

HISTOGENESIS OF MOUSE CEREBELLUM IN MICROWELL CULTURES

Cell Reaggregation and Migration, Fiber and Synapse Formation

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ABSTRACT

A microwell culture system was developed for analysis of cell movements and interactions during nervous system histogenesis. Cells from trypsinized 7-day-old mouse cerebellum reaggregated within hours into clusters which later developed interconnections consisting of either sheets of migrating cells and cell processes or cables of fiber bundles with cells migrating along their surfaces. Granule cells in several stages of differentiation, basket and/or stellate neurons, some larger neurons, and two types of neuroglial cells were identified in reproducible, nonrandom patterns by scanning and transmission electron microscopy. Axonal and dendritic processes, both with growth cones, and numerous synapses were generated in vitro.

KEY WORDS mouse cerebellum · microwell cultures · cell identification · histogenesis · reaggregation · migration

The construction of a complex organ results from a patterned proliferation and subsequent redistribution of cells of several types, apparently determined epigenetically by orderly developmental sequences of external signals and cell surface interactions. Genetic endowment ultimately controls both the production of the signalling molecules and the receptor mechanisms. Among the model systems suitable for analysis of such events, the immunological system serves as one prototype, in which undifferentiated precursor cells migrate via the circulation and assemble in specific organs after induction of differentiation (15, 26, 48, 65). Reaggregation systems provide another model, especially suitable for analysis of the events that come after migration, when dif-

ferent classes of cells interact to construct an organ (for review, see reference 38). Construction of a vertebrate nervous system, probably the most complex of organs, involves both migration and specific rearrangement of cells in an unusually precise three-dimensional pattern that appears to specify many of the functional attributes of the system (51, 59, 60).

Within the nervous system, the cerebellum has received particular attention because it is composed of a relatively limited number of cell types in a highly stereotyped geometric pattern (23, 42). The main events in genesis of that pattern have been worked out (1-4, 22, 25, 27, 30, 35, 37, 39, 40, 49, 54, 58, 59, 62, 66, 70), in part with the aid of mutations that perturb cellular development in mice (51, 58, 59). Yet, it would be useful to have a simplified in vitro system in which cell assembly could be analyzed and controlled. Ideally, one would want a culture

system which permits cell-cell interactions leading to reaggregation and migration processes comparable to those occurring under in vivo conditions, and suitable for use with developing cerebellar tissue of individual normal and mutant animals.

Existing cerebellar culture systems meet this need only in part. They have served especially well for study of synapse formation, membrane excitability, neurotransmitter synthesis, and myelination. However, each culture system has important limitations. In most dissociated cell cultures single cells attach to artificial substrates and form relatively uncoordinated monolayers (6, 9, 14, 20, 33, 34, 36, 41, 55, 57, 63, 64, 69). Organotypic culture systems allow the most reliable cell identifications and interactions (10, 29, 45, 46, 47, 67, 68), but are almost as inaccessible as in vivo tissues, particularly for analysis and manipulation of cell surface molecules during development. Suspension reaggregates have yielded relatively little developmental information on cerebellum (for review, see reference 56). The mouse, which offers better genetic control than most conveniently available laboratory animals, has been used relatively little for tissue culture studies (6, 20, 36, 41); rat, chick, and man having proved in general to be more amenable species for central nervous system (CNS) culturing (11, 12, 19, 63).

The inaccessibility in vitro of the critical morphogenetic events of cell migration and cell rearrangement led us to the present work in which we describe a microsystem designed to investigate molecular aspects of cell interactions during development of the mouse cerebellum. The results indicate that single cell suspensions of early postnatal mouse cerebellum quickly reorganize and form reproducible patterns in vitro. The reaggregates express few of the topographic features displayed by cerebellum in vivo, but do show the survival and apparent interaction of particular cerebellar cell types. The histogenetic events of cell migration, process formation, and synaptogenesis are documented in the present study as a baseline for experimental manipulation of these events, to be described in subsequent communications.

MATERIALS AND METHODS

Media

Ca⁺⁺- and Mg⁺⁺-free isotonic solution (CMF) was

prepared according to Barkley et al. (5). Basal medium Eagle (Grand Island Biological Co., Grand Island, N. Y.) supplemented with glucose (0.25%) will be referred to as EG. The culture medium consisted of EG supplemented with glutamine (2 mM), penicillin (25 U/ml), streptomycin (25 U/ml), and horse serum (10%) (Grand Island Biological Co.). Trypsin and DNase were obtained from Worthington Biochemical Corp. (Freehold, N. J.) (code TRL and DP, respectively).

Mice

Postnatal day 7 (P7) C57BL/6J mice, originally derived from The Jackson Laboratory (Bar Harbor, Me.), were obtained from matings in our own breeding colony maintained on a 12-h alternating light-dark schedule.

Preparation of Single Cell Suspensions

Preparation of single cell suspensions was performed as described by Barkely et al. (5). The cerebellum or, in a few cases, tissue from other CNS regions from P7 C57BL/6J mice was dissected out and immersed in CMF under sterile conditions, washed three times in CMF and incubated in 1% trypsin in CMF (14 min, 20°C, pH 7.2). Trypsin was removed by washing three times in CMF, and the tissue was transferred into EG containing 0.05% DNase, where it was triturated with fire-polished long-tip Pasteur pipettes with decreasing pore sizes. When necessary, the resultant cell suspension was incubated at 4°C for 4 min to sediment residual tissue clumps, and the supernate was transferred to a centrifuge tube. Cells were collected by centrifugation in a Sorvall table centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.) at 600 rpm for 8–10 min at 4°C. The pellet was resuspended in culture medium. At the end of these procedures, 85–95% of the cells were viable as determined by trypan blue exclusion.

Tissue Culture Conditions

Single cell suspensions were plated in micro titer plates (Falcon 3034, BioQuest, BBL & Falcon Products, Cockeysville, Md.) at a concentration of 5×10^4 to 1×10^5 cells per 10 μ l medium per well and incubated at 36°C in a moist chamber containing 5% CO₂. No change of medium was made in cases where samples were taken at 24 h, 72 h, and 6 days in vitro. Many thousand cultures were examined, each at several time-points up to 10 days in vitro. About 100 cultures were maintained up to 35 days, with weekly changes of medium. Medium changes consisted of removal of 5 μ l of old medium and replacement with an equal volume of the same medium. Cytological studies were confined to the first 10 days in vitro and were carried out on more than 50 cultures.

Time-Lapse Cinematography

Cultures of P7 cerebellum were maintained for 48 h in an incubator containing 5% CO₂. For time-lapse cinematography, cultures in their original wells were sealed with vacuum grease to minimize gas exchange and transferred to a 36°C room. Kodak 16-mm reversal film 7276, PXR 449 was used. Pictures were taken at a frequency of eight frames per minute at $\times 100$.

Preparation of Cultures for Light and Transmission Electron Microscopy

Cultures were fixed in microwells for 30 min at room temperature after 24 h, 72 h, and 6 days in vitro by adding 6 ml of 3% glutaraldehyde in CMF to the whole microwell plate. After three washes in CMF, the cultures were postfixed in 1% OsO₄ in CMF for 30 min, followed by seven washes in glass-distilled water to remove phosphates. Cultures were then stained en bloc with 1% uranyl acetate in H₂O for 1 h at room temperature in the dark, washed three times in water, and dehydrated progressively through 50, 70, 95%, and three changes of 100% ethanol for 10 min each. Epon was added to each well and changed five times at intervals of 1 h, 1 h, 1 h, overnight, and 2 h at room temperature, and was then polymerized for 2 days at 60°C. The blocks were removed mechanically and mounted on Epon stubs. Sections 1 μm thick were stained alkaline toluidine blue and examined by light microscopy. Ultrathin sections were stained for 2 min in 2% uranyl acetate and for 5 min in lead citrate (5 $\mu\text{g}/\text{ml}$) and examined on uncoated grids by electron microscopy.

Preparation of Cultures for Scanning Electron Microscopy

Fixation and dehydration procedures were as described in the previous paragraph. After dehydration in ethanol, cultures were critical-point dried with liquid CO₂ and coated with carbon and gold palladium (7).

RESULTS

Pattern Formation

Within each microwell a reproducible series of events commences as soon as cells are seeded and reaches a stable configuration by the third day in culture. The three major components, overlapping in time are (a) reaggregation, (b) outgrowth of specific cells and cell processes to form irregular sheets between reagggregates, and (c) outgrowth of straight cellular processes to form cables between reagggregates, with cells lying along the cables. Collectively, these events will be referred to as "pattern formation."

The first step in pattern formation observed in microcultures of cerebellar cells of P7 mice is the spontaneous and apparently random aggregation of suspended single cells (Fig. 1) into cell clumps (Fig. 2). Most cells become incorporated within the first 3–4 h in vitro, about 10–15 clumps of various sizes forming per microwell. At initial cell densities $>1 \times 10^5/\text{well}$, fewer and larger aggregates are observed but become necrotic within a few days, whereas at cell densities less than $2 \times 10^4/\text{well}$, small aggregates form and the subsequent cell behaviors to be described below for optimal aggregates are rarely seen. Thus, the optimal density for the culture conditions described here was found to be $5\text{--}10 \times 10^4$ cells per microwell.

It is of special interest to note that the cells rapidly settle to the bottom surface of the dish (Fig. 1) but then always form reagggregates before becoming attached to the culture dish. This observation suggests that under given culture conditions, the affinity of one cell for another is greater than that of cells for the tissue culture substratum.

Between 18 and 24 h after seeding the cells, the reagggregates become interconnected by two types of bridging formations. One consists of a sheet of flat cells attached to the well onto which cells migrate and cell processes grow outward from the reaggregate. The other consists of long, straight fiber bundles with additional cells positioned along them (Figs. 3, 4, 20, and 21). Proof that these cells are actively migrating was obtained by time-lapse cinematography (Fig. 5) as will be described in more detail elsewhere. The number of connections of both types and the number of cells migrating on them increase until the third day in culture, at which point a stable pattern is reached (Figs. 3 and 4). Under the described conditions these interconnecting fiber bundles, together with cells on top, are characteristic of cerebellum but not of other CNS regions tested (cerebrum and retina).

Once the described pattern is established, the size and shape of the reagggregates and their interconnections have been maintained for periods up to 1 mo in culture.

Cell Types in the Reagggregates

(a) The most numerous type was a small, strongly stained cell about 4–6 μm in diam and organized in clusters. As seen by light micros-

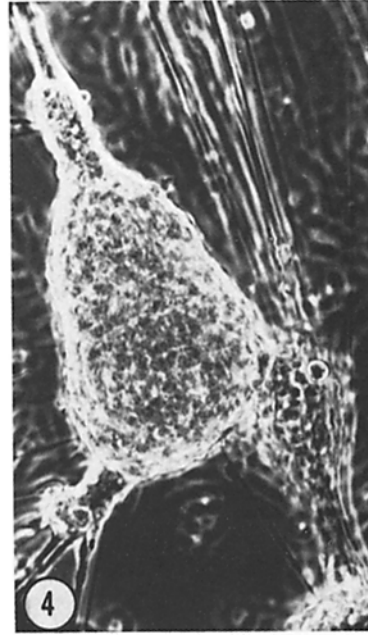
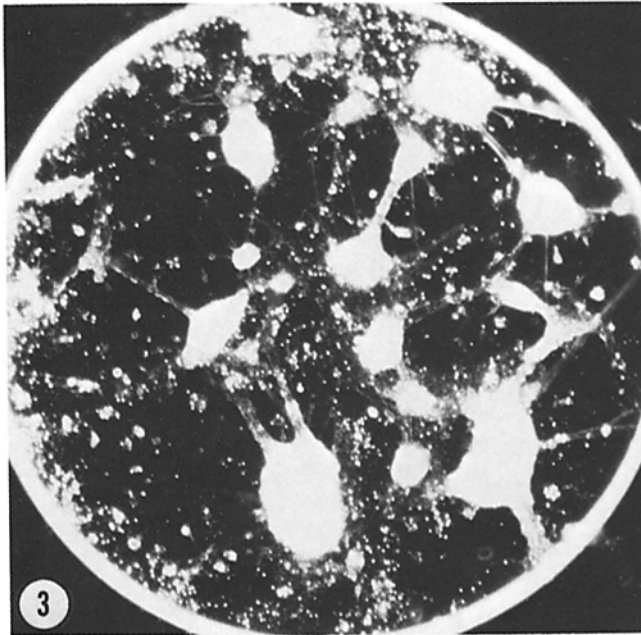
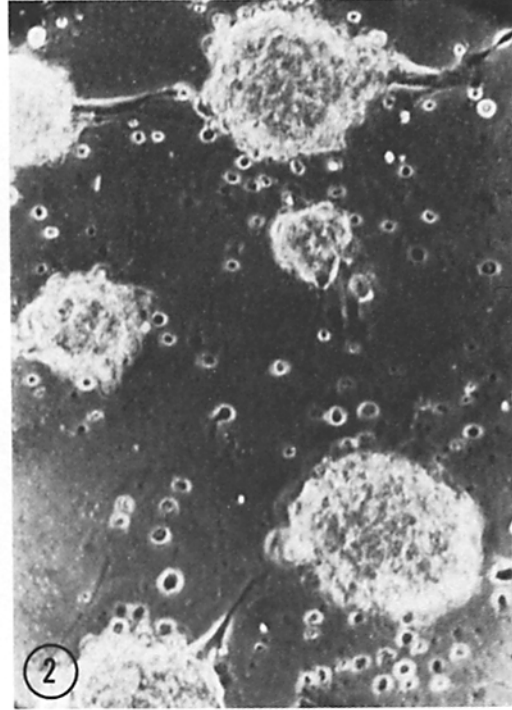
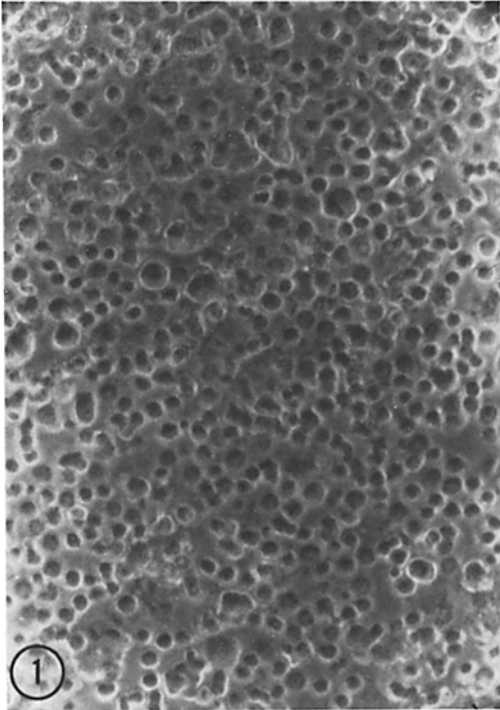
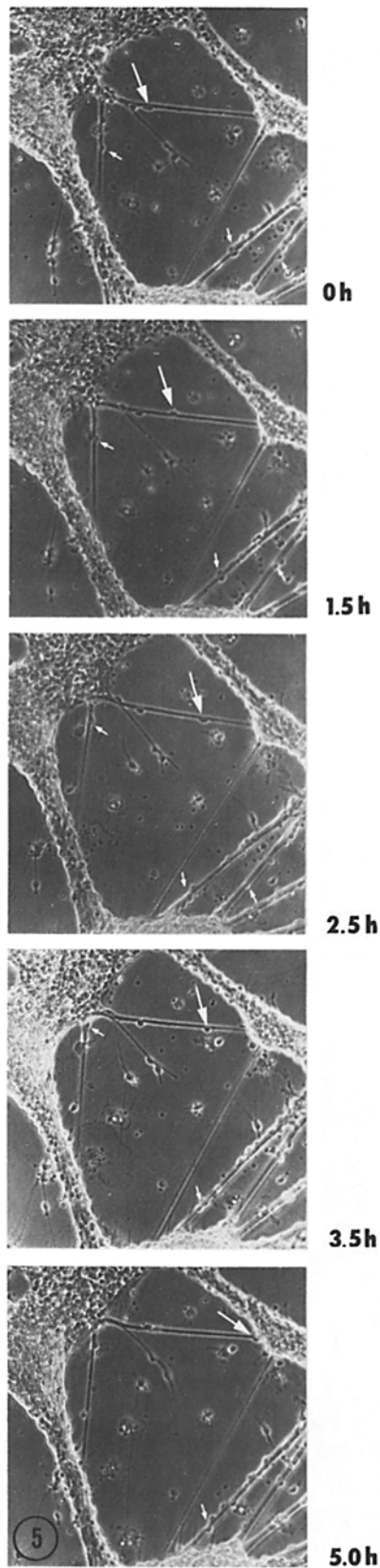


FIGURE 1 Microwell photographed immediately after seeding with 5×10^4 cells in $10 \mu\text{l}$ of culture medium (P7, postnatal day 7). $\times 560$.

FIGURE 2 The same well at 16 h in culture. Cells have formed reagggregates of various sizes. Fiber outgrowth has begun. Only a few cells remain single. $\times 560$.

FIGURE 3 The same well at 72 h in culture. The entire well is visualized with a $\times 2.5$ objective and $\times 10$ ocular. Reagggregates and both coarse and fine fiber outgrowths are seen. $\times 120$.

FIGURE 4 Same as Fig. 3. This field illustrates a single reaggregate and both patterns of outgrowth. See text for details. $\times 560$.



copy in 1- μm sections, clusters were distributed all through the reaggregate after 21 h in vitro. Later these clusters were located further toward the center of reaggregates and after 6 days in vitro most of the clusters were concentrated at the centers (Fig. 6). In electron micrographs these cells were closely packed, with no neuronal or glial processes inserted between their apposed somas (Fig. 7). The nucleus, occupying almost all of the cell body, was oval-shaped before the cells clustered, but changed to an angular form after apposition of the cell bodies. During this transition, the nucleus showed a consistent and characteristic form with large patches of condensed chromatin and finer flocculent condensations of heterochromatin associated with the nuclear membrane and dispersed through the nucleoplasm. Small, round, or elongate mitochondria were sparsely distributed in the scanty cytoplasm, along with free ribosomes, polyosomes, and cisterns of rough endoplasmic reticulum (ER). After 24 h in culture, the Golgi apparatus was not prominent, and only rare coated vesicles were detected close to Golgi cisterns. By 72 h, the Golgi apparatus had become more developed and small coated vesicles were observed, as well as occasional multivesicular bodies. These cells persisted in clusters throughout the entire culture period of 6–10 days. The large number of these cells from the earliest stages in vitro, and their cytological features allow their identification as presumptive granule cell neurons (see reference 42 for their in vivo counterparts).

(b) At the surface of the reaggregate were smooth, flat cells and oval-shaped cell bodies of undefined types, embedded in a net of unoriented cell processes and filopodia (Fig. 8).

(c) Just internal to these elements lay a second type of small cell that differed from those described above by not forming clusters (Fig. 9). Their cell bodies were 5–7 μm in diam. The nuclei were usually round on the side facing the surface of the reaggregate and lobulated at the opposite pole, although some cells of this type apparently did not develop lobulated nuclei (Fig. 10). The nuclei were lightly stained and the chromatin was dispersed, forming only small condensates. The cytoplasm was more voluminous near the lobulated, inward-directed side of the nucleus and contained small mitochondria, rough ER, a few cisterns of smooth ER, few microtubules, and incompletely developed Golgi

FIGURE 5 A series of time-lapse photographs of cells (arrows) observed for 5 h as they migrate along cable-like structures. $\times 150$.

apparatus. A tapered dendrite-like process containing free polysomes, cisterns of smooth ER, mitochondria, and microtubules projected toward the interior of the reagggregates from the pole of the cell with the lobulated aspect of the

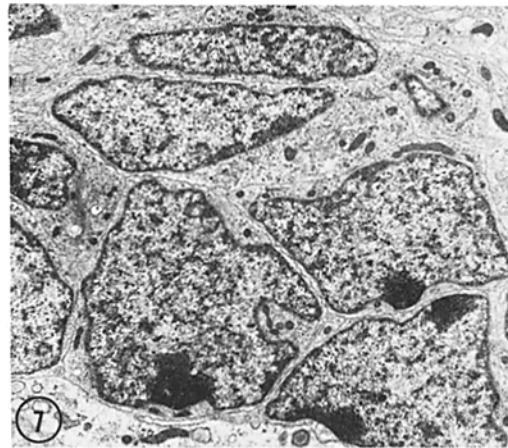
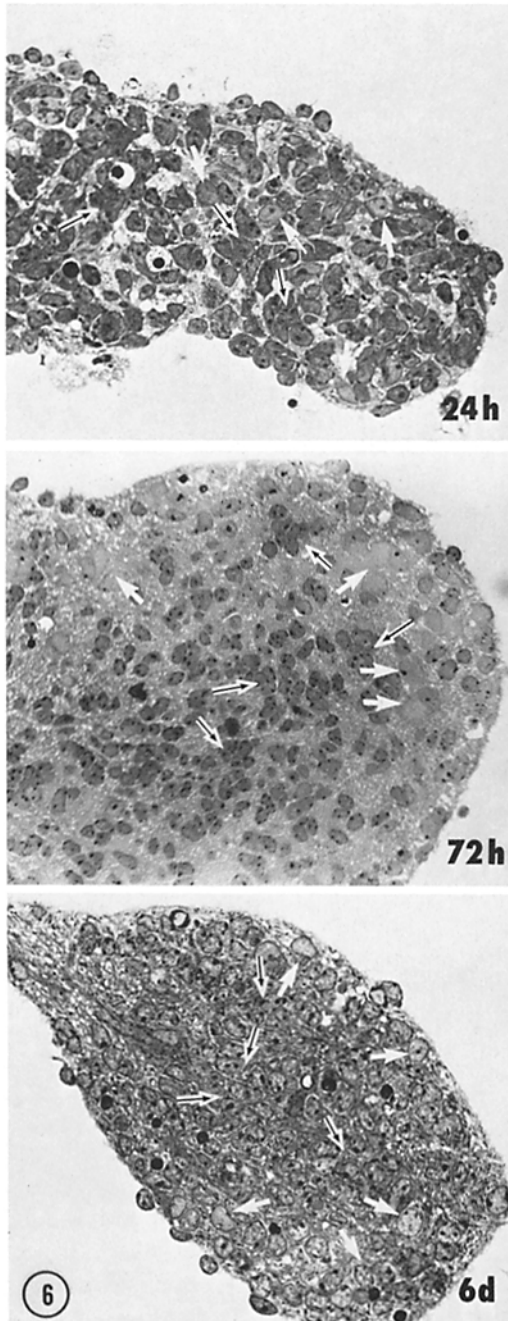


FIGURE 7 Granule cells within a reaggregate after 6 days in vitro. The majority of such cells are found more in the center of reagggregates after ~50–72 h in vitro. Their somas are in direct apposition with one another. The nucleus, occupying most of the cell body, is electron-dense and shows a distinctive distribution of heterochromatin. Large patches of condensed chromatin are associated with the nuclear membrane. $\times 7,000$.

nucleus and the larger volume of cytoplasm (Fig. 9). Axo-somatic synapses were observed on several of these cells (Figs. 9 and 10).

(d) A small number of large neurons was detected (Fig. 11). The cell bodies were 9–12 μm in diam and somewhat irregular in surface contour. The lightly staining nucleus was round or oval in shape with a few lobulations. Chromatin was more or less homogeneously distributed and only one nucleolus was observed per cell. The cytoplasm was increased in volume both in absolute terms and relative to nuclear volume, compared to the previously described cell types, and was localized mainly on the apical side of the nucleus. Darkly stained elongated

FIGURE 6 In 1- μm sections of reagggregated P7 cerebellar cells 24 h, 72 h, and 6 days in vitro, the predominant cell type was small and darkly stained, and usually had two nucleoli; they have organized in clusters (dark arrows). These clusters were distributed throughout the reaggregate after 24 h in culture but were located progressively more in the centers of reagggregates with time in culture. Larger cells with more lightly stained nuclei and only one nucleolus did not form clusters, but tended to align parallel to the surface in the outer zone of the reagggregates (white arrows). $\times 600$.

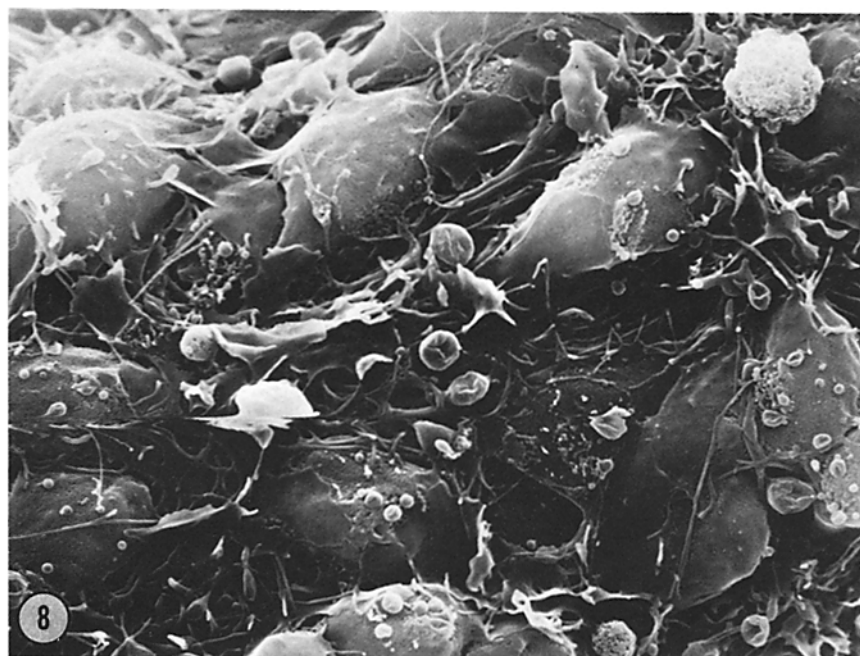


FIGURE 8 Surface of a reaggregate after 72 h in culture. Smooth, flat cells and oval-shaped cell bodies are embedded in a meshwork of unoriented filopodia and fibers. Scanning electron micrograph. $\times 2,000$.

mitochondria were located predominantly in the apical part of the cell. A well-developed Golgi apparatus, including coated vesicles and multivesicular bodies was found close to the lobulations of the nucleus. Rough ER and free polysomes were distributed throughout the cytoplasm, as were randomly oriented microtubules and occasional neurofilaments. Axo-somatic synapses contacted all parts of the cell body (Fig. 12), including somatic spines (Fig. 13). A dendritic process arose from the apical pole, and typically contained free polysomes, a few cisterns of smooth ER scattered along its peripheral zone, and longitudinally oriented microtubules and mitochondria. The surface membrane of the dendrite was smooth, without spines. Synaptic contacts were observed occasionally.

(e) Two further cell types, probably nonneuronal, were distinguished from those described above by their staining characteristics and their cytoplasmic organelles.

1. Darkly staining small round or oval-shaped cells with electron-dense nucleus and cytoplasm. Small round or oval-shaped mitochondria, rough ER, and large amounts of closely packed free

ribosomes and polysomes were the main contributors to the high cytoplasmic density. No synaptic connections were detected (Figs. 14 and 15). They match descriptions of oligodendroglial cells *in vivo* (43).

2. Larger, lightly stained cells with electron-lucent nuclei containing homogeneously distributed chromatin. A small rim of condensed chromatin was associated with the nuclear membrane except where it was interrupted by more electron-lucent condensations around the nuclear pores, as described by Palay and Chan-Palay (42). The relatively large volume of cytoplasm contained fibrils indistinguishable from those commonly seen in astrocytes (Figs. 16-18).

Outward-Migrating Cell Types

Reaggregates become interconnected by two different formations.

PATTERN I: Flattened cells of undetermined origin first provide a substrate attached to the bottom of the culture well (Fig. 19). As demonstrated by time-lapse cinematography (E. Trenkner, manuscript in preparation), cells migrate from reaggregates in wide, directed streams

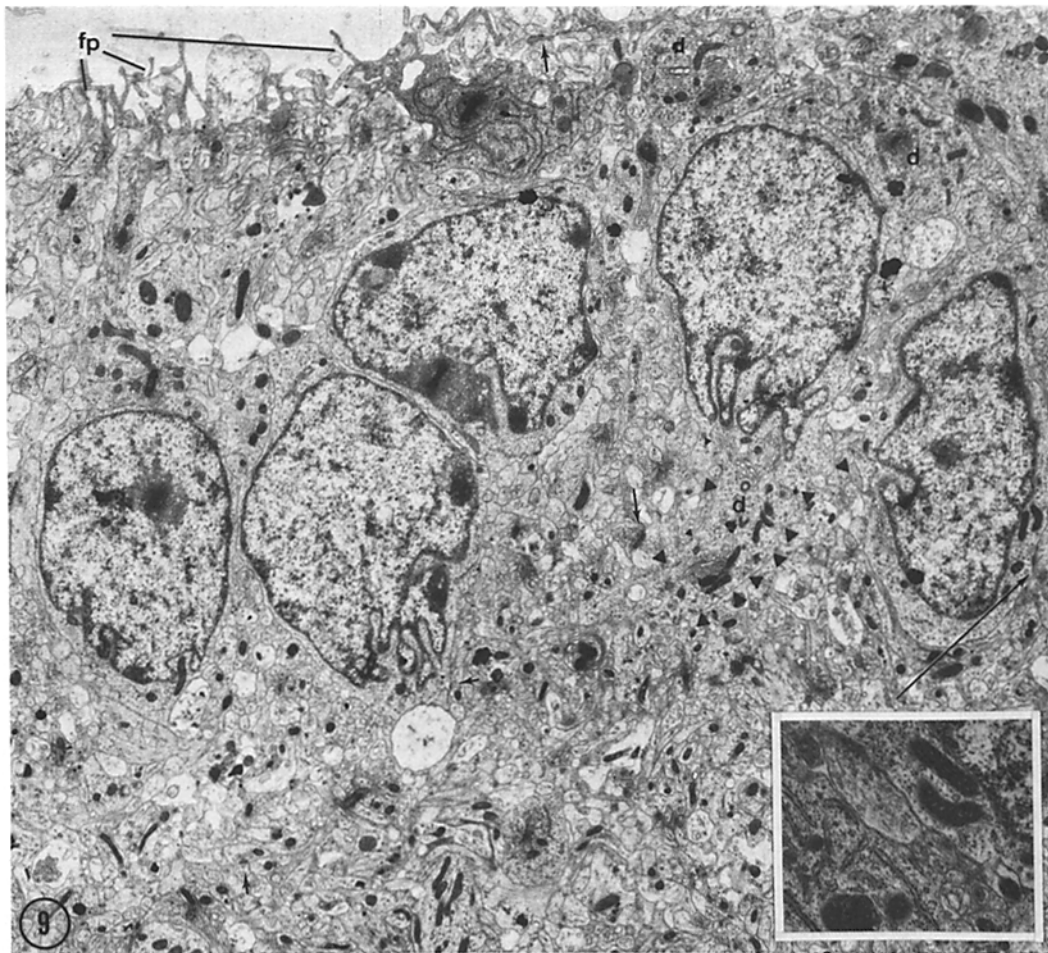


FIGURE 9 Interneurons in reagggregates after 6 days in vitro. These cells tend to align at the outer zone of reagggregates with their dendrites (*d*) projecting toward the center. Axosomatic synapses (arrows) were observed on several of these cells. Filopodia (*fp*) were observed at the surface of reagggregates. $\times 12,000$. *Inset* shows an axosomatic synapse on an interneuron. $\times 32,000$.

along the upper surfaces of the flattened cells (Figs. 3 and 4; Fig. 20, *I*). Fibers project both from perikarya within the reaggregate and from the migrating cells themselves and are distributed in parallel arrays along the migration paths.

PATTERN II: Cable-like fiber bundles, not attached to the dish, also form bridges between reagggregates (Figs. 5 and 20, *II*; Figs. 21-23). Each cable is composed of many thin fibers oriented in parallel (Figs. 24 and 25). A given bundle increases in thickness with time in culture as it gains further increments of fibers. Small cells with oval-shaped somas migrate from the reagggregates along the surfaces of the cables (Figs. 5 and 21-23) and by 4-6 days assemble

approximately midway between two reagggregates.

The cell types distributed within the reagggregates, as described above, appeared in general quite different from those migrating outward, whether along cell surfaces (Pattern I) or fiber surfaces (Pattern II). Few cell types, perhaps only one, exhibited migratory behavior, and appeared as follows:

(*a*) Cells utilizing the surfaces of flattened cells as a substrate (Pattern I) were small with spindle or oval-shaped nuclei. They were often aligned in tandem and were oriented in parallel to the fibers interspersed among them (Fig. 27). The nuclei typically contained two to three

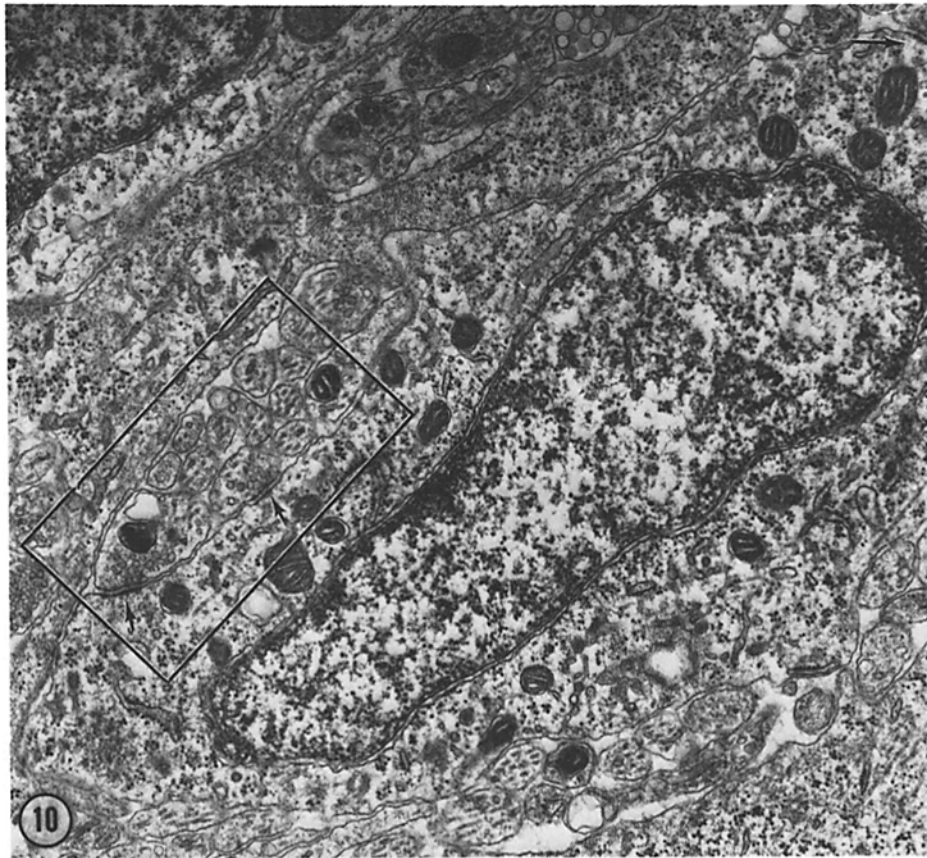
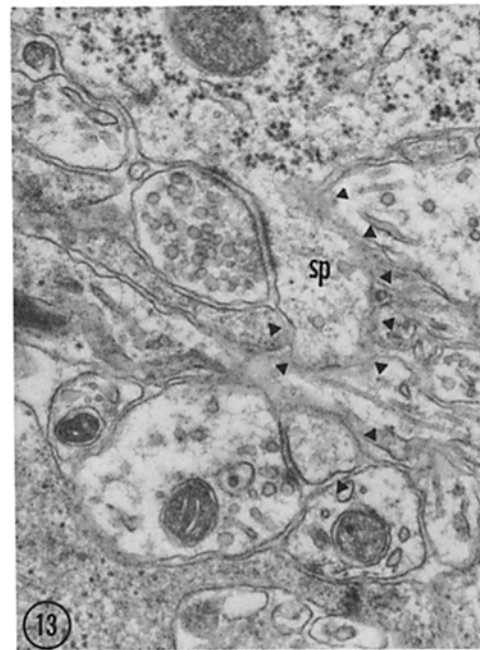
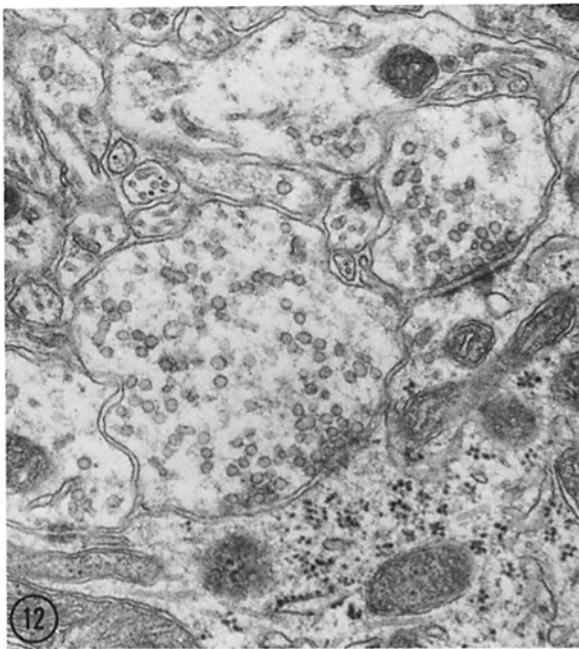
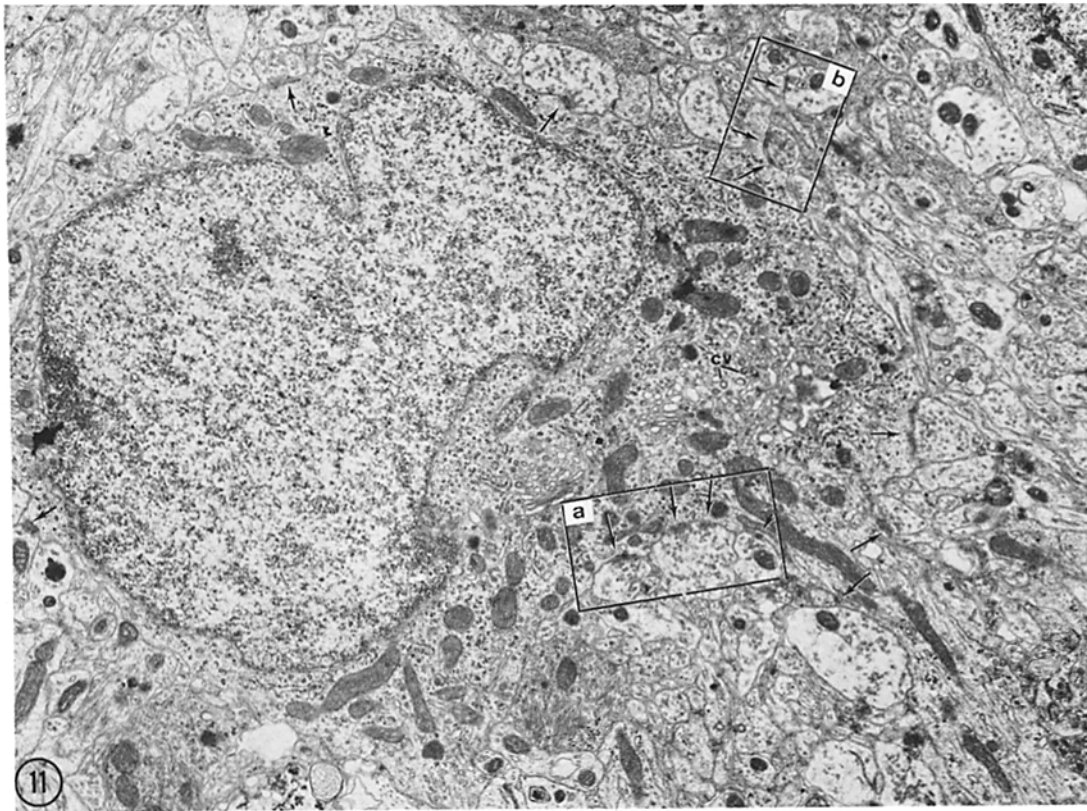


FIGURE 10 An elongated interneuron from another culture after 6 days in vitro, with several synaptic terminals on its soma (arrows, within rectangle). $\times 24,000$. The area within the rectangle is enlarged below, to show synaptic morphology. $\times 35,000$.

darkly stained condensed chromatin patches in a given EM section. The cytoplasmic volume was small along the long sides of the perikaryon, but was more extensive at each end, so that the cell as a whole displayed a bipolar form. These cells

were polarized, in that cytoplasm on one side was rich in free ribosomes and polysomes but lacked other organelles, while cytoplasm on the other side contained mitochondria, multivesicular bodies, and well-developed Golgi apparatus.



Occasionally a cilium was observed. Occasionally, nests of small neurons indistinguishable from those within the reagggregates (Figs. 7 and 20) and isolated astrocytes, but no medium-sized or large neurons were observed in Pattern I.

(b) Figs. 21–23 illustrate cell types associated with the cable-like formations (Pattern II). While migrating along fiber surfaces (Fig. 5) these cells were indistinguishable in nuclear or cytoplasmic features from those participating in Pattern I. Once assembled midway between two reagggregates, the cells displayed more round than elongated cell bodies. The cytoplasm was scanty in volume and contained small round mitochondria, sparsely distributed polysomes, little rough and smooth ER, and a poorly developed Golgi apparatus. The side of the cell contacting fibers typically was long and straight, while the opposite, free surface was domed. No specialized contacts between cell body and underlying fibers were detected, though the area of direct apposition was large.

Fiber Outgrowth

Fibers, either dispersed or assembled into bundles, were present within the reagggregates and extended between them. By 24 h in culture, a network of fibers and some fascicles were present. Their orientation appeared to be random since transverse, oblique, and longitudinal profiles through fibers and fascicles were found in a given section (Figs. 9 and 10). Fibers frequently reached the surface of reagggregates. In contrast, fibers which grew out of reagggregates and participated in either of the two migration patterns were oriented parallel to each other (Figs. 24 and 25). The majority of fibers observed were $\sim 0.1\text{--}0.2\ \mu\text{m}$ in diam and many microns in length. Three to five straight microtubules were longitudinally oriented and extended a considerable length within each fiber. Rare polysomes,

some smooth ER, and occasional mitochondria were present, but otherwise the cytoplasm was clear. Varicosities were observed and contained a few mitochondria and small vesicles.

A second type of fiber was detected within reagggregates and within the surface of reagggregates. In contrast, fibers which grew out of reagggregates and participated in either of the two migration patterns were oriented parallel to each other (Figs. 24 and 25). The majority of fibers observed were $\sim 0.1\text{--}0.2\ \mu\text{m}$ in diam and many microns in length. Three to five straight microtubules were longitudinally oriented and extended a considerable length within each fiber. Rare polysomes, some smooth ER, and occasional mitochondria were present, but otherwise the cytoplasm was clear. Varicosities were observed and contained a few mitochondria and small vesicles.

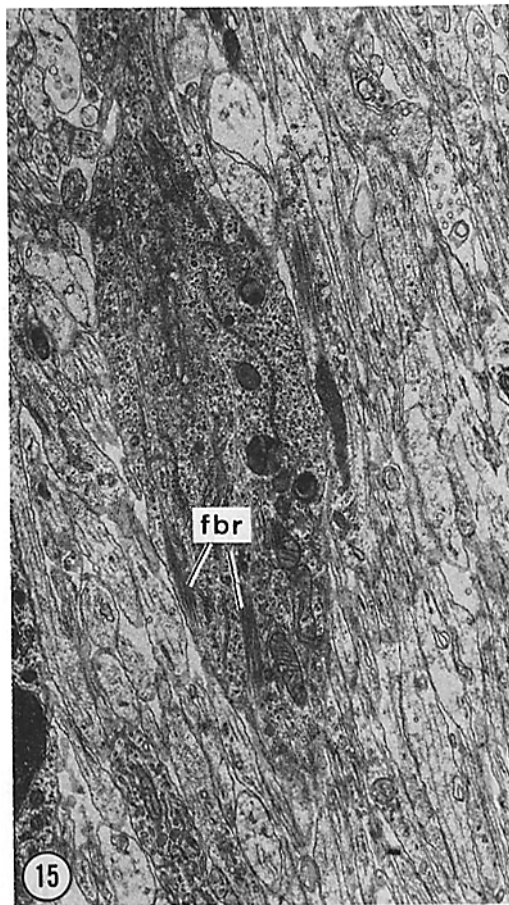
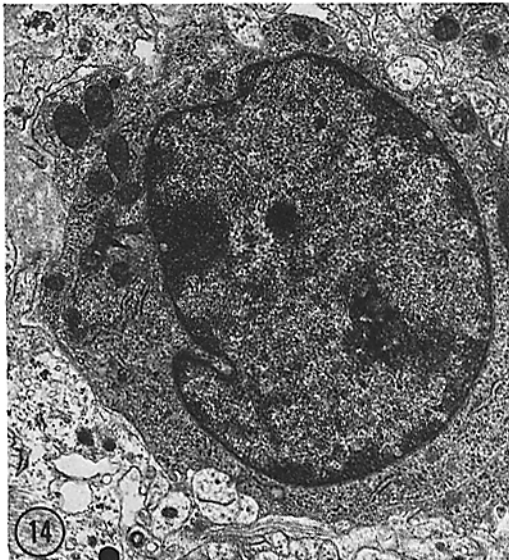
A second type of fiber was detected within reagggregates and within the cellular outgrowth zone (Pattern I), but never in the cable-like pattern. These fibers were more electron-dense than the previous type, for the cytoplasm contained polysomes, rough ER, longitudinally oriented large mitochondria, coated vesicles, and a few microtubules and neurofilaments (Figs. 9, 10, 27, and 31). Their diameters were at least twice those of fibers described above.

A third type of fiber was distinguishable from the others by its high content of lysosomes and packed bundles of fibrils. The cytoplasm of fibers in one subclass within this category was electron-lucent and contained a few microtubules, sparse elongated mitochondria, and a few ribosomes (Fig. 18). A second subclass was electron-dense and contained closely packed ribosomes and polysomes, rough ER, and round mitochondria, as well as prominent fascicles of fibrils (Fig. 15). Neither subclass was found in the cable-like formations.

FIGURE 11 Large neuron observed in reaggregate after 6 days in vitro. The outline of the perikaryon is irregular. Coated vesicles (*cv*) are present in the cytoplasm. Axosomatic synapses are present at several sites on the cell body (arrows). The fields enclosed in rectangles (*a*) and (*b*) are enlarged in Figs. 12 and 13, respectively. $\times 8,400$.

FIGURE 12 Enlargement of rectangle (*a*) from Fig. 11, reversed in orientation. Two axosomatic synapses are shown, each with multiple subsurface collections of vesicles and membrane thickenings. $\times 30,000$.

FIGURE 13 Enlargement of rectangle (*b*) from Fig. 11, reversed in orientation. A somatic spine (*sp*) is outlined by arrowheads and receives two synaptic contacts. $\times 30,000$.



After 24 h of culture, numerous growth cones were observed within reaggregates, and were found later in the migration patterns up to 6 days. Two types of growth cones were distinguished: (a) growth cones filled with large empty vesicles (Figs. 29–31); at the earliest stages such vesicles were observed in expanded parts of the perikaryal cytoplasm where no other organelles were seen (Figs. 29 and 30). After fiber outgrowth had progressed, a few mitochondria migrated into growth cones, and the segment of the process proximal to the growth cone showed axonal features (Fig. 31). (b) Growth cones filled with large vesicles and other cytoplasmic organelles (free ribosomes and polysomes, occasionally multivesicular bodies, mitochondria, coated vesicles, and sometimes elements of Golgi apparatus). This type of growth cone was found in association with a fiber more suggestive of a dendritic type as judged by its cytoplasmic organelles (Fig. 28).

Synapse Formation

Although synapses are known to be present in the mouse cerebellum at P7 (30, 32), no synapses were found immediately after formation of reaggregates. After 24 h in vitro, a few puncta adherentia were found, i.e., a symmetrically thickened patch on the cytoplasmic side of each cell surface membrane and a parallel disposition of membrane segments demarcating an intercellular space of uniform width with some electron-opaque material within it (Fig. 32). Not necessarily related to these surface specializations, the first definite synapses were detected after 72 h in vitro and their number per unit area increased with time in culture. Therefore, all synapses observed were either regenerated at previous synaptic sites or were newly synthesized in vitro. Apparent synaptic contacts were identified between neurite terminals and shafts, neurites and

FIGURE 14 Oligodendroglia cell body in a reaggregate after 72 h in vitro. The cell is almost round and shows characteristically electron-dense nucleoplasm and cytoplasm. $\times 9,600$.

FIGURE 15 Process of an oligodendroglial cell observed in the mixed cell and fiber outgrowth zone (migration pattern I). The process and soma both contain densely packed free ribosomes, lysosomes, Golgi apparatus, microfilaments, and also densely packed bundles of fibrils (*fbr*). $\times 11,600$.

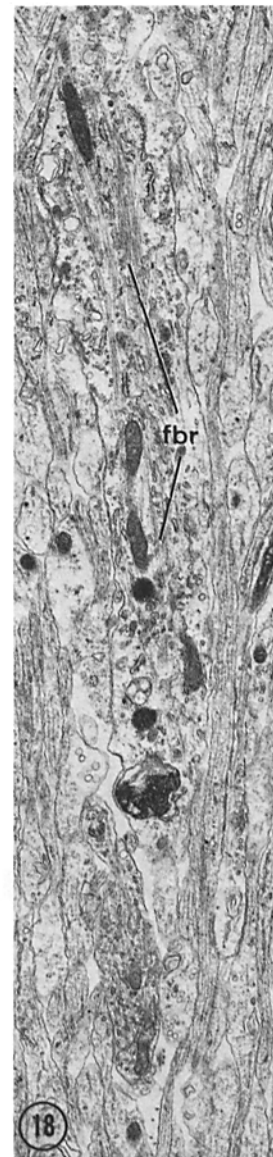
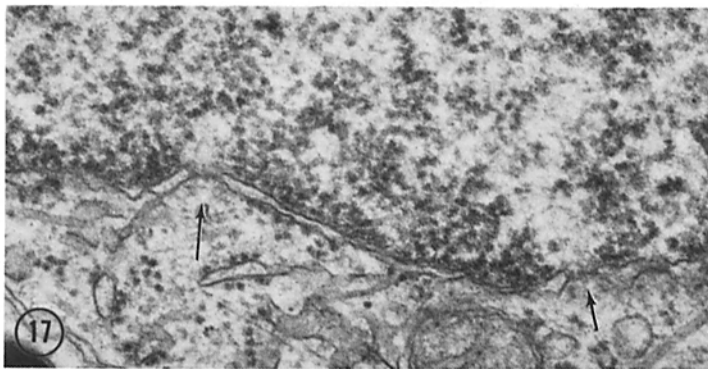
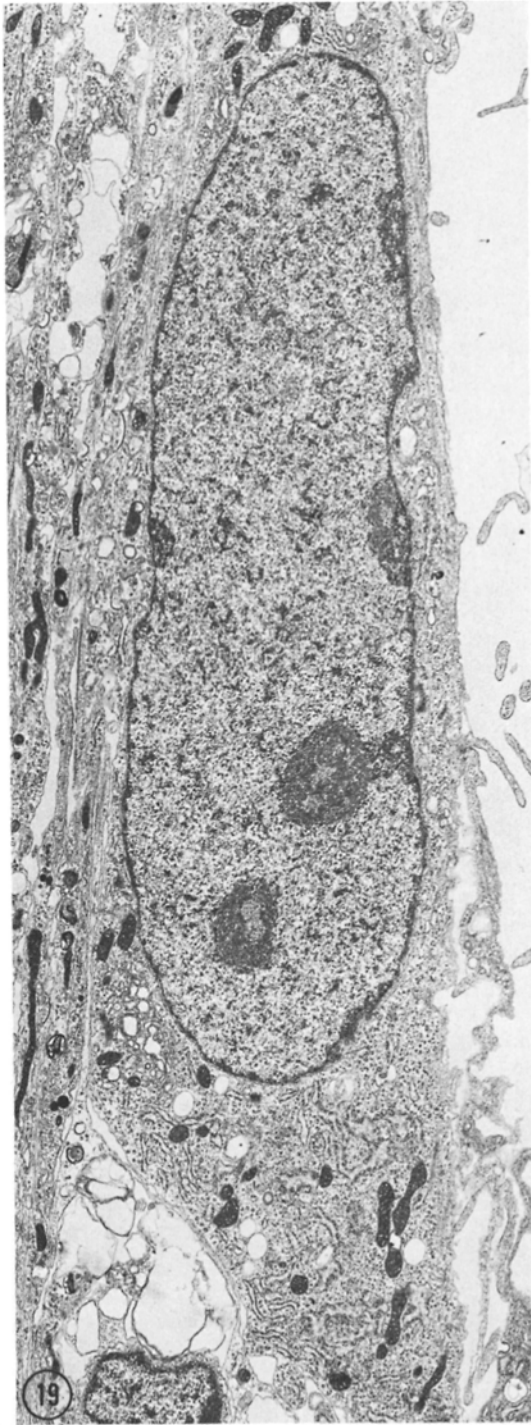


FIGURE 16 Astrocyte in a reaggregate after 72 h in vitro. The cell has a lightly stained nucleus indented on one side. A small rim of condensed chromatin is associated with the nuclear membrane. The cytoplasm contains astrocytic fibrils (*fbr*). $\times 10,000$.

FIGURE 17 Enlargement of area within the rectangle in Fig. 16 showing that the rim of the nuclear chromatin is virtually discontinuous opposite the nucleopores. $\times 80,000$.

FIGURE 18 Astrocytic process in migration pattern I. The process is irregular in contour, in contrast to neurites, and is electron-lucent, with elongated mitochondria, fibril bundles (*fbr*), and very few ribosomes. $\times 21,000$.

cell somas, and between varicosities along axon-like fibers (Figs. 33 and 34). Most of these were found within the reagggregates and less frequently



along the cable-like formations. No synaptic contacts were observed in the more cellular outgrowth summarized as Pattern I.

The main structural features of most synaptic contacts within the reagggregates consisted of various lengths of parallel surface membranes, thickenings on the cytoplasmic side of pre- or postsynaptic membrane or both, a widened cleft between the apposed segments of surface membrane, and electron-dense material in the cleft (Figs. 12 and 34). Vesicles, usually small and round, were assembled close to the membrane thickening on the presynaptic side (Figs. 9, 12, 13, 32, and 34). The majority of the synapses were classifiable as Gray type I, on the basis of asymmetrical membrane thickenings and concentration of round vesicles just beneath the presynaptic membrane, but it should be emphasized that the considerable variation in membrane thickness and vesicle distribution makes such a classification somewhat arbitrary. Contacts observed in the cables showed minimal membrane thickenings and no obvious segments of specially apposed membrane, but were demarcated by accumulations of small, round vesicles on the cytoplasmic side of one surface membrane (Fig. 24).

DISCUSSION

The present culture method involves initial dissociation of the developing cerebellum into a suspension of single cells which are then allowed to reassemble into three-dimensional reagggregates. The sheets of cells and fibers, and the cables, that come to interconnect the reagggregates are constrained to a plane parallel to the base of the culture well. Our primary question about the method is whether the cell behavior *in vitro* might illuminate normal cerebellar development and enhance prospects of elucidating the cellular events that govern it, or whether it represents a series of biological phenomena with little relationship to developmental events *in vivo*. Let us review the main features of the

FIGURE 19 The initial cellular outgrowth from reagggregates, onto which other cells and processes will subsequently migrate, consists mainly of flattened elongated cells with no specialized morphological features, as in this representative example from a culture 72 h *in vitro*. The chromatin distribution, high proportion of rough endoplasmic reticulum, and filopodial extensions from the surface suggest, but do not establish, an endothelial type of cell. $\times 8,900$.

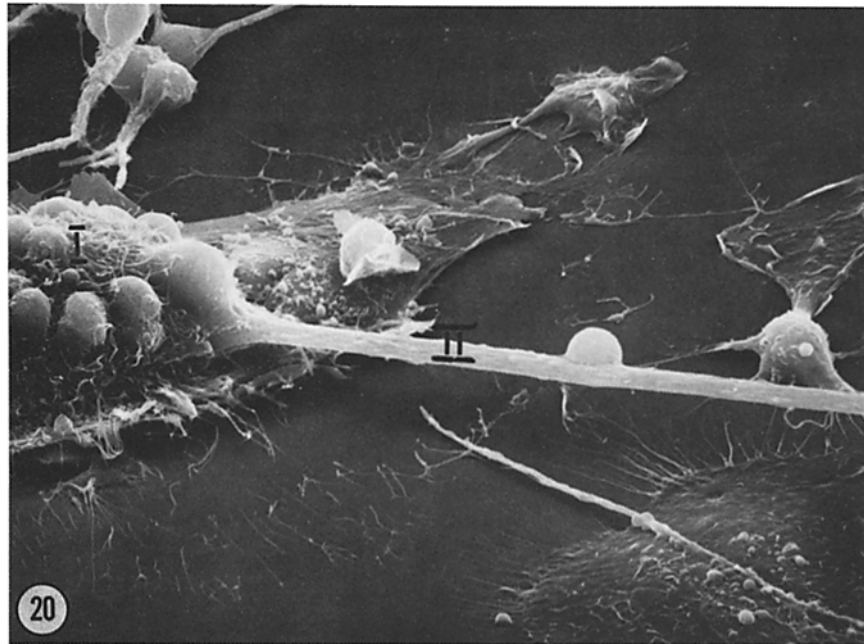


FIGURE 20 Migration patterns, formed after 72 h in vitro. Small neurons are attached to a broad flattened cell which is attached to the surface of the culture dish (*I*). An oval shaped cell has migrated along a cablelike structure (*II*) which is connecting migration pattern I and a reaggregate out of the field to the right. $\times 840$.

microwell system and the behavior of cerebellar cells in it with this question in mind.

The Microwell Culture System

The advantages of culturing cells in microwells as opposed to 35-mm dishes are numerous. First, one or two cerebella (depending upon the age of the mouse) suffice for a full experiment so that variability among animals is eliminated. Second, because of the small size of the wells, little time is needed for the cells to attain effective survival conditions. Third, culture parameters such as microenvironment, conditioning, etc., are the same throughout a given microwell, in contrast to larger dishes. Fourth, the reproducibility of the system can be assayed easily since the 60 wells available per plate are quickly scanned and compared with one another. Fifth, the culture area is small (1 mm in diam), so that it is relatively easy to follow the development of the entire pattern, either in the living state (including time-lapse cinematography) or in fixed and sectioned specimens. Finally, only 10 μ l of culture medium is required, which allows economical

use of valuable reagents such as antibodies, enzymes, etc.

A limitation to the culture system, though one that can be turned to advantage, is that pattern formation, as defined above, is very much dependent upon culture conditions. As shown elsewhere (E. Trenkner, manuscript in preparation) different types and sources of serum in the culture medium alter both the growth and the attachment behavior of dissociated cerebellar cells. In general, cells grown in the presence of horse serum attach to one another and reproducibly form patterns I and II, whereas those grown in fetal calf serum attach preferentially to the culture dish surface and form apparently random monolayers.

Identification of Cell Types

The difficulty in identification of immature cell types of the central nervous system in vitro has been one of the main limitations of neural tissue culture. Virtually no distinctive cytochemical markers have been available in vitro or in vivo. Instead, in the intact developing cerebel-

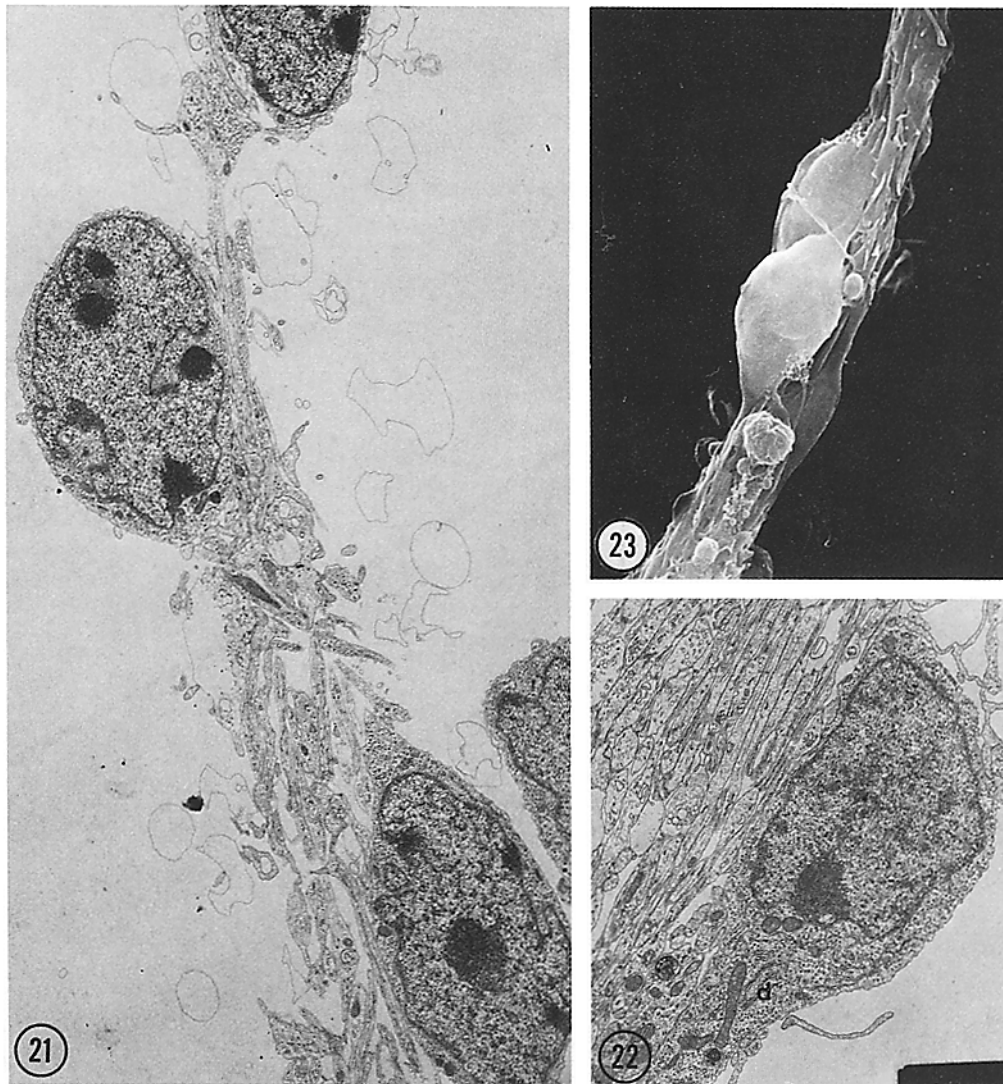


FIGURE 21 Cells and processes in migration pattern II. The cells have oval nuclei with areas of uncondensed and condensed chromatin similar in distribution to those of granule cells. The perikaryon is small and contains only few cytoplasmic organelles. $\times 7,000$.

FIGURE 22 Another similar cell with processes (*d*) leading away from the reaggregate. No specialized contacts were observed between cell body and adjacent neurites. $\times 7,000$.

FIGURE 23 Scanning electron micrograph of two cells migrating along a cablelike bundle of neurites (migration pattern II). $\times 2,000$.

lum, the cell types are recognized by their sizes and shapes, internal cytological features, migratory patterns, and above all, by their relationships to neighboring cells (23, 42). However, the size, shape, and migratory behavior of a given cell are strongly influenced by the cellular

organization of its local milieu (50, 58), so that when the tissue is dissociated and the cells allowed to reassemble, it comes as no surprise that classification of cell types will be a painstaking and, at best, only partially successful enterprise. Relative cell size, nuclear chromatin distri-

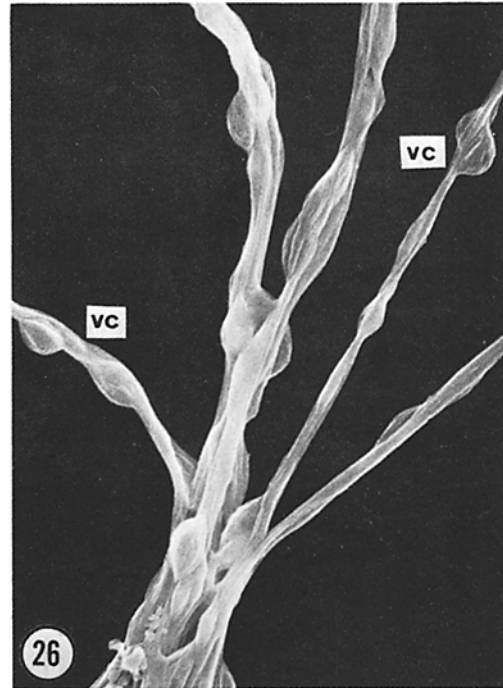
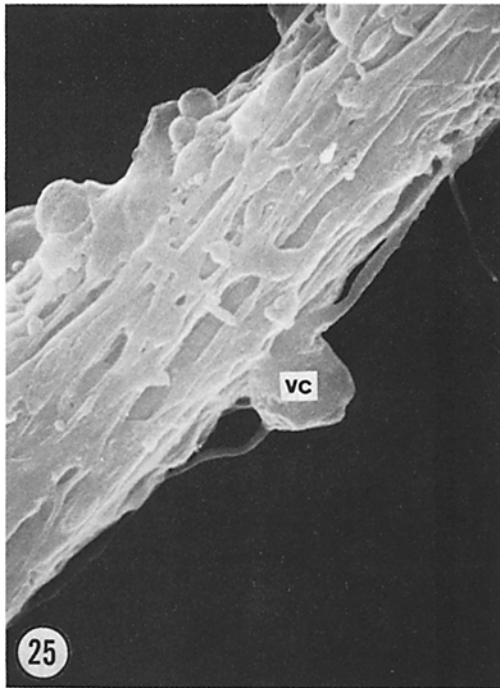
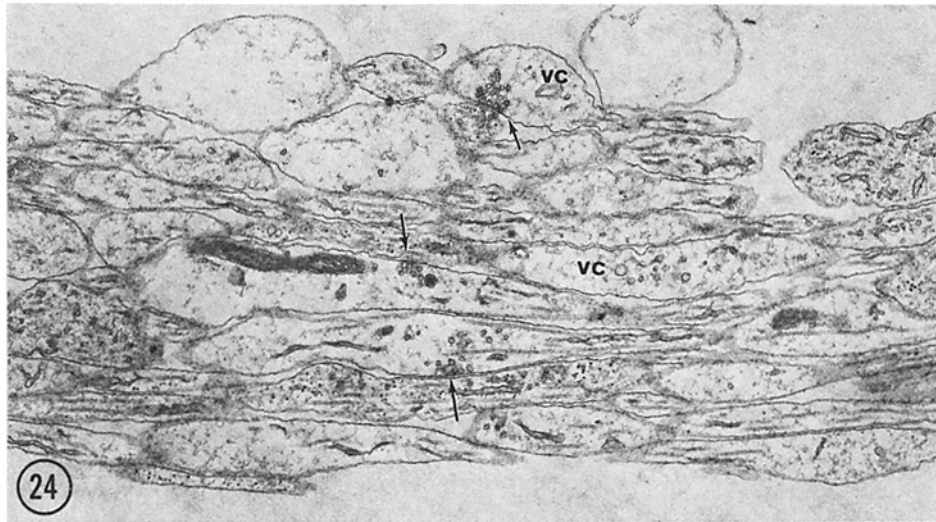


FIGURE 24 Fiber formation in migration pattern II after 72 h in vitro. Transmission electron micrograph of parallel fibers containing few straight microtubules (three to five per fiber), few polysomes, smooth endoplasmic reticulum, and few mitochondria. Varicosities (vc) along these fibers (characterized by focal accommodations of vesicles) form specialized contacts with varicosities along other fibers or with fiber shafts (arrows). $\times 20,000$.

FIGURE 25 Scanning electron micrograph illustrating that the cablelike formations are composed of many fibers oriented in parallel, with intermittent varicosities (vc) along their length. For more detailed structure of the varicosities, see Fig. 24. 72 h in vitro. $\times 10,000$.

FIGURE 26 Scanning electron micrograph showing individual fibers with varicosities (vc) splayed out from a cablelike bundle. 72 h in vitro. $\times 10,000$.

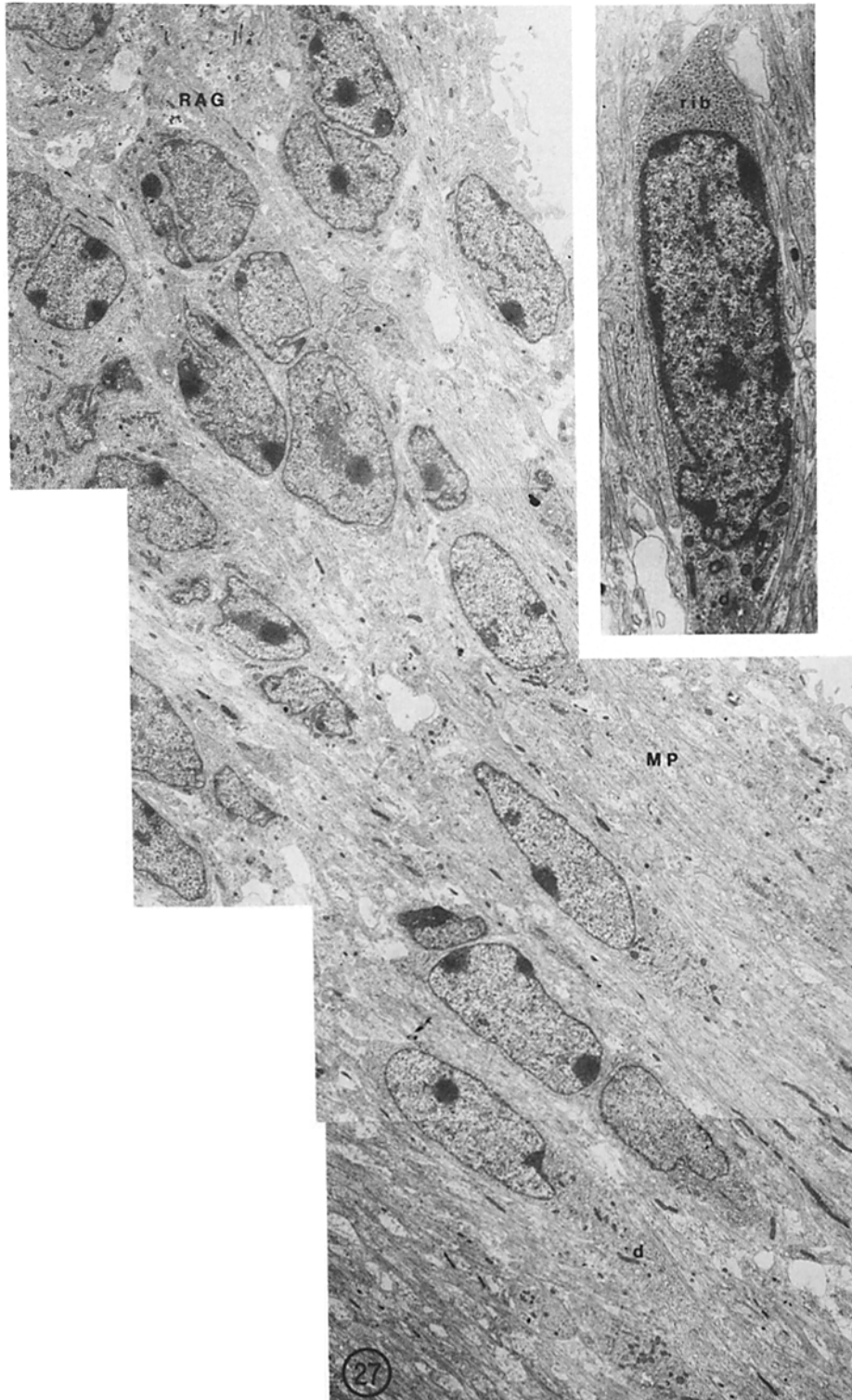


FIGURE 27 Migration pattern I after 6 days in vitro. Fiber configurations are characteristically unoriented within the reagggregates (*RAG*) but the fibers are oriented parallel to each other in the migration pattern (*MP*). Migrating cells follow each other in tandem, the process with the less differentiated morphology (*d*) leading away from the reaggregate. $\times 3,000$. The inset at the upper right illustrates a migrating granule cell. Its trailing pole contains ribosomes (*rib*) and polysomes exclusively, whereas the leading pole exhibits organelles more characteristic of conventional cell cytoplasm. $\times 7,000$.

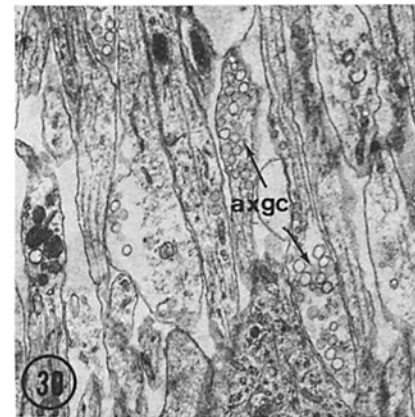
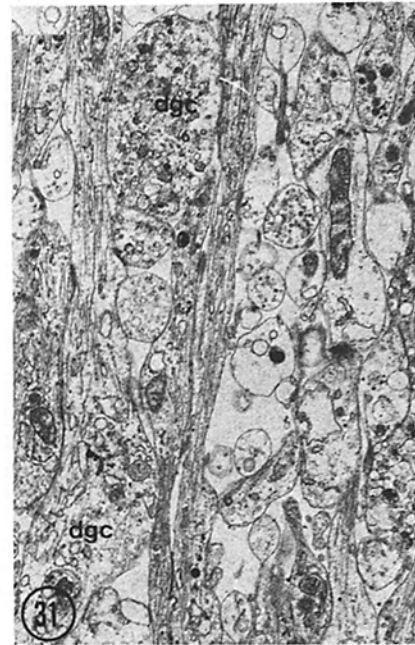
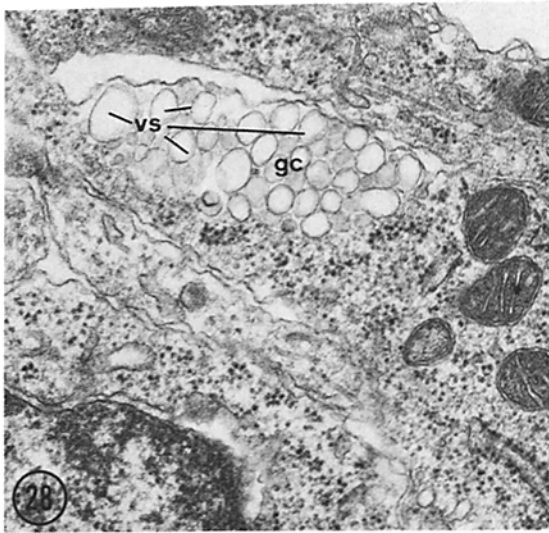


FIGURE 28 The first suggestion of growth cones (*gc*) are seen at 24 h in vitro, when some cells within the reaggregates display focal areas filled with large empty vesicles (*vs*) just beneath the surface membrane of the soma and clearly demarcated from the rest of the cytoplasm. No other cytoplasmic organelles are found in the incipient growth cone. $\times 55,000$.

FIGURE 29 Another cell within a reaggregate after 24 h in vitro. The incipient growth cone (*gc*), with its characteristic vesicles (*vs*) of various sizes, forms a bulge, or mound, at the perikaryal surface. $\times 55,000$.

FIGURE 30 Fiber outgrowth has begun at 24 h in vitro, and growth cones with essentially the same internal organization as those illustrated in Figs. 28 and 29 are frequent at the tips of the growing axons (*axgc*). $\times 12,000$.

FIGURE 31 Dendritic growth cone in a pattern I outgrowth zone after 72 h in vitro. In addition to the large vesicles, perikaryal organelles (ribosomes and multivesicular bodies) are prominent in growth cones at the tip of dendritelike fibers (*dgc*). $\times 12,000$.

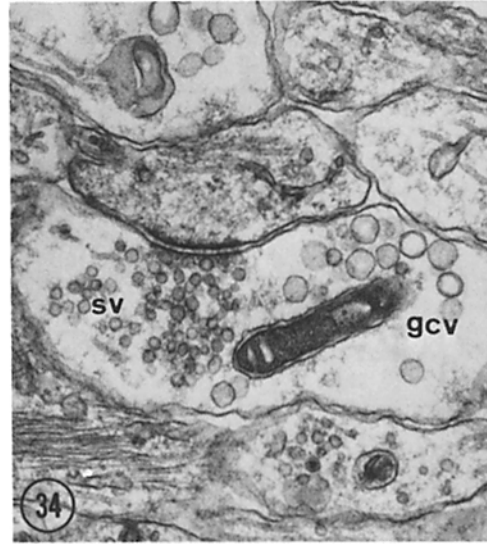
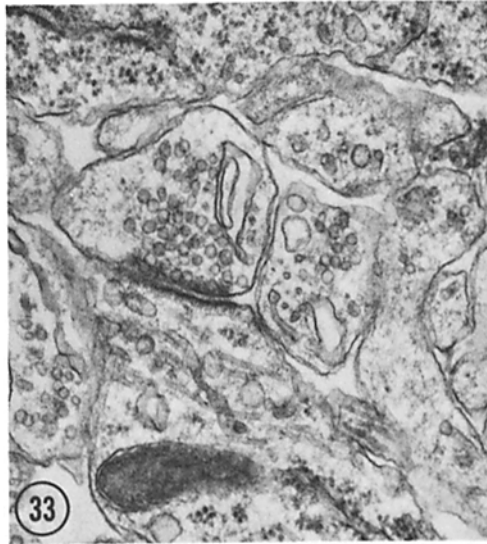
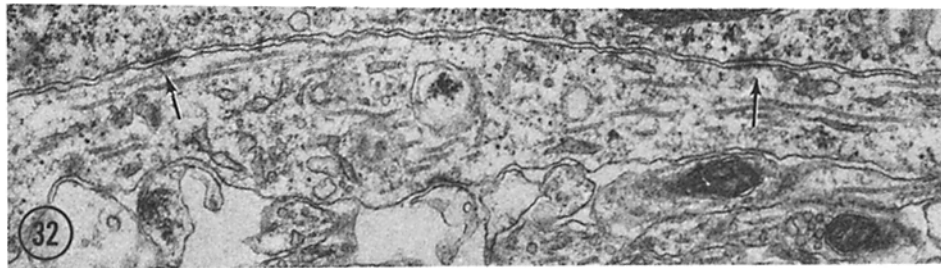


FIGURE 32 After 24 h *in vitro*, specialized contacts between cells are developed and consist of symmetrical thickenings of both membranes and a parallel alignment of the membrane segments involved in these thickenings (arrows). $\times 18,000$.

FIGURE 33 After 72 h the first synapses meeting conventionally accepted morphological criteria are developed. $\times 40,000$.

FIGURE 34 Synapse in a reaggregate after 6 days *in vitro* exhibiting thickenings of both cell membranes, a widened synaptic cleft, accumulation of synaptic vesicles (*sv*) in the presynaptic component and some growth cone vesicles (*gcv*) in the adjacent segment of the presynaptic element. If the growth cone is correctly identified, and if elongation of the neurite is continuous in time, it follows that a synapse can be formed very soon after a given segment of neurite is generated. $\times 40,000$.

bution, and formation of synapses serve as the most reliable features, and our study agrees with many previous ones in its identifications based on these criteria (2-4, 30, 42, 45, 49, 51, 53, 66, 68, 70).

SMALL NEURONS: The dominant class of small cells in the mature cerebellum is composed of granule cell neurons. These very numerous and unusually shaped cells have a unique developmental history *in vivo*. They arise from precursors which proliferate in the external granular layer on the external surface of the immature

cerebellum. In the mouse they are generated over a 3-wk span, from about embryonic day 13 through the first two postnatal weeks. At P7, the external granule cells constitute nearly half the total cerebellar cell population (1, 22, 25, 40). Cells continuously leave this proliferating pool and a young granule cell neuron, soon after becoming permanently postmitotic, takes a position in the deepest strata of the external granular layer where it assumes a bipolar shape with its fibers extending parallel to the surface in the longitudinal plane of the cerebellar folium, the

transverse plane of the brain as a whole (2, 4, 25, 32, 40, 42, 49, 54). These extended fibers begin to differentiate into the "parallel fiber" segments of the granule cell axons, while at about the same time, the soma becomes transposed radially inward within an additional cytoplasmic process, across the molecular and Purkinje soma layers to reach the (internal) granular layer. The critical cell-cell relationships during this remarkable transformation in granule cell shape are with (a) the radially oriented Bergmann fiber of the modified astrocyte of the cerebellar molecular layer, along the surface of which the inward-directed process and somatic region of the young granule cell neuron appear to move inward, (b) the outward-growing dendritic processes of the maturing Purkinje cell, which make contact with and come to receive synaptic inputs from the elongating parallel fiber axon of the granule cell, and (c) the mossy fiber inputs, arising outside the cerebellar cortex, which make contact with the somatic region of the transforming granule cell and develop a large synaptic specialization in relation to granule cell dendrites in the granular layer.

In the cultures as well, granule cell neurons constitute the predominant cell type (see the first two sections under Results). The identification is based on the large numbers, small cell size, pattern of nuclear heterochromatin, sparse volume of cytoplasm, tendency to cluster with cell bodies in direct apposition, and migratory behavior. The geometric constraints of the culture system as currently used, preclude the orthogonal arrangement of parallel fibers and Bergmann fibers characteristic of the immature cerebellum *in vivo* (40, 49), and do not encourage young granule cells to assume a transient tripolar shape and translocate their somas within the radially directed process. Kinetic studies will be required to establish whether the microwell cultures contain granule cell precursors of the external granule layer, postmitotic granule cell neurons, or both. Also, we are not yet clear whether cells in the various developmental stages at P7 (the time the cultures are set up) simply persist in expressing the properties they have already acquired, once separated from their usual intercellular relationships, or whether they continue to differentiate *in vitro*.

The group of relatively small cells illustrated in Figs. 9 and 10, limited to a zone close to the upper surface of the reaggregates, appears to

represent a second class of neurons. They are reminiscent of cells previously described as remaining close to the primary explant and distinguishable from migrating cells in rat cerebellar cultures (47). Two major features distinguish these neurons from granule cells. First, the nucleus is usually indented at one pole, is electron-lucent and its chromatin is evenly rather than patchily distributed; occasionally a nucleolus is observed. Second, axosomatic synaptic junctions on their cell bodies are observed after 72 h *in vitro* (Figs. 9 and 10). Although ectopic granule cells can form axosomatic synapses in mutant mice (31), indentations and electron-lucent appearance of the nuclei suggest that the cells in question are interneurons of the molecular layer, the basket and/or stellate cells (43, 50).

LARGE NEURONS: The Purkinje and Golgi II cells of the cerebellar cortex are relatively large neurons (42), as are neurons of several shapes in the deep cerebellar nuclei (16). All of these are generated during the embryonic period in the mouse, and have attained essentially their mature positions by P7 (60). In the micro cultures, large neurons are not numerous (18), and are confined to the edge of the reaggregates, with dendritic processes projecting inward. None were seen in either pattern evolving in the migration areas.

Classification of these cells is difficult. On some large cells, synapses are observed along the cell body and, in some cases, on a somatic spine (Fig. 11). Axosomatic synapses and somatic spines are unusual features for Golgi II cells at the stage of development under study here (4); that cell type would seem to be poorly represented in the cultures. Purkinje cells, however, are known to show such properties during development (30, 32, 45, 70). Deep cerebellar neurons in the adult rodent have smooth somas with numerous axosomatic inputs (17), but have not been studied developmentally with reference to possible transient somatic spines.

GLIAL ELEMENTS: Two types of glial cells were observed in our microsystem. One falls into the category of oligodendroglia as judged on the basis of dark round nuclei and electron-dense cytoplasm packed with free ribosomes and numerous stacks of rough ER (43); they also show, in contrast to most oligodendroglial cells *in vivo*, compact bundles of fibrils in the cell processes (Figs. 14 and 15). The other cell type meets criteria for astrocytes (43), including the specialized astrocyte of the cerebellar cortex, the Golgi

epithelial cell (42). It is a relatively lightly stained cell (Fig. 16) with a nucleus lacking chromatin condensations except for small patches associated with the nuclear membrane and separated from each other at the sites of nucleopores (Fig. 17). The perikaryon contains few fibrils, and electron-lucent cell processes containing lysosomes, elongated mitochondria, and a few fibrils are present in the cell-attached migration pattern, oriented generally parallel to axons and dendrites (Fig. 18). The flattened cells growing out from reaggregates as a prelude to pattern I of fiber formation are probably endothelial cells or fibroblasts rather than glial cells (Fig. 19).

Comparison of Cell Behavior In Vitro and In Vivo

Despite the failure to attain the topographic organization of cerebellum *in vivo*, the cells in microwell cultures do express three critical morphogenetic behaviors.

1. **FIBER OUTGROWTH:** The initial morphological step in fiber outgrowth is the formation of an area of cytoplasm filled with large, electron-lucent vesicles close to the plasma membrane at one pole of the perikaryon; it is the incipient growth cone and has been described in cells of rat cerebellum *in vivo* (21, 28) and in explant cultures from cerebellum and other regions (8, 9, 13, 20, 47, 55; for review, see references 12, 19, 44, 61). Similar cytoplasmic differentiation was observed in our system as early as 24 h in cultures (Figs. 28 and 29). During formation of the migration patterns, fibers typically grouped into fascicles and growth cones were found at the tips of apparent axonal and dendritic fibers (Figs. 30 and 31). Growth cones were still detected after 6 days *in vitro*, indicating that fiber outgrowth probably occurs throughout the analyzed culture period.

Cinematography has shown growth cones to be very actively motile, the motility dependent upon substrate-fiber interactions under *in vitro* conditions (8, 9, 34, 61; E. Trenkner, manuscript in preparation). Direction of fiber growth appears to follow certain charge differences along the substrate (34). As pointed out above, the microculture conditions described here favor cell-cell interactions over cell-dish surface interactions. The fiber outgrowth patterns differ according to whether the fibers are growing on the surfaces of flattened cells (Figs. 20 and 27) or in

longitudinal contact solely with one another, forming cable-like configurations directed toward nearby reaggregates (Figs. 3, 20, 21, and 27) but not attached to the surface of the culture dish. The time and mode of attachment of cables to nearby reaggregates and the mode of elongation of cables is under study by time-lapse cinematography.

Most of the fibers generated in these cultures resemble the descriptions of mature parallel fibers (32, 42), consisting of narrow shafts containing few (two to five) microtubules oriented in the long axis of the fiber, and varicosities distributed at irregular intervals and containing small round vesicles, occasional mitochondria, and smooth ER. However, some fibers contain prominent ribosomes and, in addition, specializations were observed between varicosities and adjacent fiber shafts or between two varicosities (Fig. 24). These are difficult to relate to the available data on maturing cerebellar axons *in vivo*.

While the cable-like pattern appears to be constructed of one class of fiber only, the widespread, cell-attached pattern shows various fiber types. As shown in Figs. 27 and 31, fibers were found containing microtubules and neurofilaments, elongated, longitudinally oriented mitochondria, and ribosomes; growing tips occasionally exhibit multivesicular bodies. These fibers, with their various organelles, meet the *in vivo* characteristics of growing dendrites (43), though the cautionary note should be added that migrating young neurons may extend leading cytoplasmic processes that are neither dendritic nor axonal.

2. **CELL MIGRATION:** Cell migration serves in developmental biology in general to bring cells into contact with new neighbors with the consequent expression of new properties, and is a prominent event in the construction of vertebrate central nervous systems (59, 60). Its occurrence *in vivo* has been validated and quantified by autoradiographic methods with tritiated thymidine, but in our microwell cultures, cell migration has been documented directly and unequivocally by time-lapse cinematography (Fig. 5). The cells migrating along the cables were examined by transmission and scanning electron microscopy, and were found to possess the small size and nuclear features of immature granule cell neurons. Like migratory cells *in vivo*, they displayed bipolar shapes, and the internal features

of the two processes were comparable to those described for leading and trailing processes of migratory young neurons *in vivo*. Further, the cell size and nuclear features were similar to those of granule cell neurons in the reagggregates themselves, and such cells were not found on cables when embryonic midbrain or cerebral cortex (lacking granule cells) were cultured in microwells (Hatten and Sidman, manuscript in preparation). Further, microwell cultures of cerebellum at P0, a stage when external granule cells are present but show little migratory activity, and few mature granule cells have formed, develop cables without associated migratory cells (E. Trenkner, manuscript in preparation).

3. **SYNAPSE FORMATION:** Synapse formation has been described in cultures of prenatal mouse brain (6, 20), including cerebellum (41). The present study demonstrates synapse formation in postnatal (P7) mouse cerebellar cultures, originating from single cell suspensions (Figs. 9-13, 27, and 34). Our observations confirm the results of others obtained in cerebellar explant or monolayer culture systems (6, 14, 41, 45-48, 67, 68) and *in vivo* (3, 4, 24, 32, 35, 39, 40, 42, 52, 59, 62, 64). After 24 h *in vitro*, desmosome-like contacts were observed (Fig. 32) and after 72 h, asymmetrical synapses (similar to Gray type I) containing small round vesicles in the presynaptic part were abundant (Figs. 33 and 34). Almost all synapses observed either after 72 h or in increasing numbers after 6 days were of the same type. Their sources remain to be determined. We have not detected flattened vesicles in any presynaptic endings, under the conditions of fixation, dehydration, and embedding that we used.

Perspectives

The microculture system of immature mouse cerebellar cells presented in this study reproduces certain cell behaviors described for *in vivo* development of the cerebellum and eliminates other. First, cells that had become integrated into a tissue before preparation of a single cell suspension will form reagggregates in which cells sort out and assemble in characteristic patterns, though the patterns bear little geometric resemblance to the normal intact cerebellum. Second, even in this relatively simplified system, cell interactions are prominent; for example, cells migrate extensively, and always in direct contact

with other cells or processes, opening the prospect of investigating functions of cell surfaces during development by immunological and biochemical methods. Third, this system gives practical, analytical access to individual young mice bearing mutations that perturb critical steps in cerebellar development. Finally, in common with some other culture systems, growth cone formation, fiber outgrowth, and synapse formation can be manipulated experimentally.

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¹ For a complete description see: del Cerro, M. P., and J. R. Swarz. 1976. Prenatal development of Bergmann glial fibres in rodent cerebellum. *J. Neurocytology.* **5**:669-676.

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