departments of [1]Neurobiology and Behavior and [2]Neurology, State University of New York at Stony Brook, Stony Brook, New York 11794

Immunofluorescence and immunoperoxidase techniques were used to localize a cell surface chondroitin-sulfate proteoglycan antigen, termed NG2, in the developing and adult rat cerebellum. In the adult, both polyclonal and monoclonal anti-NG2 antibodies labeled cells throughout the cerebellar cortex, with the labeled cells being especially prominent in the molecular layer. The labeled cells had small, irregularly shaped cell bodies from which thin highly branched processes radiated in a stellate array. The NG2-labeled cells were not labeled with antibodies against glial fibrillary acidic protein (GFAP), vimentin, or S-100 protein, intracellular markers for astrocytes. However, electron microscopic immunocytochemical analysis of NG2 immunoreactive cells revealed a cell morphology consistent with that of protoplasmic astrocytes. Labeled cell bodies contained a thin rim of organelle-poor cytoplasm surrounding a euchromatic nucleus. Thick processes originating from the cell soma tapered to form thin branches with highly irregular surface contours that extended between adjacent neuronal elements.The labeled processes did not form synapses in the neuropil, and no synaptic profiles onto anti-NG2-labeled cell bodies or processes were observed. Thus, we conclude that the NG2 antigen is a cell surface marker for a class of smooth protoplasmic astrocytes.

Immunoreactive cells were seen in the developing cerebellum beginning at embryonic day 16. The number of labeled cells increased during the early stages of cerebellar development, reaching a peak at about postnatal day (PND) 4 or 5 and declining thereafter. In the developing cerebellum, labeled cells lying within the forming molecular layer resembled the cells seen in the adult, whereas cells lying deeper within the folia had an immature appearance with fewer processes and less branching. This apparent gradient of morphological maturation suggests that an interaction with parallel fibers in the developing molecular layer may play a role in the terminal cytodifferentiation of the NG2-labeled smooth protoplasmic astrocytes.

Received Aug. 19, 1986; revised Feb. 4, 1987; accepted Mar. 25, 1987.

We thank Dr. W. B. Stallcup in whose laboratory the initial stages of this work were carried out. We also thank Drs. B. Boss, D. Dahl, and D. Paulin for their gifts of antibodies. This work was supported by NIH Grants NS 21198 (to J.M.L.) and NS 19714 (to J.P.C.).

Correspondence should be addressed to Dr. Levine at the above address.

[a] Present address: E. I. du Pont de Nemours and Company, Medical Products Department, Experimental Station, Bldg. 400/3444, Wilmington, DE 19898.

The mammalian CNS consists of between 10^9–10^{12} cells, of which $9/10$ are glial cells and $1/10$ neurons. Within these 2 broad cell classes, there may be hundreds and perhaps thousands of different cell types (Solnick et al., 1984). Neuronal and glial cells are usually distinguished on the basis of their size, morphology, location, biochemical, and electrophysiological properties. Recently, it has become apparent that different cell types within the CNS also differ in their repertoire of cell surface molecules, some of which are potent antigens. For example, subpopulations of horizontal cells in the carp retina have been identified on the basis of their different surface antigens (Young and Dowling, 1984), and antibodies against surface components of subpopulations of mammalian neurons and glial cells have also been reported (Sommer and Schachner, 1981; Wood et al.; 1982, Hockfield and McKay, 1983).

We have described previously a cell surface antigen of brain termed NG2 (Wilson et al., 1981; Stallcup et al., 1981), a high molecular weight chondroitin-sulfate proteoglycan found on the surfaces of several cell lines that have properties of both neurons and glial cells (Wilson et al., 1981) and on embryonic rat brain cells in primary culture (Stallcup, 1981). Antibodies against the NG2 antigen also label cells throughout the brain using conventional immunohistochemical techniques (Stallcup et al., 1983). Although the cells recognized by the anti-NG2 antibodies have a characteristic stellate morphology, their exact identity has not been established.

The experiments reported here were initiated to characterize the cells within the cerebellar cortex that react with anti-NG2 antibodies using light and electron microscopic immunohistochemistry. We chose to examine the distribution of the NG2 antigen in the cerebellum because the cortical layers contain relatively few cell types, all of which have been well described (Palay and Chan-Palay, 1974). The development of the cerebellum in the rat has also been well documented (Altman, 1972a–c). The cerebellum therefore provides a useful model system for analyzing the developmental expression of cell surface antigens. We report here that anti-NG2 antibodies specifically label a subpopulation of smooth protoplasmic astrocytes in the cerebellar cortex. The anti-NG2-labeled cells have a stellate morphology, exhibit few if any cytoplasmic filaments and are found at high frequency in the molecular layer of the cerebellar cortex. In order to define the developmental history of this class of protoplasmic astrocytes, we analyzed the expression of the NG2 antigen during pre- and postnatal cerebellar development. These developmental studies suggest that interactions between parallel fibers of the developing molecular layer and nascent NG2-la-

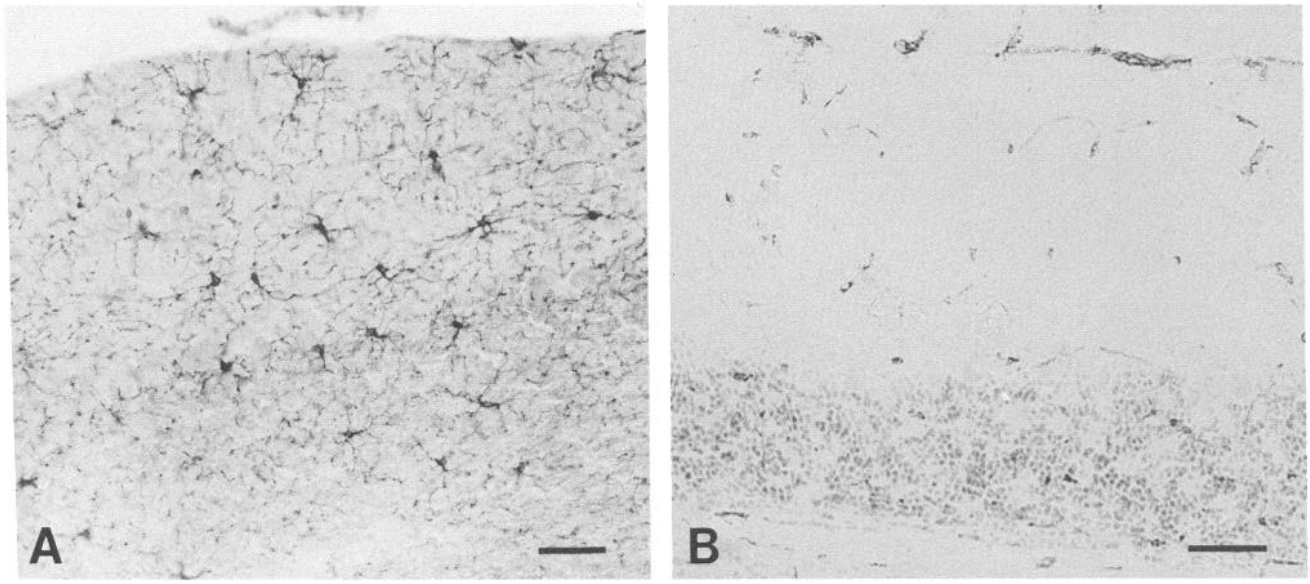

Figure 1. Specificity of anti-NG2 staining. Frozen sections, 30 μm, of adult cerebellum were labeled with monoclonal anti-NG2 (*A*) or antibody that had been absorbed 3× with B49 cells (*B*) using an avidin-biotin HRP system as described under Materials and Methods. Bar, 50 μm.

beled cells may play a role in regulating the morphological differentiation of smooth protoplasmic astrocytes.

Materials and Methods

Antibodies. The derivation and specificity of rabbit polyclonal anti-NG2 and mouse monoclonal anti-NG2 antibodies have been described (Wilson et al., 1981; Stallcup et al., 1983). The cell absorptions used to produce the original rabbit anti-NG2 antibodies resulted in a monospecific but weak antiserum. Therefore, we prepared additional rabbit anti-NG2 antibodies by immunizing rabbits with the NG2 proteoglycan purified by affinity chromatography on columns of mouse monoclonal anti-NG2 Sepharose. Rabbit antibodies against glial fibrillary acidic protein (GFAP) were a generous gift of Dr. D. Dahl. A monoclonal antibody against the S-100 protein (Haan et al., 1982) was a gift from Dr. B. Boss, and a human monoclonal antibody against vimentin (Dellagi et al., 1982) was a gift from Dr. D. Paulin. In control experiments, the anti-NG2 antibodies were absorbed 3 times with an equal volume of packed B49 cells, the cell line against which the antibodies were originally raised.

Immunocytochemistry. We used both immunofluorescence and immunoperoxidase (Hsu et al., 1981) techniques to localize the NG2 antigen. Adult rats were anesthetized with ether and perfused intracardially with 4% paraformaldehyde at variable pH as described (Berod et al., 1981). Tissue blocks were immersed overnight in 25% sucrose in 0.1 M carbonate/bicarbonate buffer, pH 10. Sections were cut using either a Lab Tek II cryostat or a freezing microtome. Brains from embryonic or young postnatal rats were fixed by immersion in 1% paraformaldehyde, and 0.1% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 3 hr at 4°C. After cryoprotection in 25% sucrose, the tissue was cut in either a horizontal or frontal plane using a Lab Tek II cryostat. The sections were washed and labeled as described previously (Levine et al., 1984). Rabbit anti-NG2 antibodies were used at 1/50 dilution, rabbit anti-GFAP at 1/100, and 2 different mouse monoclonal antibodies against the NG2 antigen (D31.10 and D120.43) were used at 70 and 15 μg/ml, respectively. In fluorescence experiments, we used fluorescein-labeled goat anti-mouse IgG and IgM (Tago) and either fluorescein- or rhodamine-conjugated goat anti-rabbit IgG prepared in our laboratory. For detection of vimentin antigens, a rhodamine-labeled goat anti-human IgM antibody (Tago) was used. The immunoperoxidase reaction product was visualized with diaminobenzidine (DAB) and hydrogen peroxide. Sections were examined with either a Leitz or Zeiss microscope and photographed on Kodak Tri-X film.

Electron microscopy. The rabbit polyclonal anti-NG2 antiserum was used for electron microscopic localization of immunoreactive cells and processes in the adult cerebellum. Following deep sodium pentobarbital-induced anesthesia, animals were perfused transcardially with 30–50 ml of physiological saline followed by 300–500 ml of paraformaldehyde–lysine–periodate fixative (McLean and Nakane, 1974). The brain was removed and postfixed in the same solution for 1 hr at 4°C and then passed through several changes of 0.1 M sodium phosphate buffer over the course of 30 min. Serial 100 μm sections of the cerebellum were cut with a vibratome in either the coronal or sagittal plane and placed in the anti-NG2 antiserum diluted to 1/1000 with 0.1 M phosphate buffer containing 1% normal goat serum. The tissue was incubated in the primary antiserum for 24–48 hr at 4°C. In some cases, the tissue was subjected to a 1 hr preincubation in antiserum that contained 0.1% Triton X-100 to enhance antibody penetration. The tissue sections were then washed in three 10 min changes of phosphate buffer and placed in a 1/50 dilution of goat anti-rabbit IgG for 30 min at room temperature. This incubation was followed by several buffer washes over 30 min and a 45 min incubation in rabbit peroxidase–antiperoxidase complex, diluted to 1/100. DAB was used as a chromogen, and the immunoperoxidase complex was visualized by the addition of hydrogen peroxide. The DAB reaction product was intensified and stabilized with a 1 hr postfixation in 1% osmium tetroxide, 1.5% potassium ferricyanide in 0.1 M phosphate buffer. The tissue was then washed, dehydrated, and flat-embedded in plastic resin as described previously (Card et al., 1981). Immunoreactive cells were first identified in 0.5 μm sections cut with a Reichart Ultracut ultramicrotome (American Optical) prior to identification in ultrathin sections. Immunoreactive cells and processes were examined and photographed with a Jeol 100cx transmission electron microscope.

Results

The distribution of cells exhibiting NG2-like immunoreactivity in the adult rat cerebellum is shown in Figure 1*A*. Numerous immunoreactive cells and processes were present throughout all regions of the cortex, but labeled cells were most prevalent within the molecular layer. Figure 1*B* shows a control section labeled with monoclonal anti-NG2 antibodies that were absorbed with B49 cells, the cell line against which the monoclonal antibodies were raised. This absorption completely removed the ability of the antibody to label cells in tissue sections. As an additional control, we labeled sections with supernatants from P3 × 63.Ag8.6.5.3 mouse myeloma cells. This reagent did not label the sections using either fluorescence or immunoperoxidase techniques.

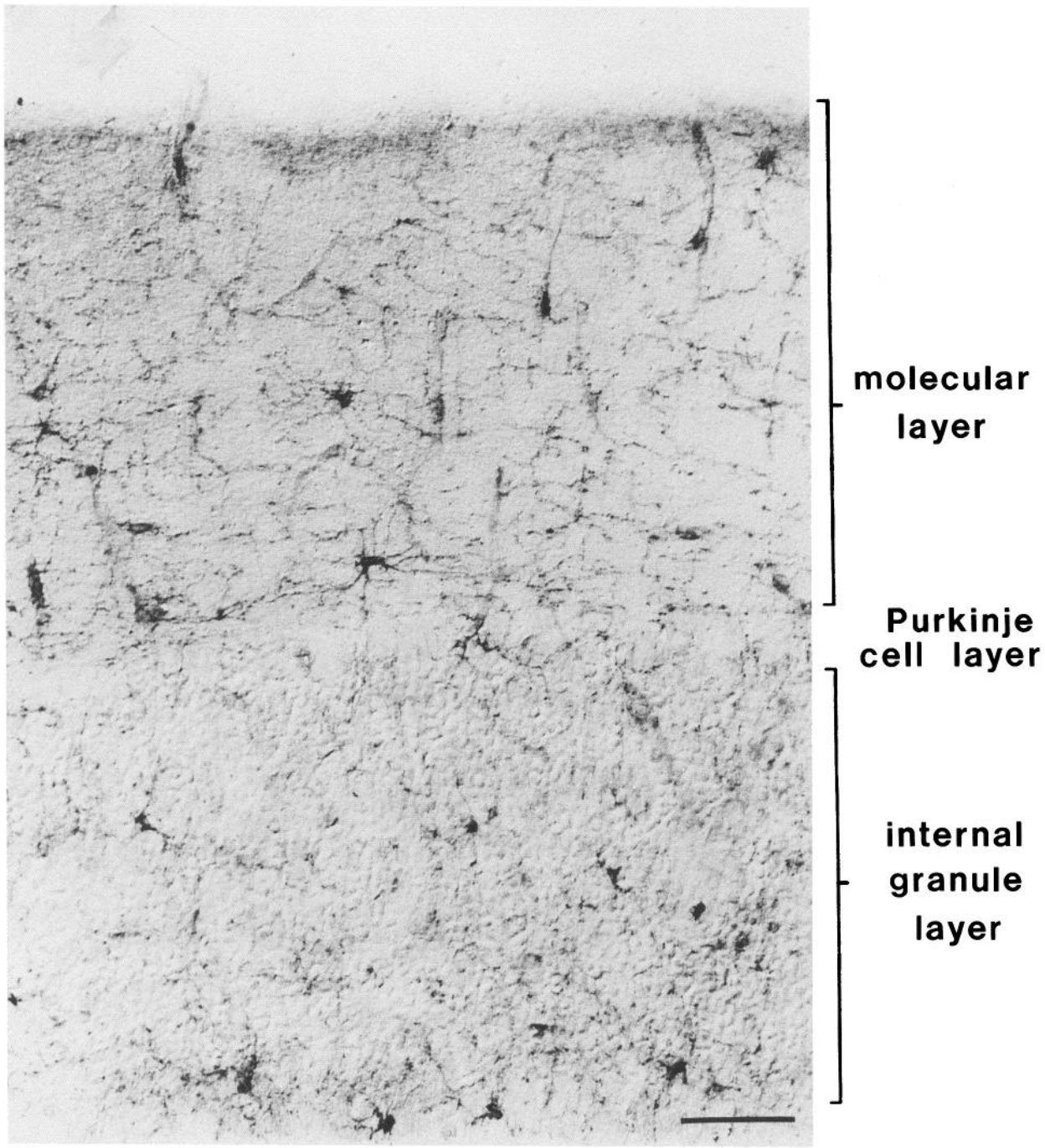

Figure 2. Distribution of anti-NG2 immunoreactivity in adult cerebellum. Frozen sections, 30 μm, were labeled with monoclonal anti-NG2 antibodies as in Figure 1. The cortical layers are indicated at right. Differential interference optics. Bar, 50 μm.

Figure 2 is a higher power view of anti-NG2 labeling of the cerebellar cortex, and Figure 3 shows the appearance of individual NG2-labeled cells. Reaction product was concentrated at the cell perimeter, suggesting that the antibody is binding to the surface of the cells. The NG2 immunoreactive cells had the following features: (1) perikarya were regularly spaced throughout the expanse of the molecular layer, (2) the cell bodies were small (cell area determined by planimetry was 53 $\pm$ 18 μm^2, $n = 26$) and had irregular shapes, and (3) the perikarya gave rise to radially oriented processes with branches extending into the surrounding neuropil. Although the NG2-labeled cells were most prominent within the molecular layer, they were also found infrequently in the Purkinje cell layer, the internal granule cell layer, and the white matter underlying the cerebellar cortex.

To determine whether the NG2-labeled cells contained intracellular markers for astroglial cells, we performed double-label experiments using either rabbit anti-GFAP or human anti-vimentin antibodies and mouse monoclonal anti-NG2 antibodies. Figure 4 shows that the NG2-positive cells of the molecular layer were not labeled with either the anti-GFAP or the anti-vimentin antibodies. The most prominent labeled feature of the molecular layer was the glial fibrillary acidic protein

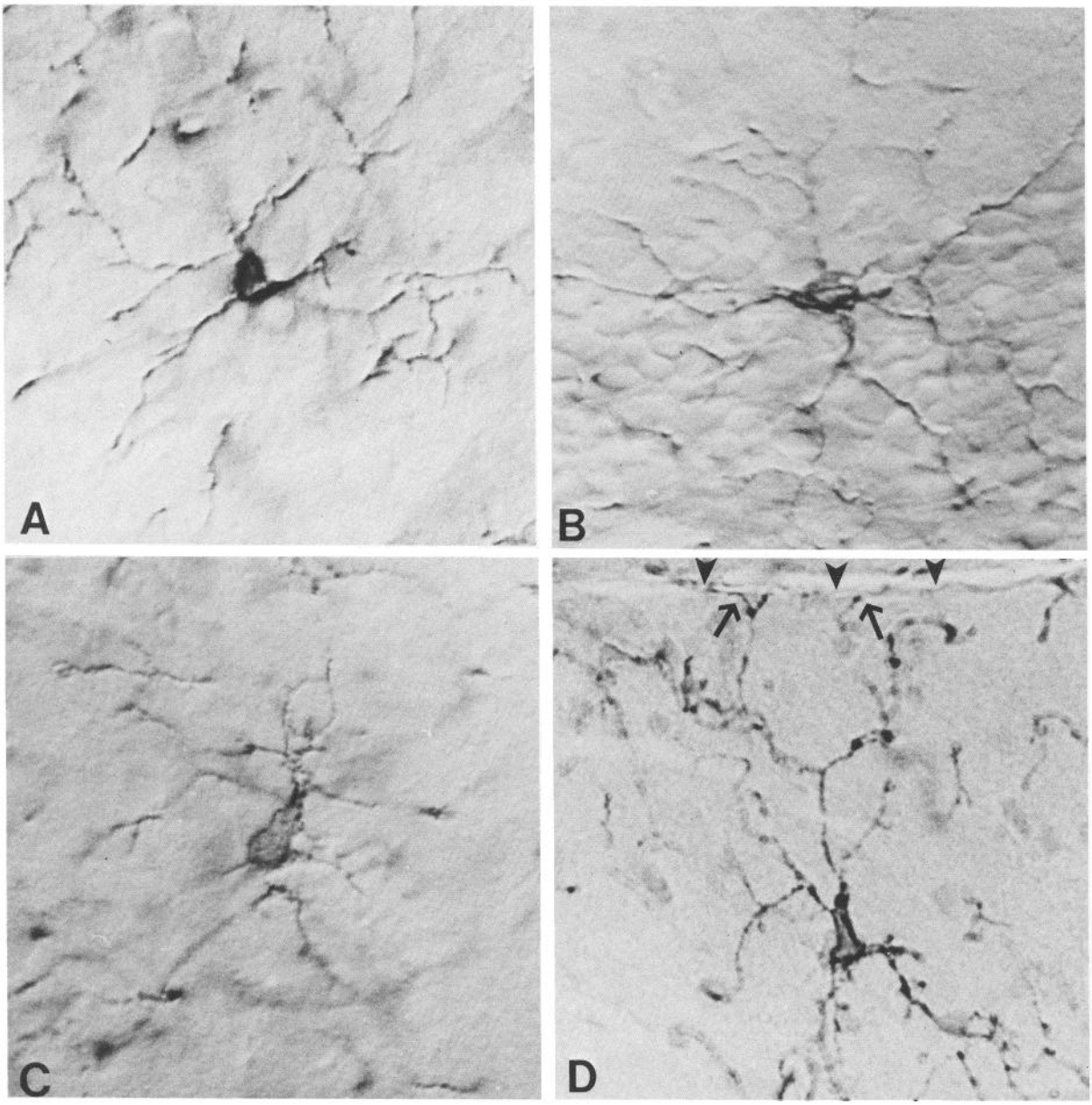

Figure 3. Appearance of individual anti-NG2-labeled cells. Frozen sections, 30 μm, were labeled as in Figures 1 and 2. *A,* Cell lying in the middle of the molecular layer. *B,* Labeled cell lying at the level of the Purkinje cell bodies. *C,* Labeled cell lying in the outer molecular layer. *D,* NG2-labeled cell lying in the outer molecular layer. Labeled processes (*arrows*) ascend to the pial surface (*arrowheads*). *A–C,* Differential interference optics, ×950. *D,* Bright-field optics, ×750.

(GFAP) and vimentin-immunoreactive Bergmann glial fibers. The anti-GFAP antibodies stained astrocytes in the internal granule layer and white matter, but these cells were not labeled with the anti-NG2 antibodies. Similar results were obtained in double-label experiments using rabbit anti-NG2 antibodies and a mouse monoclonal antibody against the S-100 protein (data not shown). Thus, the NG2-labeled cells of the cerebellum do not contain detectable amounts of several intracellular markers commonly used to identify astrocytes.

Despite the lack of detectable intracellular markers characteristic of astroglial cells, the NG2-positive cells of the cerebellar molecular layer had several morphological features characteristic of astrocytes. Immunoreactive cells lying in the outer molecular layer had processes that ascended to the pial surface (Figs. 1 and 3). NG2-labeled processes were also observed in close association with blood vessels, often appearing to make contact with the endothelial cells (data not shown).

Although the light microscopic techniques used above were adequate for describing the general features of the NG2-labeled cells, these techniques are insufficient to identify positively the phenotype of the cells. Therefore, we extended our localization studies to the ultrastructural level. The fine structural characteristics of cells and processes exhibiting NG2 immunoreactivity are shown in Figures 5–7. The cells were characterized by a spherical to oval soma. The nucleus was the dominant feature of the perikaryon and was surrounded by a thin rim of organelle-poor cytoplasm. The nucleus contained a prominent nucleolus and had aggregates of heterochromatin at the inner surface of

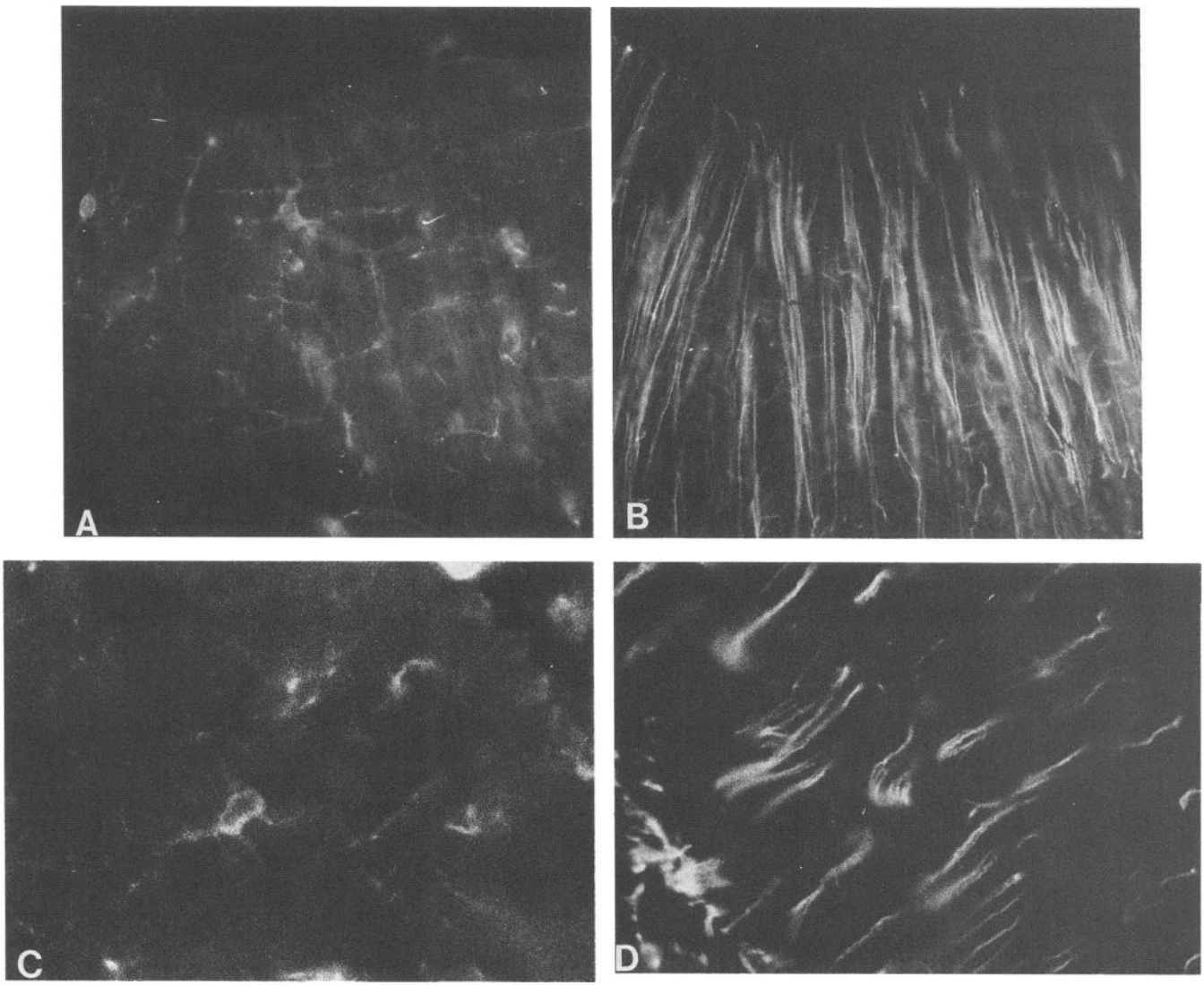

Figure 4. Double-label fluorescent staining of the adult cerebellum. *A,* Cryostat section labeled with mouse monoclonal anti-NG2 antibodies and fluorescein-labeled goat anti-mouse IgG. *B,* Same section labeled with rabbit anti-GFAP antibodies and rhodamine-conjugated goat anti-rabbit. ×280. *C,* Cryostat section labeled with rabbit anti-NG2 and fluorescein-conjugated goat anti-rabbit IgM. *D,* Same section labeled with human anti-vimentin and rhodamine-conjugated goat anti-human IgM. ×620. Both the anti-GFAP and anti-vimentin antibodies label the Bergmann glial fibers. The NG2-positive cells are not labeled with either anti-GFAP or anti-vimentin.

the nuclear envelope. The small amount of cytoplasm in each cell contained scattered organelles, but was conspicuously poor in filaments. This was also true of the processes that arose from the immunoreactive perikarya. Processes such as the ones shown in Figure 7 routinely gave rise to thin branches that extended throughout the neuropil and interdigitated among the neuronal profiles (Fig. 6). Although synaptic profiles were evident throughout the molecular layer (Fig. 6, arrows), no such profiles were seen contacting the NG2-labeled processes, and the labeled processes themselves did not give rise to synaptic profiles. These features have been described for protoplasmic astrocytes (Peters et al., 1976).

To study the development of protoplasmic astrocytes, we localized the NG2 antigen by indirect immunofluorescence in the embryonic and early postnatal cerebellum. Small numbers of anti-NG2-labeled cells were first observed in the cerebellar anlage at about embryonic day (E) 16. These labeled cells (Fig. 8*A*) had 1 or 2 elongated processes with few, if any, branches. The processes extended from one side of the cell as if the cell were migrating across the developing cerebellum. Over the next 4–5 d of prenatal development, during which time the external granule layer (EGL) formed, the number of NG2-labeled cells increased progressively. Figure 8*B* shows several immunoreactive cells and processes lying below the EGL in the E20 cerebellum. Although the processes of the labeled cells were more numerous than at E16, the processes had an immature appearance with relatively few branches. Neither labeled cell bodies nor processes were seen within the EGL. These studies demonstrate that at least some of the NG2-labeled cells are generated very early in cerebellar development and suggest that they originate in areas other than the nascent EGL.

The expression of the NG2 antigen in the developing postnatal cerebellum is shown in Figure 9. At postnatal day 3 (PND3; Fig. 9*A*), the anti-NG2 antibodies labeled many cells and processes throughout the expanse of each developing folium. The branching pattern of the processes was more complex than at

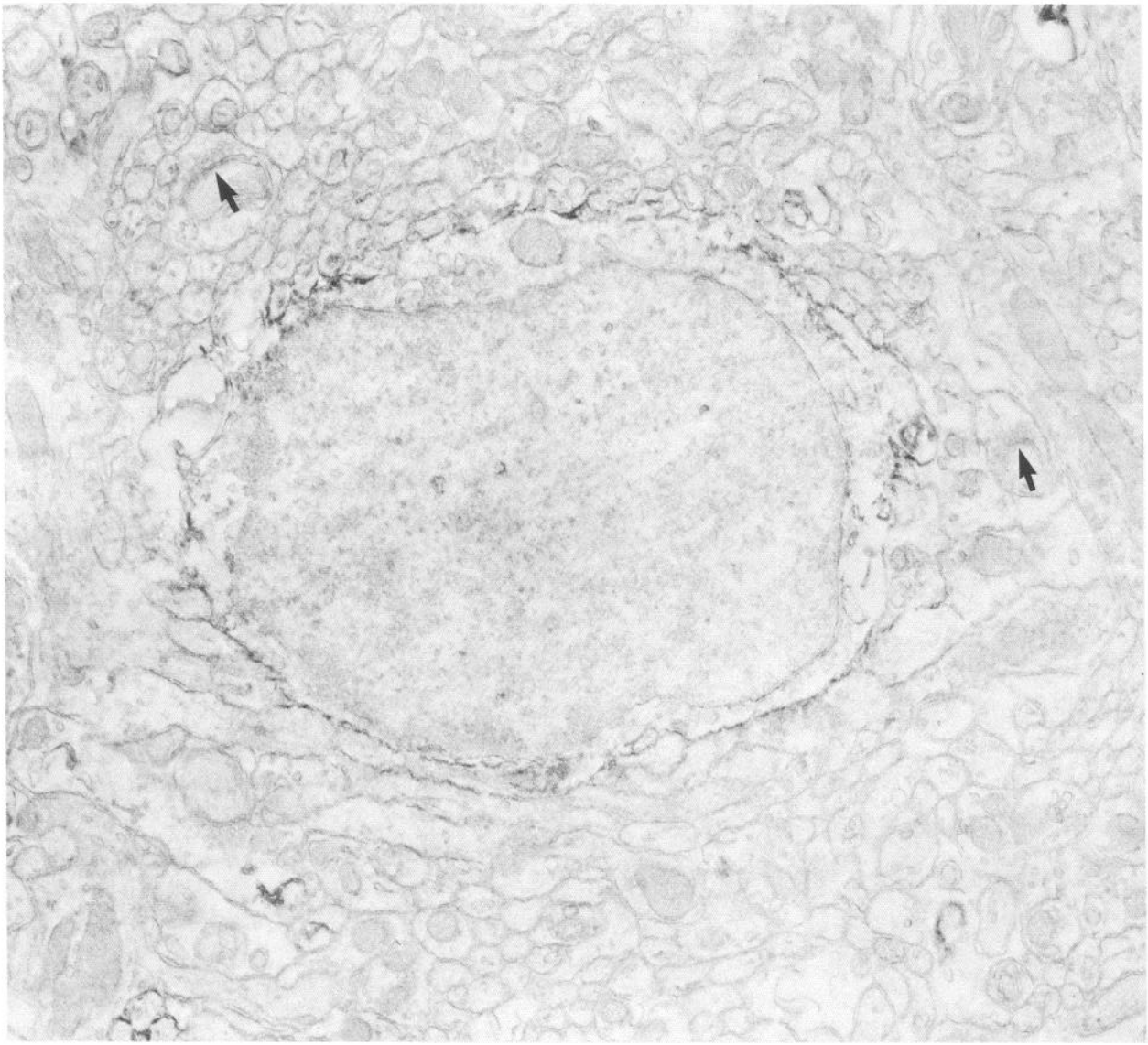

Figure 5. Ultrastructural characteristics typical of NG2-immunoreactive astrocytes in the molecular layer of the adult rat cerebellum. Immunoperoxidase reaction product discretely associated with the external limiting membrane defines the boundaries of the cell among the numerous parallel fibers in the molecular layer. The nucleus of these small cells characteristically fills the majority of the cell soma and is surrounded by a thin rim of organelle-poor cytoplasm. While synaptic contacts are evident in the surrounding neuropil (*arrows*), they never involve immunoreactive cells or processes. ×21,800.

E20 but far simpler than in the adult. Three or four short processes radiated from round cell bodies. At PND5 (Fig. 9*B*), the density of labeled cells and processes had increased. No labeled perikarya and only a few scattered processes were seen within the EGL. By PND7 (Fig. 9*C*), some of the labeled cells had a morphology resembling that seen in the adult. Cell bodies that lay immediately below the developing molecular layer had processes that extended within the molecular layer, branching repeatedly. The morphology of cells lying deeper within the developing folia was less complex, with sparsely branching processes extending radially from the soma. Over the next 2 weeks of development, labeling within the more central regions of the folia declined. Concurrently, cells within the molecular layer began to take on their adult appearance, so that by PND15 (Fig. 9*D*), cells with a characteristic stellate morphology were seen within the molecular layer.

Discussion

Astrocytes and oligodendrocytes comprise the macroglia of the CNS. The 2 major classes of astrocytes are the fibrous and protoplasmic astrocytes, although other specialized forms such as the Müller cells of the retina and the Golgi epithelial cells of the cerebellum have been identified. In the cerebellar cortex, protoplasmic astrocytes have been further classified as either velate or smooth (Chan-Palay and Palay, 1972; Palay and Chan-Palay, 1974). Velate astrocytes, which include the Golgi epithelial cells, have thin veillike processes that often divide the neuropil into compartments or, in the case of the Bergmann fibers, cover the surfaces of the Purkinje cells. This cell type is especially prominent in the internal granule layer (Chan-Palay and Palay, 1972). Smooth protoplasmic astrocytes, which have a stellate morphology, have been seen only rarely in Golgi-

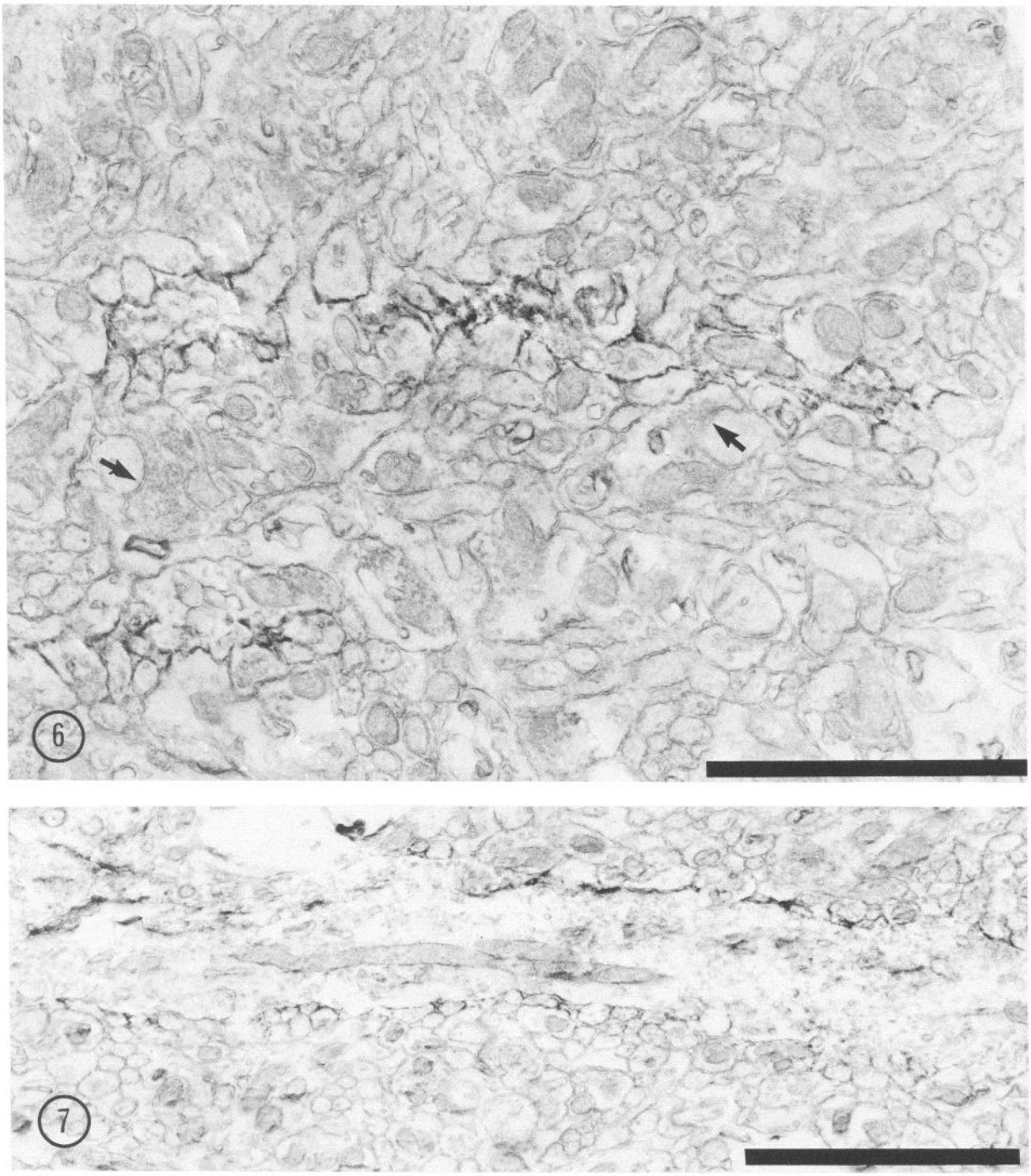

Figure 6. The thin, terminal branches of NG2 immunoreactive cells are characteristically interdigitated among numerous unmyelinated axons and dendrites in the molecular layer of the adult cerebellum. Once again, the immunoperoxidase reaction product is associated with the external limiting membrane and highlights the irregular shape and tortuous course of these processes through the molecular layer. Axospinous synaptic contacts are evident in the neuropil (*arrows*) but never involve the immunoreactive processes. Scale bar, 2.5 μm.

Figure 7. Thick processes, such as the one illustrated, arise from the soma of NG2-immunoreactive cells and give rise to the thin terminal processes illustrated in Figure 6. The processes abut directly upon the unmyelinated axons in the molecular layer, but synaptic contacts between these processes and axon terminals were never observed. Scale bar, 2.5 μm.

impregnated sections of the molecular layer. However, use of specific antisera against the NG2 antigen has allowed us to identify a class of stellate-appearing astrocytes that are most prevalent within the molecular layer. On the basis of the data presented above, we conclude that the NG2 proteoglycan antigen is a surface marker for smooth protoplasmic astrocytes.

This conclusion is based on the light and electron microscopic appearance of the cells identified by labeling with the anti-NG2 antibodies. These cells have oval, euchromatic nuclei surrounded by a thin rim of organelle-poor cytoplasm. Thick processes radiate from the cell soma in a stellate array, tapering and branching to form thinner processes with irregular surface con-

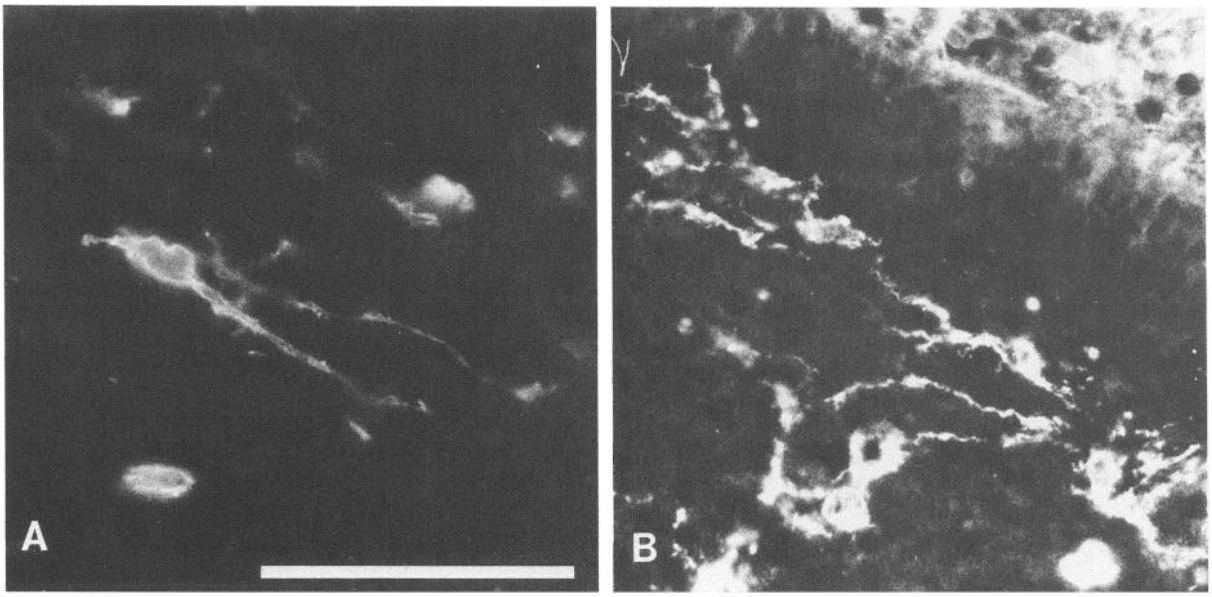

Figure 8. Anti-NG2 labeling of cells within the embryonic cerebellum. Cryostat sections were labeled with rabbit anti-NG2 and fluorescein goat anti-rabbit. *A*, E16; *B*, E20. Bar, 50 μm.

tours that interdigitate within the parallel fibers of the molecular layer. At both the light and electron microscopic level, we find no evidence that these thin NG2-labeled processes form sheets or veils around Purkinje cell bodies; rather, the stellate appearance of the cells strongly resembles the smooth protoplasmic astrocytes described by Palay and Chan-Palay (1974). Consistent with their astrocytic nature, the NG2-labeled cells have processes that appear to make contact with the pial surface and blood vessels. Our anti-NG2 antibodies also label cells within the internal granule layer, mostly at the level of the Purkinje cell bodies and at the interface of the granule layer and the underlying white matter. In addition, similar cells are labeled throughout the brain, being especially prominent in the cerebral cortex (Stallcup et al., 1983). Thus, it is likely that the NG2 antigen is a general cell surface marker for a class of smooth protoplasmic astrocytes *in vivo*.

The identification and characterization of glial cells have been studied recently using macromolecular markers such as GFAP or vimentin for astrocytes (Bignami and Dahl, 1972; Ludwin et al., 1976; Ghandour et al.; 1980, Bovolenta et al., 1984) and galactocerebroside or sulfatide for oligodendrocytes (Schachner et al., 1981; Ranscht et al., 1982). *In vivo* the NG2-labeled cells contain neither detectable GFAP filaments nor vimentin-like intermediate filaments, which may explain both why this class of astrocytes has not been recognized in previous immunohistochemical studies of the cerebellum and our preliminary identification of the NG2-labeled cells as interneurons (Stallcup et al., 1983). Despite the lack of detectable glial filaments, the ultrastructural characteristics of the cells labeled at their surfaces with the anti-NG2 antibodies supports our identification of these cells as a subclass of astrocytes. They have neither the nuclear nor cytoplasmic features of oligodendrocytes, and we have found no synaptic contacts on NG2-labeled cell bodies or processes. It is also unlikely that the NG2-labeled perikarya represent a class of immature or developing glial cell since we find the cells throughout the molecular layer of adult animals and can, in the material prepared from young, postnatal rats, identify immature-appearing cells (i.e., lacking the complex branching pattern seen in the adult) that do carry the NG2 antigen or cross-reactive molecules on their surfaces. In tissue sections prepared from late-term embryos, many of the NG2-positive processes were labeled with the anti-vimentin antibodies (data not shown). Since the adult cells are vimentin negative, the morphological maturation of smooth protoplasmic astrocytes is accompanied by a change in intermediate filament protein expression, as is the case for other classes of astrocytes (Schnitzer et al., 1981).

Fibrous and protoplasmic astrocytes are distinguished on the basis of their morphology and location. Fibrous astrocytes are usually found in white matter and have long, radially arranged cylindrical processes with smooth surface contours and contain dense bundles of glial filaments (Peters et al., 1976). Protoplasmic astrocytes are generally found in gray matter and tend to have thick processes with a variable content of glial filaments. The cells we have identified using the anti-NG2 antibodies have characteristics of both types of astrocytes. First, the NG2-labeled cells are found with high frequency in the molecular layer. Such an axon-rich area would be expected to contain mostly fibrous astrocytes. Second, our immunofluorescence and ultrastructural analysis shows that the NG2-labeled cells contain few if any bundles of glial filaments, a characteristic of protoplasmic astrocytes. Third, the processes of the NG2-labeled cells have the irregular surface contours expected for a protoplasmic astrocyte but a length and branching pattern that is more typical of a fibrous astrocyte. Whether the NG2-labeled cells comprise a unique cell type or represent a variant on a continuum of astrocytic forms and properties remains to be established.

Our developmental studies provide indirect information concerning the factors that may affect glial differentiation and, in particular, the distribution and morphology of the NG2-labeled cells of the cerebellum. NG2-labeled cells are first found in the cerebellar anlage at about E16, prior to the formation of the EGL or a multilayered cortical structure. The number of labeled cells increases during pre- and postnatal development, reaching a peak at about PND4 or 5. Since there are no labeled cells within the EGL at this time, it is likely that this increase in cell number is due to either local proliferation or to migration of

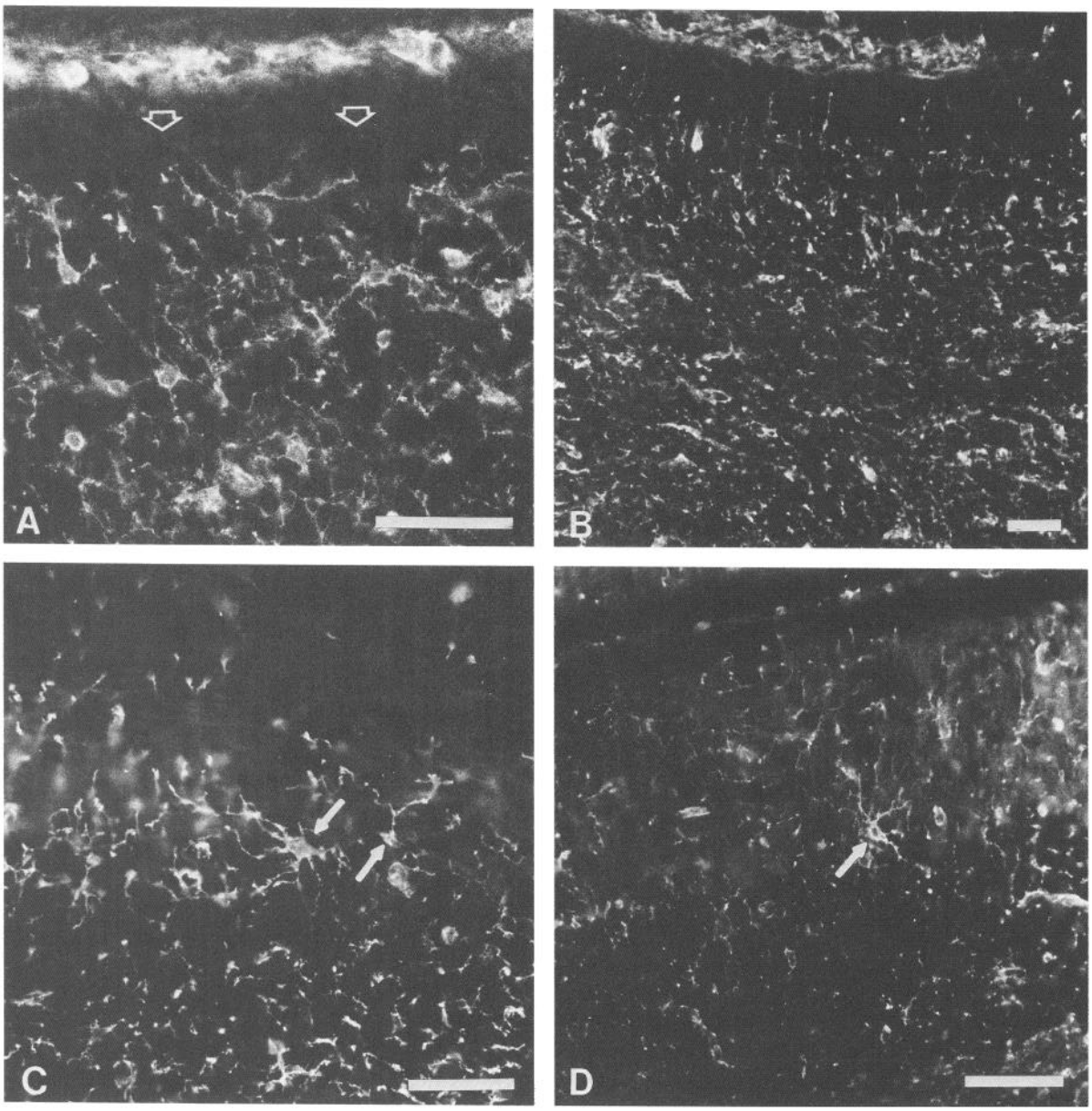

Figure 9. Expression of the NG2 antigen in the developing cerebellum. Sections were labeled with rabbit anti-NG2 and fluorescein goat anti-rabbit. *A,* Postnatal day 3. The *open arrowheads* denote the EGL. *B,* Postnatal day 4, a low-power view showing labeled processes throughout a folium. *C,* Postnatal day 7. The *arrows* point to cells that have begun to cytodifferentiate within the molecular layer. *D,* Postnatal day 15. Labeling of the internal granule layer is reduced and cells within the molecular layer (*arrow*) have begun to assume their adult form. Bar, 50 μm.

cells into the developing cerebellum from other proliferative areas such as the ventricular zones surrounding the fourth ventricle. By PND7, cells lying within the developing molecular layer have begun to assume the stellate morphology of the NG2-labeled cells in the adult, while cells lying deeper within each folium have an immature appearance with shorter processes and fewer branches. During the second week of postnatal life, the number of mature-appearing, NG2-labeled cells increases, while the density of labeling within the developing internal granule layer and white matter decreases. Finally, at about PND15–17, the staining pattern resembles that seen in the adult. This morphological gradient suggests that contact or interactions between developing NG2 cells and growing parallel fibers trigger the final morphological differentiation of the NG2-labeled cells in a manner similar to the effect of parallel fibers on the dendritic branching pattern of stellate interneurons (Rakic, 1972).

The developmental pattern of expression of the NG2 antigen in the cerebellum deduced from immunofluorescence labeling studies is consistent with the relative expression of the NG2 antigen in whole brain as deduced from quantitative absorption experiments (Wilson et al., 1981). There, relative levels of the NG2 antigen reach a peak at E19, declining thereafter by about 50% to a steady-state level that is maintained in the adult. A qualitatively similar but temporally retarded pattern of expression is seen in the cerebellum, where the major events of morphogenesis occur postnatally. We were unable to determine whether all of the immature-appearing cells that lie deep within the developing folia eventually become the stellate-appearing astrocytes of the molecular layer. It is possible that some of these immature cells die or differentiate along other cell lineages

(Stallcup, 1981). These questions can be approached using dissociated cultures of developing cerebellum, where it is possible to analyze the phenotypic development of the NG2-labeled astrocytes at the level of the single cell.

References

Altman, J. (1972a) Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer. J. Comp. Neurol. *145:* 353–398.

Altman, J. (1972b) 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.

Altman, J. (1972c) Postnatal development of the cerebellar cortex in the rat. III. Maturation of the components of the granular layer. J. Comp. Neurol. *145:* 465–514.

Berod, A., B. K. Hartman, and J. F. Pujol (1981) Importance of fixation and immunohistochemistry: Use of formaldehyde solutions at variable pH for the localization of tyrosine hydroxylase. J. Histochem. Cytochem. *29:* 844–850.

Bignami, A., and D. Dahl (1972) Differentiation of astrocytes in the cerebellar cortex and the pyramidal tracts of the newborn rat. An immunofluorescence study with antibodies to a protein specific to astrocytes. Brain Res. *49:* 393–402.

Bovolenta, P., R. K. H. Liem, and C. A. Mason (1984) Development of cerebellar astroglia: Transitions in form and cytoskeletal content. Dev. Biol. *102:* 248–259.

Card, J. P., N. Brecha, H. J. Karten, and R. Y. Moore (1981) Immunocytochemical localization of vasoactive intestinal polypeptide containing cells and processes in the suprachiasmatic nucleus of the rat: Light and electron microscopic analysis. J. Neurosci. *1:* 1298–1303.

Chan-Palay, V., and S. L. Palay (1972) The form of velate astrocytes in the cerebellar cortex of monkey and rat: High voltage electron microscopy of rapid Golgi preparations. Z. Anat. Entwickl.-Gesch. *138:* 1–19.

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

Ghandour, M. S., G. Vincendon, and G. Gombos (1980) Astrocyte and oligodendrocyte distribution in adult rat cerebellum: An immunohistological study. J. Neurocytol. *9:* 637–646.

Haan, E. A., B. D. Boss, and W. M. Cowan (1982) Production and characterization of monoclonal antibodies against the "brain-specific" proteins 14-3-2 and S-100. Proc. Natl. Acad. Sci. USA *79:* 7585–7589.

Hockfield, S., and R. D. G. McKay (1983) A surface antigen expressed by a subset of neurons in the vertebrate central nervous system. Proc. Natl. Acad. Sci. USA *80:* 5758–5761.

Hsu, S., L. Raine, and H. Fanger (1981) Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques. J. Histochem. Cytochem. *29:* 577–580.

Levine, J. M., L. Beasley, and W. B. Stallcup (1984) The D1.1 antigen: A cell surface marker for germinal cells of the central nervous system. J. Neurosci. *4:* 820–831.

Ludwin, S. K., J. C. Kosek, and L. F. Eng (1976) The topographical distribution of S-100 and GFA proteins in the adult rat brain: An immunohistochemical study using horseradish peroxidase-labelled antibodies. J. Comp. Neurol. *165:* 197–208.

McLean, I., and P. Nakane (1974) Periodate-lysine-paraformaldehyde fixative: A new fixative for immunoelectron microscopy. J. Histochem. Cytochem. *22:* 1077–1083.

Palay, S. L., and V. Chan-Palay (1974) *Cerebellar Cortex, Cytology and Organization,* Springer-Verlag, New York.

Peters, A., S. L. Palay, and H. Webster (1976) *The Fine Structure of the Nervous System,* Saunders, Philadelphia, PA.

Rakic, P. (1972) Extrinsic cytological determinants of basket and stellate cell dendritic pattern in the cerebellar molecular layer. J. Comp. Neurol. *146:* 335–364.

Ranscht, B., P. A. Clapshaw, J. Price, M. Nobel, and W. Seifert (1982) Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. Proc. Natl. Acad. Sci. USA *79:* 2709–2713.

Schachner, M., S. K. Kim, and R. Zehnle (1981) Developmental expression in central and peripheral nervous system of oligodendrocyte cell surface antigens (O antigens) recognized by monoclonal antibodies. Dev. Biol. *83:* 328–338.

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

Solnick, B., T. L. Davis, and P. Sterling (1984) Numbers of specific types of neurons in layer IVab of cat striate cortex. Proc. Natl. Acad. Sci. USA *81:* 3898–3900.

Sommer, I., and M. Schachner (1981) Monoclonal antibodies (01 to 04) to oligodendrocyte cell surfaces: An immunocytological study in the central nervous system. Dev. Biol. *83:* 311–327.

Stallcup, W. B. (1981) The NG2 antigen, a putative lineage marker: Immunofluorescent localization in primary cultures of rat brain. Dev. Biol. *83:* 154–165.

Stallcup, W. B., J. Levine, and W. Raschke (1981) Monoclonal antibody to the NG2 marker. In *Monoclonal Antibodies to Neural Antigens,* R. McKay, M. C. Raff, and L. F. Reichardt, eds., pp. 39–50, Cold Spring Harbor Laboratory, NY.

Stallcup, W. B., L. Beasley, and J. Levine (1983) Cell-surface molecules that characterize different stages in the development of cerebellar interneurons. Cold Spring Harbor Symp. Quant. Biol. *48:* 761–774.

Wilson, S.-S., E. E. Baetge, and W. B. Stallcup (1981) Antisera specific for cell lines with mixed neuronal and glial properties. Dev. Biol. *83:* 146–153.

Wood, J. N., L. Hudson, T. M. Jessell, and M. Yamamoto (1982) A monoclonal antibody defining antigenic determinants on subpopulations of mammalian neurons and Trypanosoma Cruzi parasites. Nature *296:* 34–38.

Young, L. H. Y., and J. E. Dowling (1984) Monoclonal antibodies distinguish subtypes of retinal horizontal cells. Proc. Natl. Acad. Sci. USA *81:* 6255–6259.