

Two Forms of Cerebellar Glial Cells Interact Differently with Neurons In Vitro

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ABSTRACT Specific interactions between neurons and glia dissociated from early postnatal mouse cerebellar tissue were studied in vitro by indirect immunocytochemical staining with antisera raised against purified glial filament protein, galactocerebroside, and the NILE glycoprotein. Two forms of cells were stained with antisera raised against purified glial filament protein. The first, characterized by a cell body 9 μm diam and processes 130–150 μm long, usually had two to three neurons associated with them and resembled Bergmann glia. The second had a slightly larger cell body with markedly shorter arms among which were nestled several dozen neuronal cells, and resembled astrocytes of the granular layer.

Staining with monoclonal antisera raised against purified galactocerebroside revealed the presence of immature oligodendroglia in the cultures. These glial cells constituted ~2% of the total cell population in the cultures and, in contrast to astroglia, did not form specific contacts with neurons. Staining with two neuronal markers, antisera raised against purified NILE glycoprotein and tetanus toxin, revealed that most cells associated with presumed astroglia were small neurons (5–8 μm). After 1–2 d in culture, some stained neurons had very fine, short processes. Nearly all of the processes >10–20 μm long were glial in origin.

Electron microscopy also demonstrated the presence of two forms of astroglia in the cultures, each with a different organizing influence on cerebellar neurons. Most neurons associated with astroglia were granule neurons, although a few larger neurons sometimes associated with them.

Time-lapse video microscopy revealed extensive cell migration (~10 $\mu\text{m}/\text{h}$) along the arms of Bergmann-like astroglia. In contrast, cells did not migrate along the arms of astrocyte-like astroglia, but remained stationary at or near branch points. Growth cone activity, pulsating movements of cell perikarya, and ruffling of the membranes of glial and neuronal processes were also seen.

Two roles have been proposed for astroglia in establishing the cellular architecture of the developing mammalian brain. First, radially oriented astroglial processes are thought to guide the migration of immature neurons from ventricular zones, where they undergo their final mitosis, to the position where they form synaptic connections (24–26, 32). Second, recent evidence suggests that after neuronal migration, astroglia organize mature neurons into compartments (21, 22).

We recently reported the development of a tissue culture system with which specific associations between astroglia and neurons harvested from developing mouse cerebellar tissue can be studied (14). Here we wish to report a more detailed study of the classes of cerebellar astroglia present in microcultures and of the specific interactions between astroglia and cerebellar neurons. In addition, we wish to determine whether the other major class of glial cell—oligodendroglia, cells that myelinate axons in the white matter but do not mediate

neuronal migrations or positioning during development—are present in the cultures and whether they interact with cerebellar neurons.

Both astroglia and oligodendroglia can be identified reliably by immunocytochemical localization of specific markers, astrocytes with antisera raised against glial filament protein (1, 3–5, 7, 19, 20, 31) and oligodendrocytes with antisera raised against purified galactocerebroside (20, 27). In the present study, we used these cell markers with the peroxidase-antiperoxidase (PAP)¹ method (33) to visualize specific interactions between neurons and glia in cultures of cerebellar cells disso-

¹ *Abbreviations used in this paper:* AbGalc, AbGF, and AbNILE, Antisera against purified galactocerebroside, the major glial filament protein, and NILE glycoprotein, respectively; CMF-PBS, calcium- and magnesium-free Tyrode's solution; PAP method, peroxidase-antiperoxidase method.

ciated from early postnatal mouse cerebellum. To confirm the identity of the cells and to visualize the specific interactions of neurons with astroglia *in vitro*, we analyzed the cultures by electron microscopy. Finally, to monitor the behavior of the neurons that associate with astroglia, we carried out time-lapse video microscopy.

MATERIALS AND METHODS

Cerebellar Cultures

All studies were carried out with C57B1/6J mice derived from a timed-pregnancy breeding colony in this department. Whole cerebellum was removed from postnatal animals at 5–7 d after birth (P5–P7). Single cell suspensions were prepared as described (11, 15, 34) and plated at a cell density of $1-2 \times 10^6$ cells/ml in glass coverslip microcultures (11) for immunocytochemistry and time-lapse video microscopy or in microwells (cat. no. 3034, Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) for electron microscopy. For either culture method, the culture surface was pretreated with poly-D-lysine (10 μ g/ml, Sigma Chemical Co., St. Louis, MO) as described (15). Plating efficiency was 85–95% (15). Cells were maintained at 35.5°C with 5% CO₂ and 100% humidity.

Immunocytochemistry

ANTISERA: Antisera against the major glial filament protein (AbGF; glial filament protein, 51,000 daltons) were raised in rabbits as described (19). In some experiments the antiserum was preabsorbed with purified glial filament protein. Monoclonal antiserum raised against purified galactocerebroside (AbGalc), an antiserum specific for oligodendroglia, was kindly provided by Dr. Barbara Ranscht (Tuebingen, Federal Republic of Germany). Antiserum against the purified NILE glycoprotein (30) (230,000 daltons; AbNILE) was the generous gift of Drs. Steven R. J. Salton, M. L. Shelanski and L. A. Greene (this department). This antiserum selectively stains most neurons, including those from cerebellum (30). Tetanus toxin and antisera raised against tetanus toxin were the generous gift of Dr. William Habig (National Institutes of Health).

INDIRECT IMMUNOCYTOCHEMICAL STAINING: The PAP method (33) was used for all experiments. After 24–48 h *in vitro*, cultures were rinsed three times with calcium- and magnesium-free Tyrode's solution (CMF-PBS) (15), fixed with paraformaldehyde (4% in CMF-PBS) for 30 min at room temperature, and washed three times with CMF-PBS. After incubation with normal goat serum (10% in CMF-PBS containing 0.1% Triton X-100) for 30 min at room temperature, primary antibody (1:500 in CMF-PBS containing 0.1 percent Triton X-100) was added and incubated overnight at 4°C. Primary antibody was removed and the cultures were rinsed three times with CMF-PBS before the addition of goat-anti-rabbit antibody (1:100 in CMF-PBS containing 0.1% Triton X-100) for 30–60 min at room temperature. After being rinsed with CMF-PBS, the cultures were incubated with PAP (rabbit) (1:100 in CMF-PBS containing 0.1% Triton X-100) for 30–60 min, rinsed twice with CMF-PBS, once with Tris buffer (0.05 M, pH 7.6) and exposed to diaminobenzidine (10 mg of diaminobenzidine was dissolved in 20 ml 0.05 M Tris buffer, pH 7.6, and 20 μ l of a 1% H₂O₂ solution was added just prior to use) for 5–10 min at room temperature.

After being rinsed three times with CMF-PBS, the coverslip was removed from the bottom of the dish with a razor blade, dehydrated through a series of ethanols and xylene, and mounted on a glass slide with Permount (Fischer Scientific Co., Pittsburgh, PA).

In experiments with AbGalc, AbNILE, or tetanus toxin, Triton X-100 was omitted from all solutions. In control experiments with AbGF, AbGF was preabsorbed with purified glial filament protein. In control experiments with AbNILE, preimmune serum was used to stain the cultures. Staining with tetanus toxin was carried out as described (8, 14).

Electron Microscopy

After 24–36 h *in vitro*, microwell cultures were washed three times with CMF-PBS and fixed with 3% glutaraldehyde (0.1 M Sorenson's phosphate buffer, 30 min, 20°C). After three additional washes with phosphate buffer, the cultures were postfixed in 1% osmium tetroxide for 30 min, washed in 0.9% NaCl for 30 min, stained en bloc with 0.5 percent uranyl magnesium acetate in 0.9% NaCl for 1 h in the dark at 4°C, and washed in saline for an additional 30 min. After dehydration in ethanols, cultures were embedded in Epok (cat. 51570, E. F. Fullam Inc., Schenectady, NY). Blocks of microcultures were removed and glued to epon stubs; thin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100S electron microscope.

Some cultures prepared for immunocytochemistry with the PAP technique were osmicated after the diaminobenzidine step and processed for electron microscopy as described.

Time-Lapse Video Microscopy

After 12–24 h *in vitro*, glass coverslip microcultures of cerebellar cells were washed one to three times with Eagle's basal medium with Hanks' Salts supplemented with HEPES buffer (20 mM), glutamine (4 mM), penicillin-streptomycin (20 U/ml) and glucose (8 mM); the medium was removed and fresh medium (50 μ l) containing 10% horse serum (Gibco Laboratories, Grand Island, NY) was then ringed with pieces of broken coverslips (no. 1, Thomas Scientific Co., Philadelphia, PA) to provide a spacer and a coverslip (no. 1, 20 mm diam, Thomas Scientific Co.) was placed over the culture. Excess medium and air bubbles were removed by blotting the edge of the coverslip with a sterile paper towel, and a ring of vaseline was applied around the perimeter with a 12-cc hypodermic syringe fitted with a 18-gauge needle. A second coverslip (no. 1, 25 mm diam, Thomas Scientific Co.) was placed over the ring of vaseline and gently tapped down with forceps (no. 5, Dumont, Oscilloscope Laboratories, Inc., West Caldwell, NJ) to seal the culture and prevent evaporation of the medium.

The culture dish was then placed on the stage of an inverted microscope (Diavert, E. Leitz Inc., Rockleigh, NJ) fitted with fiber optic illumination (series 180, Dolan-Jenner Industries, Inc., Woburn, MA), an oil-immersion phase-contrast condenser (Leitz) and a Zeiss oil immersion planapochromat 63 \times 1.4 NA phase contrast/fluorescence objective (Carl Zeiss, Inc., Thornwood, NY). The temperature was maintained at 35.5°C with a blower (model 8500, Pammotor, Burlingame, CA) heater (Leitz heater part for Leitz Diavert Environmental Chamber) mounted in a plexiglass box (10 \times 8 \times 8½ in) adjacent to the stage and controlled by an air temperature probe (model 410-J, Yellow Springs Instruments Co., Inc., Yellow Springs, OH) and a temperature controller (model 72, Yellow Springs Instruments Co., Inc.). Heat dissipation was minimized by placing a large plastic bag over both the microscope stage and heater-blower. The microscope image was recorded with a DAGE-MTI model 67 camera (DAGE-MTI, Inc., Michigan City, IN) with a newvicon tube and an NEC time-lapse video recorder (model VC-9507, Nippon Electric Co. Ltd., Tokyo Japan). An Ikegami 9-in video monitor (model PM-950, Ikegami Electronics, Inc., Maywood, NJ) was used to view the image, and a Vicon time-date generator (model V240T, Vicon Industries, Plainview, NY) was used to record the time. Photographs were made from the television monitor as described (14) with Panatomic-X film (Eastman Kodak Co., Rochester, NY).

More than 100 cultures were studied. At the conclusion of approximately one-third of the experiments, the field was scribed with a diamond knife mounted on a false objective, the culture was processed for immunocytochemistry, and the cells in that field were identified by light microscopy. In some experiments, the field was double-labeled with immunofluorescence by staining first with tetanus toxin (8, 14) and then with AbGF (14).

RESULTS

As described previously (14), after 24–48 h *in vitro*, 12–15% of the cells in cultures of cerebellar cells dissociated from early

FIGURE 1 Specific associations of cerebellar neurons with two different forms of astroglia. After 48 h *in vitro*, glass coverslip microcultures of cerebellar cells harvested from mouse cerebellum at postnatal day 7 were stained with AbGF. (a) Several dozen unstained cells nestled at or near branch points of the processes of stained cells with a stellate morphology (A). Other stained cells (B) extend longer (150 μ m) glial processes (gp) against which several unstained, phase-bright cells are apposed; one unstained cell resembles a resting neuron (rn), another a migrating neuron (mn) (compare with Fig. 9). (b) The glial process of cell B shown in a, at higher magnification. The thickened, leading process of the cell resembling a migrating neuron (mn) is contacting the stained astroglial process (gp). The end foot (ef) of the astroglial cell is densely packed with stained, glial filaments, is adjacent to the processes of stellate astrocyte (A), and appears to end on an unstained flat cell. (c) Stained astroglia with stellate morphology (A) which bind a number of presumed neurons (n) and constitute the major astroglial cell form present in the culture. (a and c) \times 348; (b) \times 868.

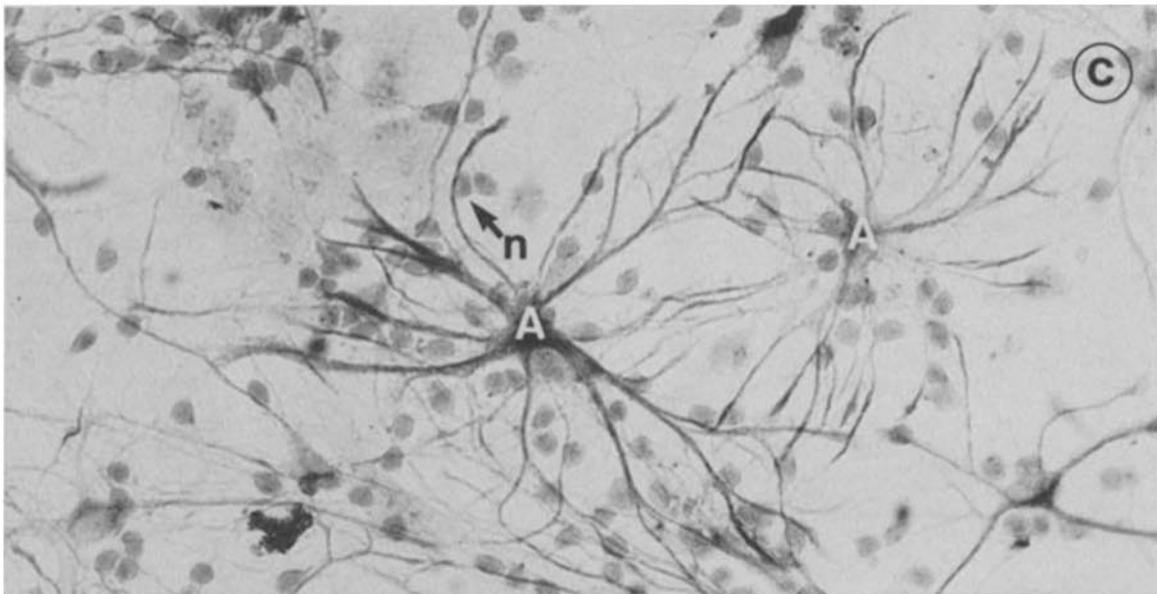
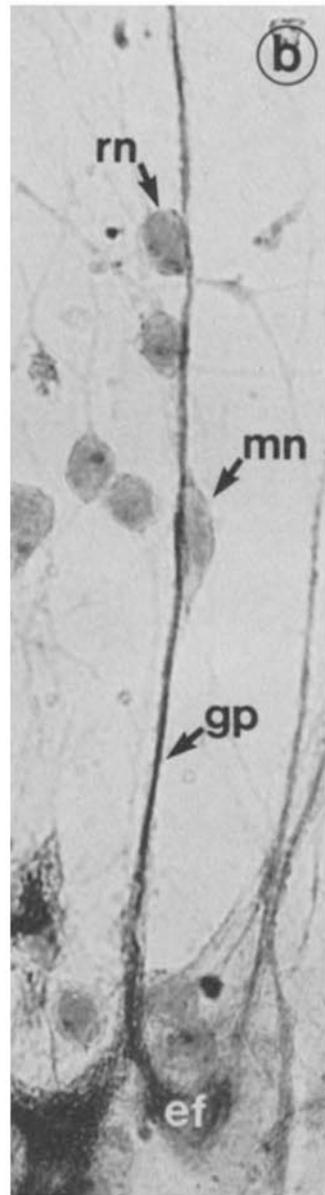
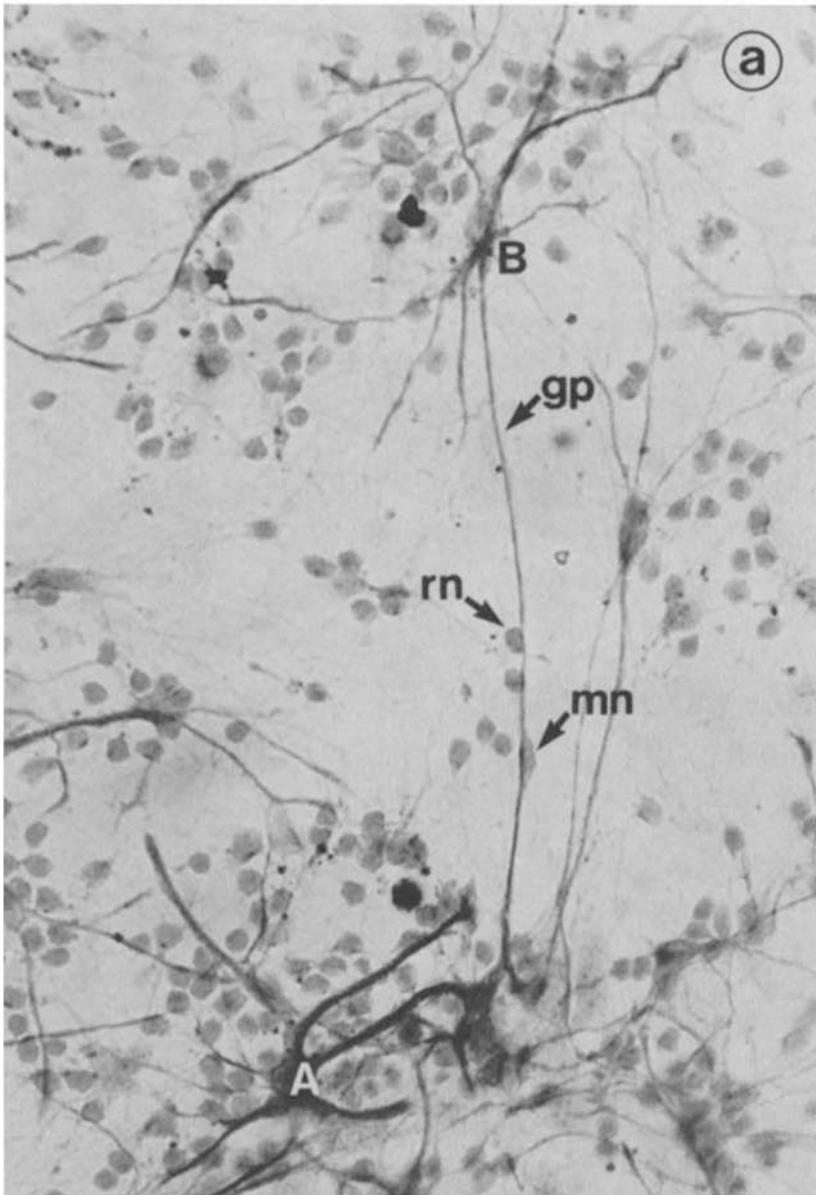


TABLE I
Characteristics of P7 Mouse Cerebellar Cells Stained with AbGF In Vitro

	Diameter of cell body	Diameter of arms	Length of processes	Number of processes	Number neurons/stained process
	μm	μm	μm		
I.	9 ± 1	1.8 ± 0.6	132 ± 38	3 ± 1	1.4 ± 1.2
II.	12 ± 3	4.7 ± 1.3	45 ± 17	6 ± 1	3.2 ± 1.4
Bergmann	6	1	130-150	4-5	2-3
Astrocytes	9	3	35-50	5-7	>5

The cellular dimensions and associations with cerebellar neurons of more than three hundred astroglial cells stained with AbGF were measured with a calibrated eyepiece. The two major forms of cells in the culture were classified as type I and type II. Values given are the mean \pm standard deviation. To compare these results with the characteristic shapes of astrocytes in vivo, we measured a few representative cells in sections of P7 mouse cerebellar tissue stained with AbGF. The number of neurons that associate with processes of Bergmann astroglia in vivo was taken from reference 25.

postnatal mouse cerebellum were stained with AbGF. Two populations of stained astroglia were observed (Fig. 1). The first, constituting ~ 10 – 20% of stained cells present in the cultures, had a smaller cell body and thinner, longer processes (Table I). Several unstained neuronal cells bound to the arms of these astroglia distal to the cell body. On the basis of their shape and pattern of association with neurons, these cells were identified as Bergmann-like astroglia (21, 24) (Fig. 1, *a* and *b* Table I).

A second type of AbGF-positive cells had a slightly larger cell body and stellate, shorter processes (Fig. 1 *c*, Table I). Among the arms of these astroglia were nestled several dozen unstained neurons with very fine, short processes (5 – $10 \mu\text{m}$). Their shape and interaction with a relatively large number of neurons resembled astrocytes of the cerebellar granular layer as well as those of white matter (21). Occasional intermediate forms were also found (Fig. 2), having both short radial arms and long Bergmann-like processes.

Staining with AbGalc revealed the presence of another class of cerebellar glial cells, oligodendroglia, which after 24 – 48 h in vitro, constituted $\sim 2\%$ of the cells in the cultures (Fig. 3). Stained cells had a large cell body 10 – $15 \mu\text{m}$ diam and numerous, short processes (20 – $30 \mu\text{m}$) radiating in all directions. In sparse cultures, stained oligodendrocytes were usually isolated from other cells. In dense cultures, stained cells were interspersed among astroglia and neurons. No unstained cells were associated with stained oligodendroglia. In addition, stained oligodendroglia did not associate with presumed astroglia.

To visualize neurons, we stained cultures with tetanus toxin or with AbNILE (8, 14, 30) (Fig. 4). The vast majority of labeled neurons were small, phase-bright cells (5 – $8 \mu\text{m}$), most of which had very fine, short processes. In general, after 24 – 48 h in culture, the processes of cells stained with AbNILE or tetanus toxin were markedly thinner and shorter than those of cells stained with AbGF. AbNILE was used routinely to avoid the potential biohazard of tetanus toxin.

The major advantage of the PAP technique was that it revealed the outlines of both labeled and unlabeled cells in the culture. However, the method was limited because it did not allow the simultaneous visualization of neurons and glia by double-labeling and because the neuronal markers we used did not distinguish among the different types of cerebellar neurons. To circumvent this limitation and to provide a second method of identifying cell types, including different classes of neurons, we processed the cultures for electron microscopy.

Three criteria were used to identify astroglia in the cultures

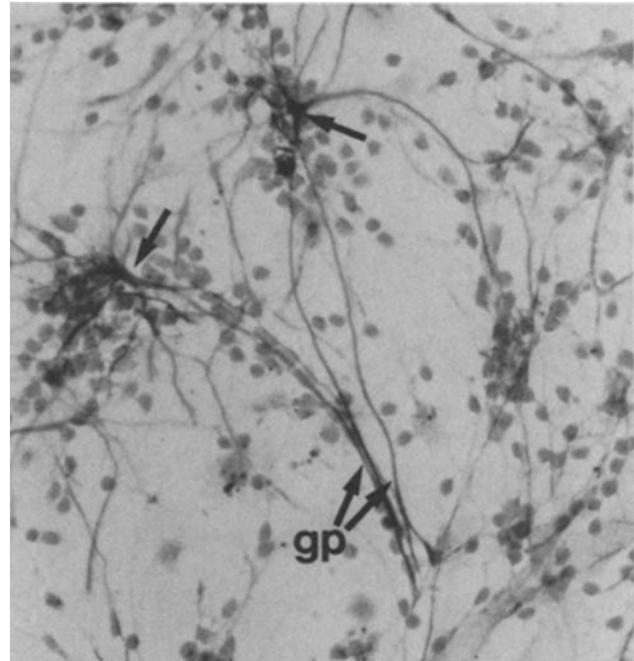


FIGURE 2 Astrocytes (arrows) that exhibit intermediate forms between Bergmann-like and astrocyte-like cells. After 48 h in vitro, glass coverslip microcultures of cerebellar cells harvested from mouse cerebellum at postnatal day 7 were stained with AbGF. Illustrated are two cells that have stellate forms as well as several long, slender arms (*gp*). $\times 248$.

with electron microscopy: first, at low power in conventional thin sections, the size, multipolar shape, and thickness of the processes provided preliminary identification (Figs. 5 and 8). Second, at higher magnification, intermediate filaments, smooth endoplasmic reticulum, elongated mitochondria, and microtubules were prominent in the cytoplasm of these large cells, which also had an indented, bean-shaped nucleus with diffuse chromatin. Third, in thin sections from cultures stained with AbGF, such cells and their processes were AbGF-positive (not shown).

Most astroglia in the cultures had a stellate profile and numerous small cells with dark cytoplasm around them. These cells resembled astrocytes of the granular layer and white matter. A few astroglia had longer, thinner processes resembling Bergmann cells which made them more difficult to characterize. The presence of intermediate filaments was used to identify such long processes as astroglial. Neuronal processes, in contrast, only contained microtubules (Fig. 7).

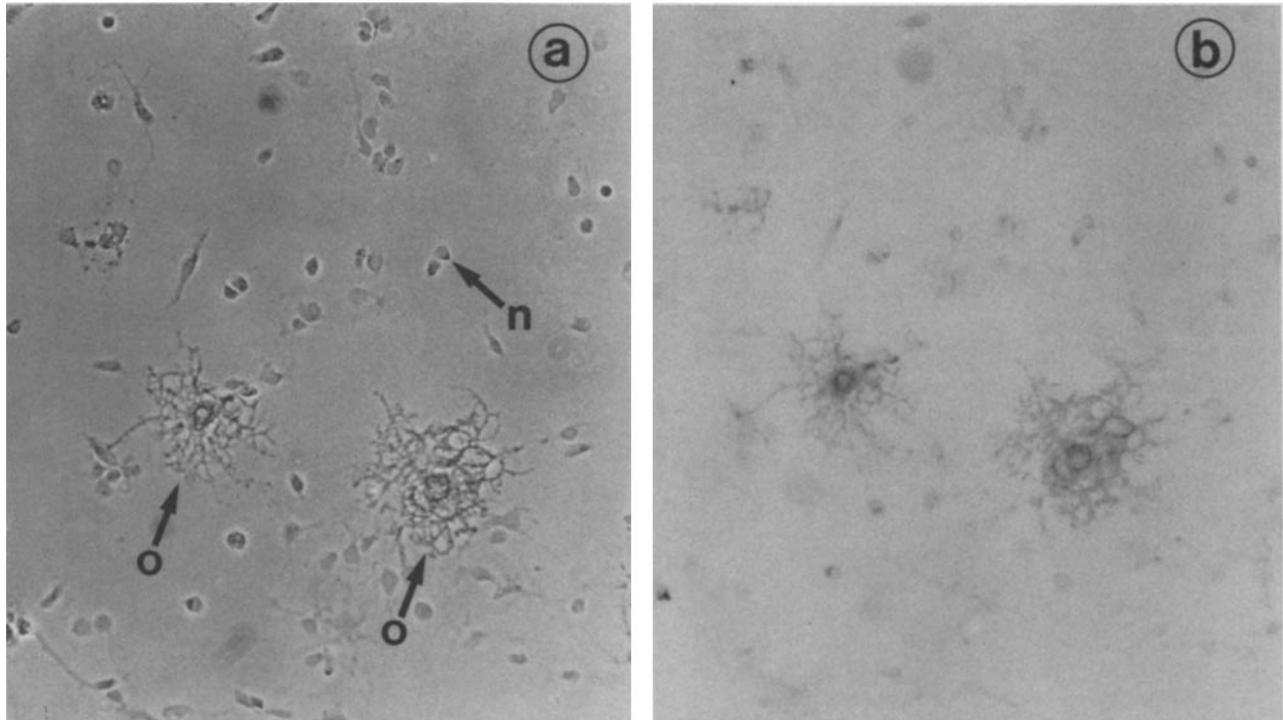


FIGURE 3 Identification of oligodendroglial cells with AbGalc. After 48 h in vitro, glass coverslip microcultures of cerebellar cells harvested from mouse cerebellum at postnatal day 7 were stained with AbGalc. (a) Phase-contrast micrograph of two stained cells, each with extensive, radial, short processes resembling oligodendrocytes (o). Smaller, phase-bright, presumed neurons (n) do not associate with stained cells. $\times 248$. (b) The same field with bright-field illumination. $\times 248$.

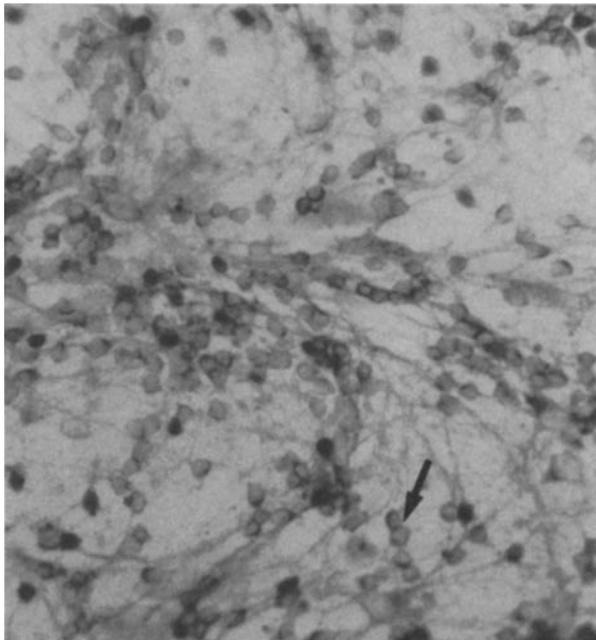


FIGURE 4 Identification of cerebellar neurons with AbNILE (30). After 48 h in vitro, glass coverslip cultures of cerebellar cells harvested from mouse cerebellum at postnatal day 7 were stained with AbNILE. Most cells in the culture, nearly all of which are phase-bright cells 5–8 μm diam with short, fine processes, show characteristic surface labeling (30), a rim of staining around the perimeter of the cell body (arrow). $\times 248$.

Immunocytochemical staining with antisera to glial filaments always stained processes with intermediate filaments, confirming their glial origin. Antisera raised against the 160,000-

dalton component of the neurofilament triplet failed to stain any cell or process type in these cultures (data not shown) after 48 h in vitro. Thus, after comparison with the light microscopic and video (see below) observations, we considered the long processes that had intermediate filaments to represent Bergmann-like astroglial fibers and those that had microtubules only (Fig. 7) to be neurites.

The cells associated with the stellate or Bergmann-like astroglia did not stain with AbGF and resembled granule neurons (21), having dark, indented nuclei rimmed with a sparse cytoplasm filled with ribosomes (Figs. 5–8). These neurons associated with both stellate (Figs. 5 and 6) and Bergmann-like glia (Fig. 7). Occasionally, larger neurons with ovoid shapes and pale cytoplasm were seen near astroglial cells. These neurons lacked intermediate filaments, and by nuclear morphology and cytoplasmic criteria, resembled larger cerebellar neurons (Purkinje, Golgi, and stellate cells) (Fig. 8). No further identification of these latter neuronal types was attempted.

The fine structure of regions of neuronal-glia contact had three notable features: The membranes of both the intermediate filament-containing glial process and neuron were ruffled (Figs. 6 and 7), a common characteristic of processes of both forms of astroglia in these cultures. Coated pits were commonly observed within glial processes (Fig. 7). Finally, junctions of the *punctum adherens* type were seen between neurons and glia, more often in the stellate astroglial variety (Fig. 5).

Stellate astrocytes were only observed in sections taken close to the surface of the dish. Neuronal cell bodies and their processes were seen in sections some distance away from the surface, indicating that the astrocytes attach to the substratum and neurons are on top of their processes.

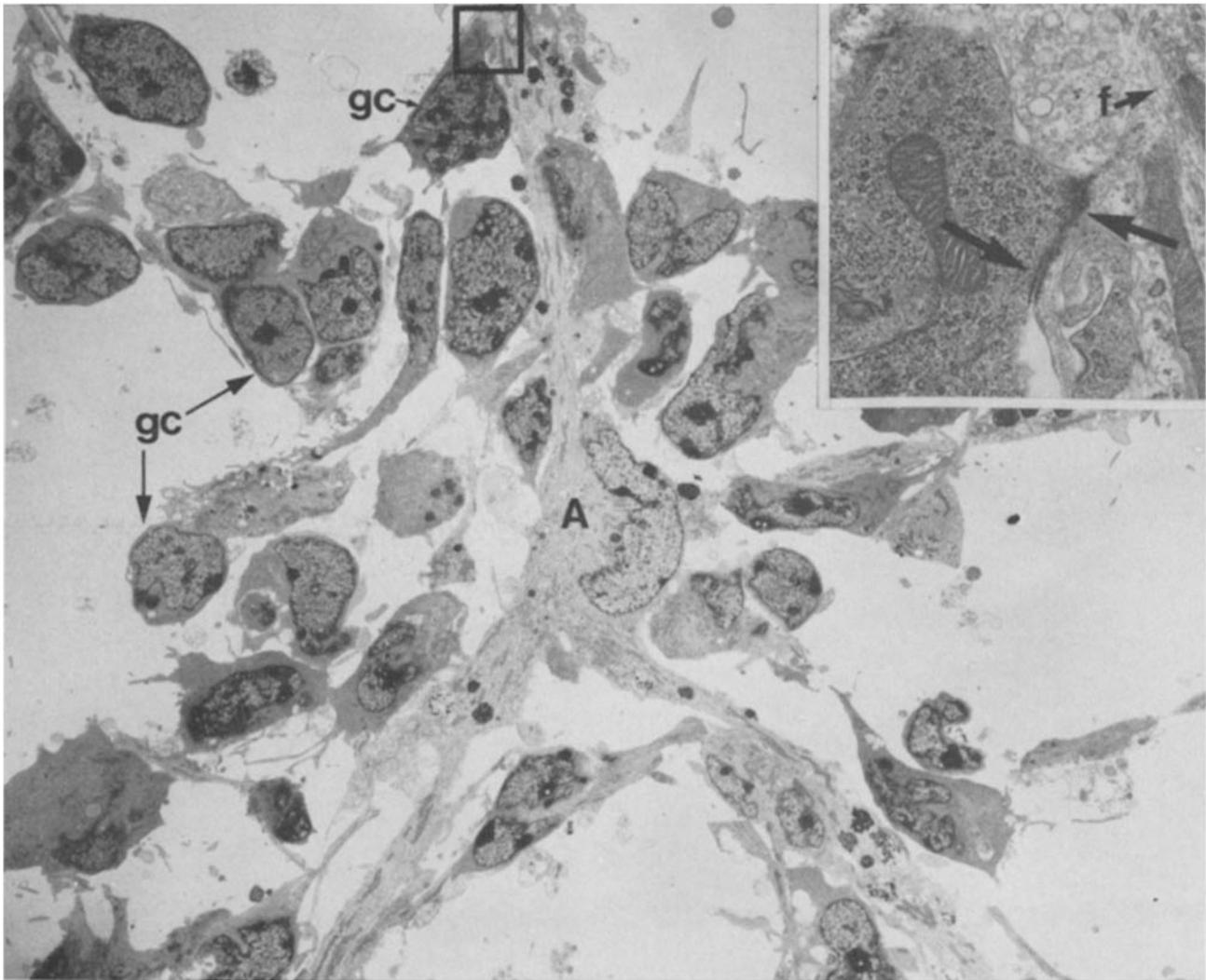


FIGURE 5 Electron micrograph of a stellate astroglial cell and the neurons associated with it, 8 h in vitro. A large, stellate cell (A) with pale cytoplasm is at the center of a cluster of smaller cells with darker cytoplasm and shorter, finer processes. Among the latter, cells 5–8 μm diam and having nucleus filled with dense chromatin and a thin rim of cytoplasm resemble granule neurons (gc) seen in vivo. $\times 2,500$. *Inset*: higher magnification of area in square, showing a granule cell (gc, small arrow in main micrograph) making a *punctum adherens* contact (arrows) with the glial process, which contains intermediate filaments (f). $\times 20,000$.

The question was then raised as to whether the behavior of neurons differed on the two shapes of astroglial cells. To address this, we made time-lapse video recordings of the cultures. The recordings revealed extensive cell movement along Bergmann-like astroglia (Fig. 9, right panel; Fig. 10). Phase-bright, presumed neurons moved by extending a thickened processes at their leading edge, progressing by an “inch-worm” motion, and rotating about the presumed glial process. The migration was rapid ($\sim 10 \mu\text{m}/\text{h}$) and bidirectional in vitro. In some experiments, neurons ran to and fro along the glial process, pausing in the region of the glial end foot or soma to reorient their leading process before starting in the opposite direction. The speed of neuronal migration and the fact that Bergmann glia were a minority among the astroglia present and generally only had a few neurons associated with them made it difficult to catch neurons in motion on Bergmann-like cells; this was also the case when analyzing the cultures with electron microscopy.

Many of the neurons in contact with Bergmann-like astroglia did not migrate during the time period the culture was

recorded. Since they did move along the glial arms later, these were termed “resting neurons”. In some cultures, several neurons were seen along a single Bergmann-like process, some resting and others migrating. Migrating neurons were able to pass stationary, resting neurons by apparently interposing between the resting neuron and the glial process (Fig. 10). In the cases where two migrating neurons were seen on a single Bergmann-like glial process, they both moved in the same direction. No examples were recorded of two migrating neurons traveling in opposite directions.

These results contrasted with the behavior of the neurons on and around astrocyte-like astroglia (Fig. 9, left panel). Although extensive movements—growth cone activity, undulation of the cell soma and process membrane, and movement of intracellular particles and organelles in the soma and processes—were seen, translocation of neuronal cells was not observed along the arms of this form of astroglial cell. In a few cases, the entire entourage, glial cell and associated neurons, moved slowly on the dish, apparently due to the movement of the glial cell.

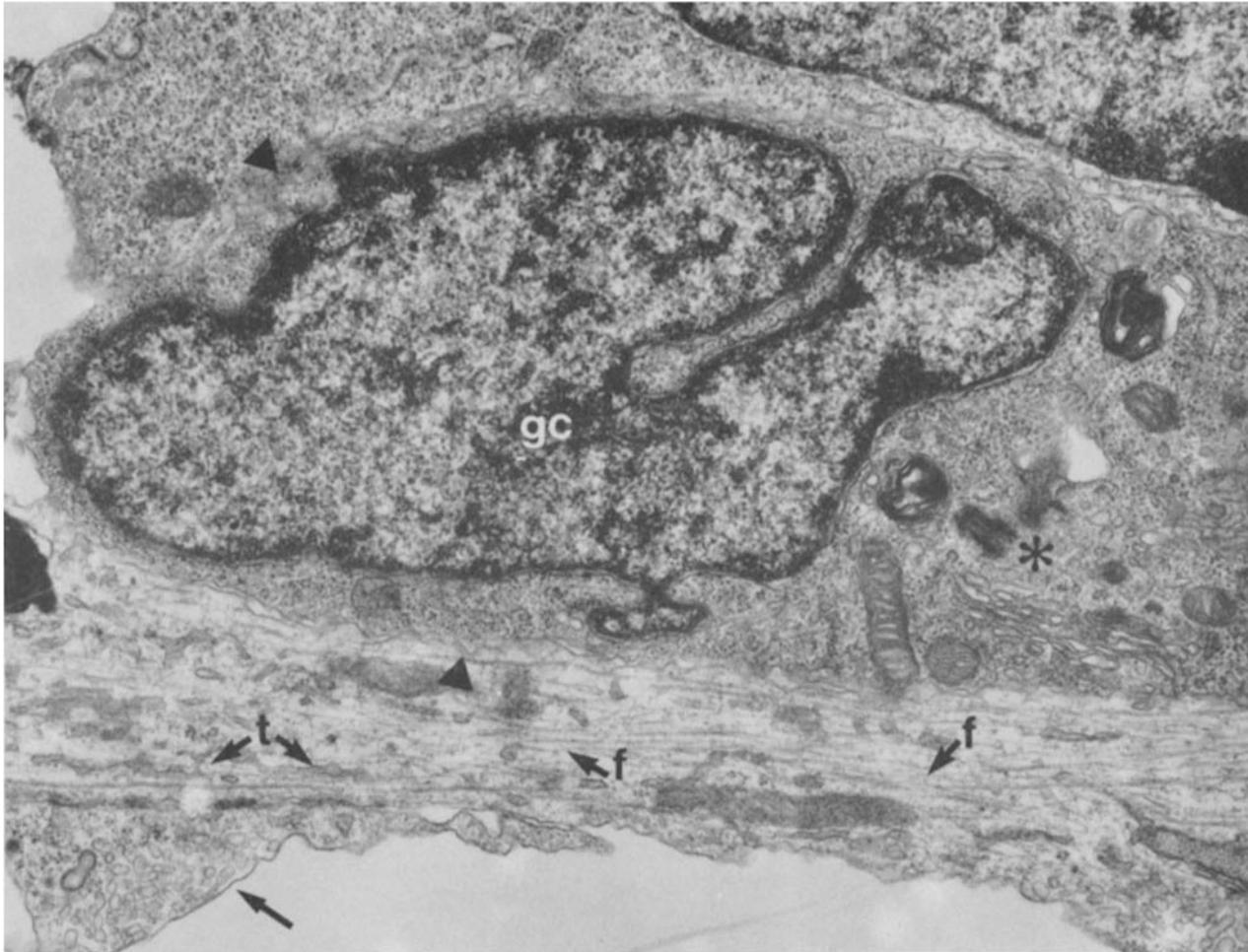


FIGURE 6 Higher power view of a granule neuron (gc) with a highly infolded nucleus containing dense heterochromatin and little cytoplasm and associating with a peripheral length of a stellate astroglial process. The soma is slightly elongated, possibly signifying that it is migrating (see Fig. 7, Fig. 9, right panel, and Fig. 10). The presence of a centriole and base of a cilium (asterisk) may also be a feature of migrating cells. The glial process contains intermediate filaments (*f*) and a smooth tubular network (*t*). Both the neuronal and glial membrane appear ruffled (arrowhead). A profile resembling a growth cone (arrow), with a variety of smooth and coated vesicles, is also apposed to the glial process. $\times 20,000$.

In about a third of the experiments, the identity of neurons and glia was confirmed by scribing the area in view and performing immunocytochemistry. In general, however, glial cell processes could be reliably discerned at high magnification by their diameter and neuronal cell bodies identified by their smaller size and phase brightness. In cases where the cells were identified by immunocytochemistry, glial cell migration along glial processes or neuronal migration along neuronal processes was not observed.

DISCUSSION

These results suggest that two classes of cerebellar glia, astroglia and oligodendroglia, are present in cultures of early postnatal cerebellar tissue, each with a different organizing influence on cerebellar neurons. Astroglia stained with AbGF appeared to interact directly with cerebellar neurons. Among stained astroglia, two subtypes were evident, Bergmann-like astroglia, which bound only two to three neurons per process and stellate astrocytes, which clustered a dozen or more neurons amongst their arms. In contrast, oligodendroglia stained with AbGalc did not associate with cerebellar neurons

or their processes in tissue culture over the time period studied.

Although early postnatal cerebellar cells stain with AbGalc *in vitro*, staining is not observed *in vivo* until later developmental stages (C. A. Mason, unpublished observation). This may reflect an "early" expression of galactocerebroside *in vitro*, or the fact that in tissue culture where staining is predominantly of the cell surface, it is easier to visualize AbGalc.

The finding that oligodendrocytes in the cultures did not associate with neurons or their processes after short periods *in vitro* is consistent with their observed role *in vivo*, namely to myelinate axons in the white matter of the cerebellum. Myelination does not begin until after the first postnatal week (C. A. Mason, unpublished observation). It is possible that some specific association(s) between identified oligodendroglia and Purkinje cell axons would occur *in vitro* if the cultures were maintained for much longer periods and if Purkinje cells survived. We did not pursue this possibility.

Although after 24 h *in vitro*, the most prominent processes in the culture were stained with AbGF, high magnification light microscopy suggested that very fine, short, unstained

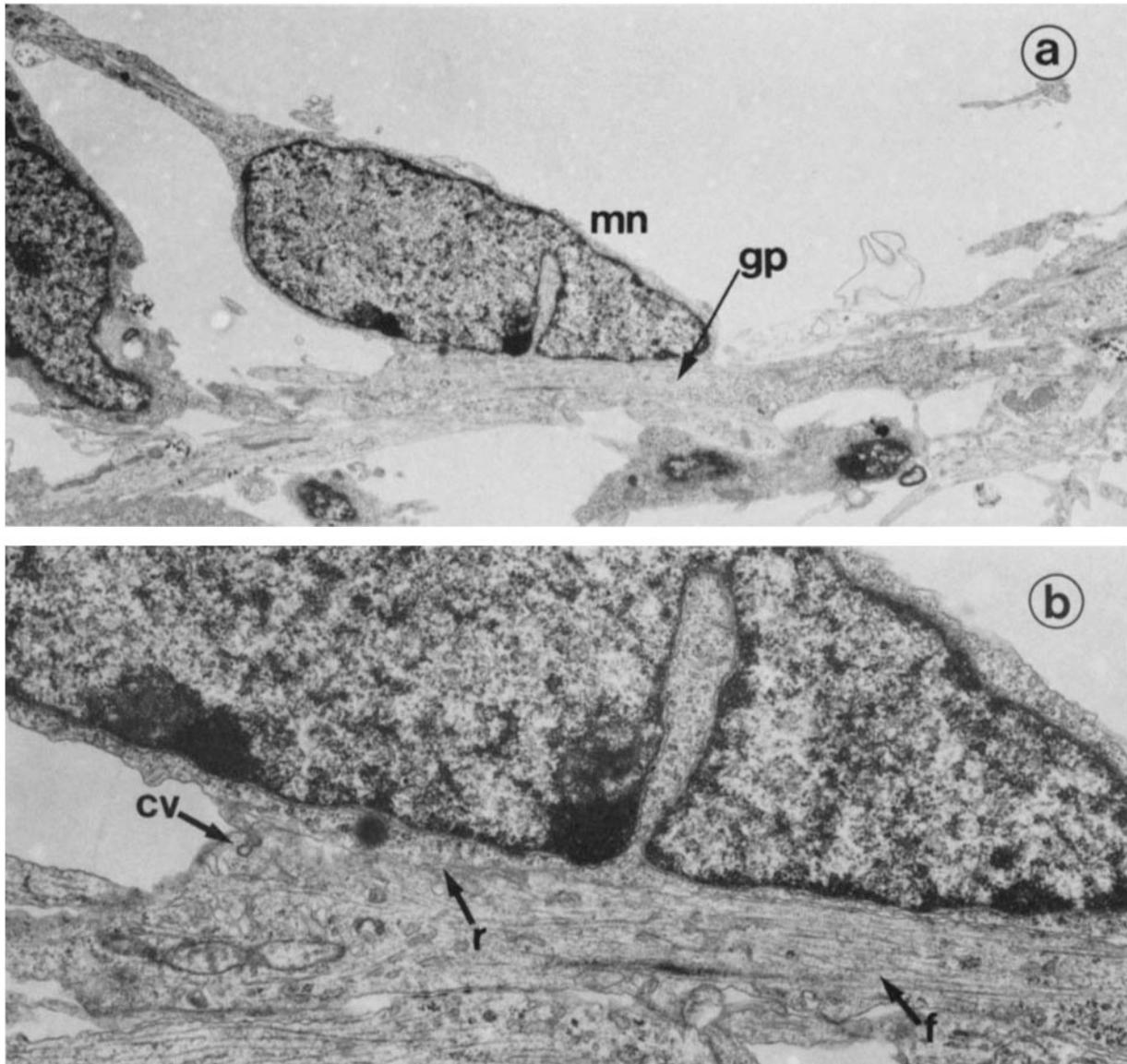


FIGURE 7 Electron microscopy of the interaction between cerebellar neurons and Bergmann-like astroglial processes (gp), 24 h in vitro. (a) Granule neuron (mn) apposed to a long thin process, assumed to be migrating by its elongated shape (cf. Fig. 9, right panel and Fig. 10). Even at low power, the thin rim of cytoplasm and blocks of heterochromatin in the indented nucleus identifies this as a granule cell. (b) At higher power, the process on which the neuron sits contains intermediate filaments (f) and a coated vesicle (cv). Note ruffling of both the neuronal and glial membranes (r). Process at lower edge of micrograph may be neuronal, since it lacks filaments. (a) $\times 5,000$; (b) $\times 15,000$.

processes, which were presumably neuronal, were also present at this time. This result was more easily observed with the PAP technique than with fluorescence microscopy (14).

The major limitation of the immunocytochemistry presented was that it did not allow the simultaneous identification of glia and neurons. The markers used for cerebellar neurons had the further limitation that they did not distinguish among the types of neurons present in the cultures. These issues were addressed by evaluating the cultures with electron microscopy. Electron microscopy also provided the first opportunity to directly compare the identification of cell types made with indirect immunocytochemistry with that made by ultrastructural characteristics alone.

The results with electron microscopy were consistent with those obtained with immunocytochemistry. Two forms of astroglia were evident in the cultures, each with a different

organizing influence on cerebellar neurons. In general, the majority of neurons that associated with identified astroglia were granule neurons, but occasionally other neurons, including large cells, contacted astroglia in vitro. We could not distinguish whether this reflected the high percentage (>90%) of granule cells in the neuronal population, both in vivo and in vitro, or a higher specificity of granule cells for astroglia.

The time-lapse video recordings demonstrated two different functional roles for the two types of cerebellar astroglia. Bergmann-like astroglia appear to promote migration, whereas astrocyte-like astroglia appear to inhibit migration and, instead tether neurons. It remains to be determined whether the binding of a certain number of neurons at a specific maturational stage induces the shape and surface properties of the astroglial cell or, conversely, whether the form and surface chemistry of the glial cell is intrinsic and

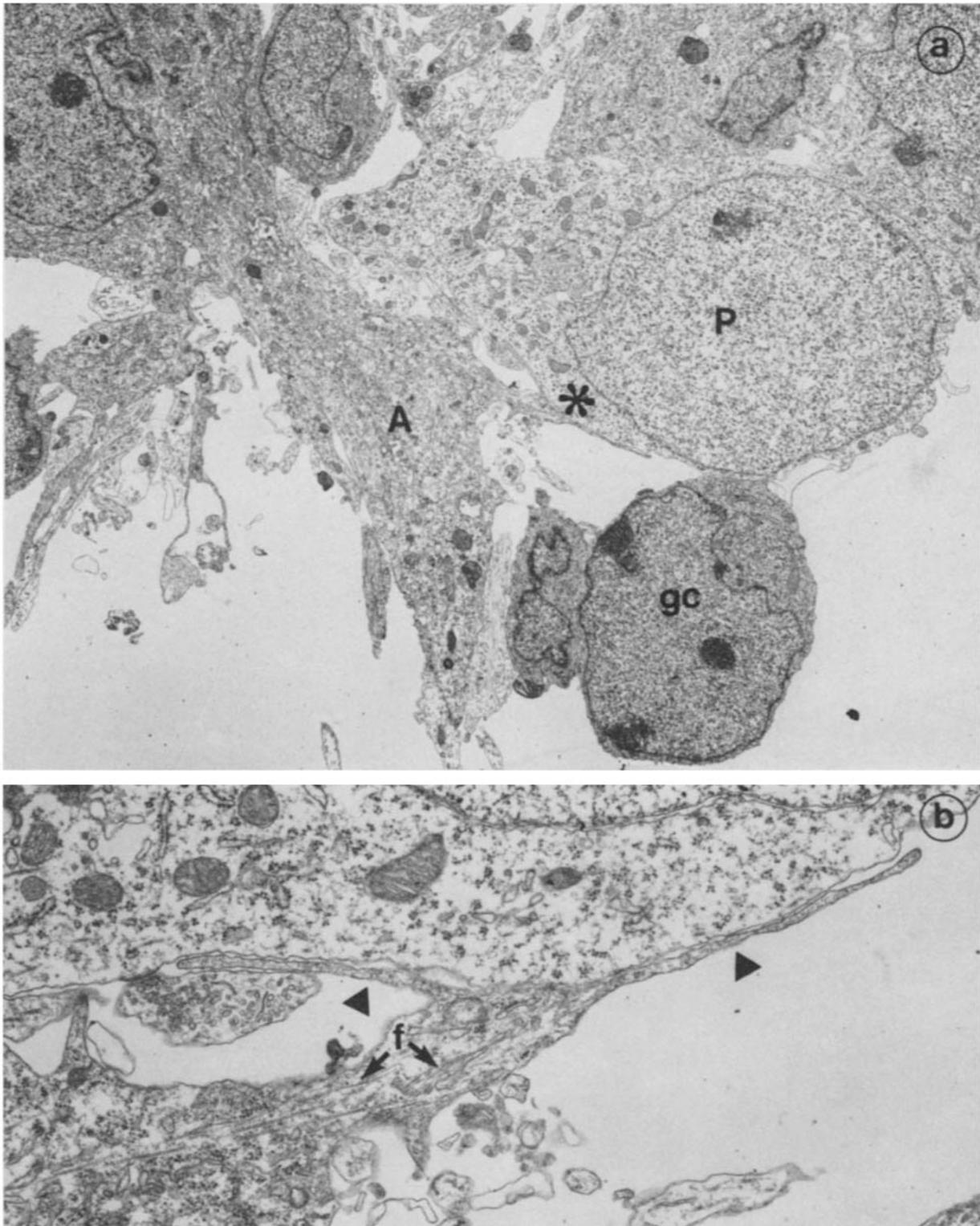


FIGURE 8 a) Low-power electron micrograph of the edge of an astrocytic nest, showing two forms of neurons seen in these cultures 24 h in vitro. Granule neurons (gc) with scant amounts of cytoplasm are the most common cell type. Larger neurons with oval shapes and abundant pale cytoplasm at one pole of the cell (P) are less frequently encountered. These resemble Purkinje cells in vivo. (b) Area in a marked by asterisk. A y-shaped process (arrowheads) of the intermediate filament-containing astrocyte (f) clasps the Purkinje-like neuron. (a) $\times 5,000$; (b) $\times 20,000$.

dictates the binding and mobility of the neurons.

Others have reported a close association of neurons with glia in tissue culture. However, these studies were made with explant preparations (6, 9, 10) or under conditions where cellular reaggregates formed (34). In addition, others have

shown the movement of neurons along thick bundles of processes that contain glial elements (34).

Cell surface elements, including those of glial cells, have been proposed to promote cellular adhesion in vitro (11-13) and to facilitate neurite outgrowth for certain types of neurons

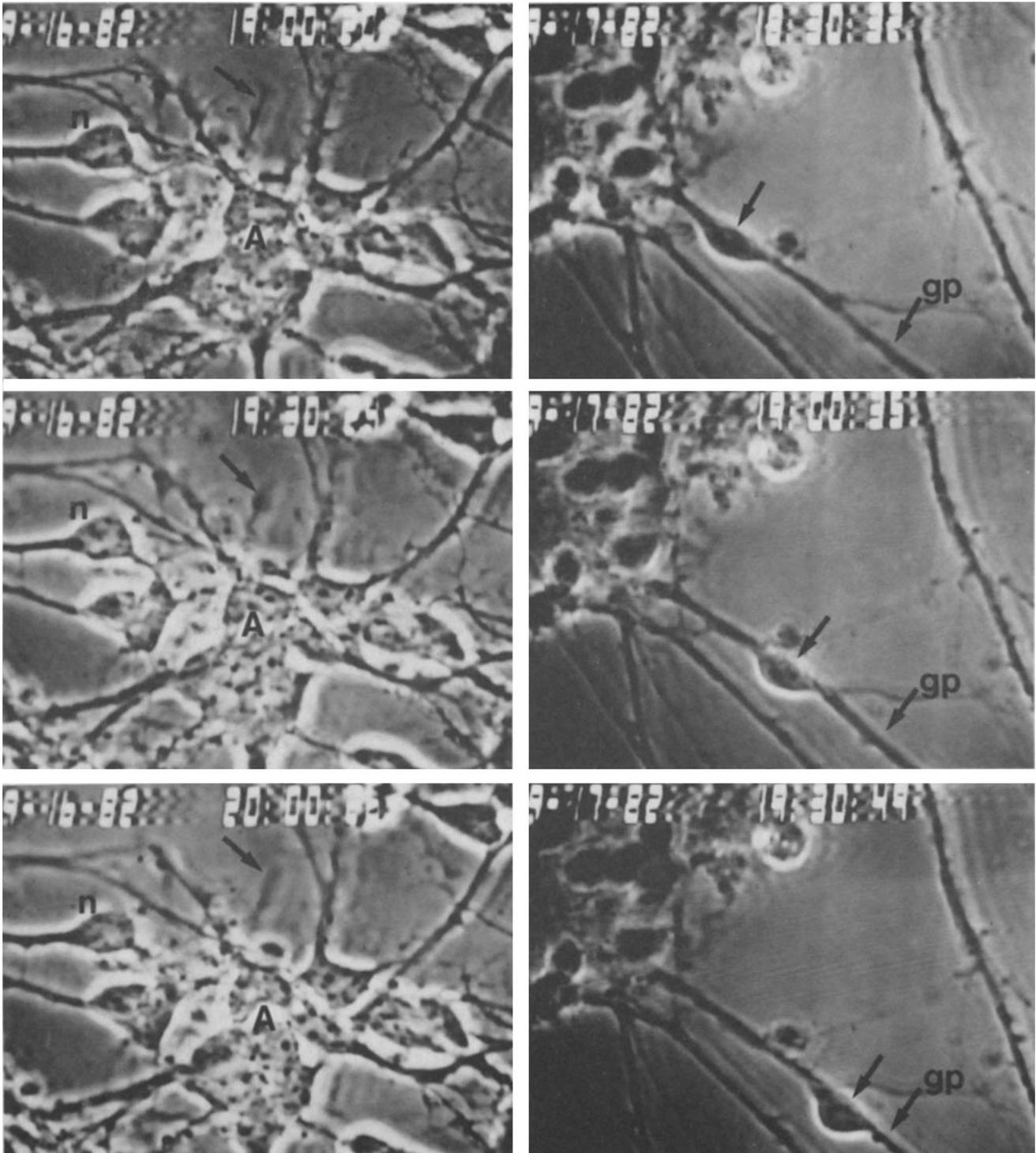


FIGURE 9 Time-lapse video microscopy of cells associated with different forms of cerebellar astroglia. After 12–24 h in vitro, glass coverslip microcultures of cerebellar cells harvested at postnatal day 7 were transferred to the video microscopy apparatus. In the left panels, the behavior of cells nestled on a stellate astroglial cell is recorded. The astroglial cell (A) is at the center of the field, its cell body obscured by phase-bright, presumed neurons (n) clustered on and around it. The latter cells are stationary during the time period studied (shown by the record of the time-date generator across the top of each photograph), but growth cone activity (arrow) as well as pulsating movements of the cell somata are evident. The right panels show the behavior of cells that appose the long, slender glial processes (gp) resembling those of Bergmann astroglia. The phase-bright cell body (arrow) moves along the process at $\sim 30 \mu\text{m}/\text{h}$. All photographs were made directly from the television monitor. Phase-contrast illumination. $\times 1,045$.

(2, 16–18, 23). The latter issue as well as the relationship of granule neurite extension to cell migration remains to be studied.

The role of *puncta adherentia* and of coated vesicles seen at the junction between cerebellar neurons and astroglia remains to be analyzed. Although coated vesicles have been

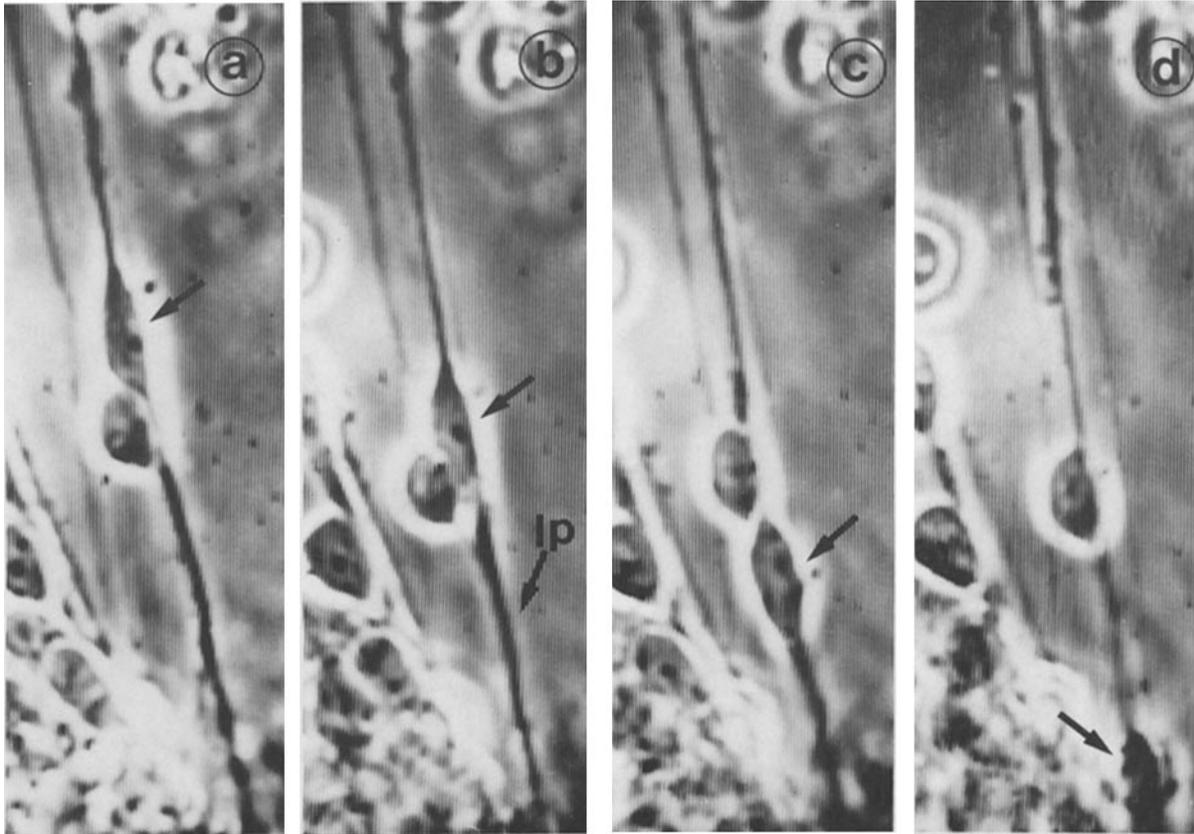


FIGURE 10 The movement of migrating cells past stationary cells apposed to glial processes. After 24 h, glass coverslip microcultures of mouse cerebellar cells harvested at postnatal day 7 were transferred to the time-lapse video apparatus and the behavior of the cells was recorded. A phase-bright cell with a thickened leading process (*lp*) is shown moving along a long process of an astroglial cell, past a rounded, phase-bright cell that remains stationary. Four time points are shown: (a) time zero, (b) after 30 min, (c) after 60 min, and (d) after 90 min. All photographs were taken directly from the television monitor. Phase-contrast microscopy. $\times 1,290$.

seen repeatedly between neural elements, including pre- and postsynaptic elements, during development (28, 29), the present results are the first suggestion that these occur between neurons and glia. Such vesicles, if they represent a vehicle for the exchange of macromolecules, could play a role in neuron-glia interactions or in the differentiation or function of one or both of these cell types.

This study demonstrates that immunocytochemistry, electron microscopy, and video time-lapse microscopy are a useful combination of techniques for correlating morphological characteristics with cellular behavior. The microcultures provide a paradigm for the regulation of specific interactions between neurons and cerebellar astroglia and should facilitate the identification of surface macromolecules involved in these specific cell contacts.

Ann M. Francois and Beth Gregory provided expert technical assistance in these studies. James Edmondson participated in the experiment presented in Fig. 9. We are especially grateful to Dr. Michael L. Shelanski for his support and criticism throughout this study and thank Dr. Paul C. Letourneau for his advice on time-lapse video microscopy.

This work was supported by National Institutes of Health (NIH) grants NS 15429 (M. E. Hatten), NS 15182 (R. K. H. Liem), and NS 16951 (C. A. Mason). M. E. Hatten and C. A. Mason are recipients of the Irma T. Hirsch Career Scientist Award and M. E. Hatten is a Sloan Research Fellow. R. K. H. Liem and C. A. Mason are recipients of an NIH Research Career Development Award.

Received for publication 22 July 1983, and in revised form 16 September 1983.

REFERENCES

1. Antanitus, D. S., B. H. Choi, and L. W. Lapham. 1975. Immunofluorescence staining of astrocytes *in vitro* using antiserum to glial fibrillary acidic protein. *Brain Res.* 89:363-367.
2. Baron-Van Evercooren, A., H. K. Kleinman, S. Ohno, P. Marangos, J. P. Schwartz, and M. E. Dubois-Dalecq. 1982. Nerve growth factor, laminin and fibronectin promote neurite outgrowth in human fetal sensory ganglion cultures. *J. Neurosci. Res.* 8:179-193.
3. Bignami, A., and D. Dahl. 1973. 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.
4. Bignami, A., and D. Dahl. 1974. Astrocyte-specific protein and neuroglial differentiation. An immunofluorescence study with antibodies to the glial fibrillary acidic protein. *J. Comp. Neurol.* 153:27-38.
5. Bignami, A., L. F. Eng, D. Dahl, and C. T. Uyeda. 1972. Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence. *Brain Res.* 43:429-435.
6. Bunge, R. P., M. B. Bunge, and E. R. Peterson. 1965. An electron microscopic study of cultured rat spinal cord. *J. Cell Biol.* 24:163-191.
7. Eng, L. F., J. J. Vanderhaeghen, A. Bignami, and B. Gerstl. 1971. An acidic protein isolated from fibrous astrocytes. *Brain Res.* 28:351-354.
8. Fields, K. L., J. P. Brookes, R. Mirsky, and L. M. B. Wendon. 1978. Cell surface markers for distinguishing different types of dorsal root ganglion cells in culture. *Cell.* 14:43-51.
9. Grainger, F., and D. W. James. 1970. Association of glial cells with terminal parts of neurite bundles extending from chick spinal cord *in vitro*. *Z. Zellforsch.* 108:93-105.
10. Guillery, R. W., H. M. Sobkowicz, and G. L. Scott. 1970. Relationships between glial and neuronal elements in the development of long term cultures of the spinal cord of the fetal mouse. *J. Comp. Neurol.* 140:1-34.
11. Hatten, M. E. 1981. Cell assembly patterns of embryonic mouse cerebellar cells on carbohydrate-derivatized polylysine culture substrata. *J. Cell Biol.* 89:54-61.
12. Hatten, M. E., and A. M. Francois. 1981. Cell assembly patterns of developing cerebellar cells on lectin-derivatized culture substrata. *Devel. Biol.* 82:102-113.
13. Hatten, M. E., M. B. Furie, and D. B. Rifkin. 1982. Binding of developing mouse cerebellar cells to fibronectin: a possible mechanism for the formation of the external granular layer. *J. Neurosci.* 2:1195-1206.

14. Hatten, M. E., and R. K. H. Liem. 1981. Astroglial cells provide a template for the positioning of developing cerebellar neurons in vitro. *J. Cell Biol.* 90:622-630.
15. Hatten, M. E., and R. L. Sidman. 1978. Cell reassociation behavior and lectin-induced agglutination of embryonic mouse cells from different brain regions. *Exp. Cell Res.* 113:111-125.
16. Lander, A. D., D. K. Fujii, D. Gospodarowitz, and L. F. Reichardt. 1982. Characterization of a factor that promotes neurite outgrowth: evidence linking activity to a heparan sulfate proteoglycan. *J. Cell Biol.* 94:574-585.
17. Letourneau, P. C. 1975. Possible roles for cell to substratum adhesion in neuronal morphogenesis. *Dev. Biol.* 44:77-91.
18. Letourneau, P. C. 1975. Cell-to-substratum adhesion and guidance of axonal elongation. *Devel. Biol.* 44:92-101.
19. Liem, R. K. H., S. H. Yen, G. D. Salomon, and M. L. Shelanski. 1978. Intermediate filaments in nervous tissue. *J. Cell Biol.* 79:637-645.
20. Mirsky, R. 1982. The use of antibodies to define and study major cell types in the central and peripheral nervous system. In *Neuroimmunology*. J. Brockes editor. Plenum Press, New York. 141-181.
21. Palay, S. L., and V. Chan-Palay. 1974. In *Cerebellar Cortex, Cytology and Organization*. Springer-Verlag, Berlin. 289-316.
22. Peters, A., S. L. Palay, and H. D. F. Webster. 1976. In *The Structure of the Nervous System*. W. B. Saunders Co., Philadelphia. 231-248.
23. Pintar, J. E. 1978. Distribution and synthesis of glycosaminoglycans during quail neural crest morphogenesis. *Devel. Biol.* 67:444-464.
24. Rakic, P. 1971. Neuron-glia relationship during granule cell migration in developing cerebellar cortex. A golgi and electron microscopic study in *Macacus Rhesus*. *J. Comp. Neurol.* 141:282-312.
25. Rakic, P., and R. L. Sidman. 1973. Sequence of developmental abnormalities leading to granule cell deficit in cerebellar cortex of weaver mutant mice. *J. Comp. Neurol.* 152:103-132.
26. Rakic, P., L. J. Stensaas, E. P. Sayre, and R. L. Sidman. 1974. Computer-aided three dimensional reconstruction and quantitative analysis of cells from serial electron microscopic montages of foetal monkey brain. *Nature (Lond.)* 250:31-34.
27. Ranscht, B., P. A. Clapshaw, J. Price, M. Noble, 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.
28. Reese, R. P. and M. B. Bunge. 1975. Origin of coated vesicles in neuronal perikarya. A study of peroxidase uptake by cultured neurons with or without neurites. *J. Cell Biol.* 27(2, Pt. 2):357a. (Abstr.).
29. Reese, R. P., M. B. Bunge, and R. P. Bunge. 1976. Morphological changes in the neurite growth cone and target neuron during synaptic junction development in culture. *J. Cell Biol.* 68:240-263.
30. Salton, S. R. J., C. Richter-Landsberg, L. Greene, and M. L. Shelanski. 1983. Nerve growth factor-inducible large external (NILE) glycoprotein: studies of a central and peripheral neuronal marker. *J. Neurosci.* 3:441-454.
31. Schachner, M., E. T. Hedley-White, D. W. Hsu, G. Shoonmaker, and A. Bignami. 1977. Ultrastructural localization of glial fibrillary acidic protein in mouse cerebellum by immunoperoxidase labeling. *J. Cell Biol.* 75:67-73.
32. Sidman, R. L., and P. Rakic. 1973. Neuronal migration, with special reference to developing human brain. A review. *Brain Res.* 62:1-35.
33. Sternberger, L. A. 1979. In *Immunocytochemistry*. John Wiley and Sons, New York.
34. Trenkner, E., and R. L. Sidman. 1978. Histogenesis of mouse cerebellum in microwell cultures. *J. Cell Biol.* 75:915-940.