CEREBELLAR GRANULE CELLS IN VITRO

A Light and Electron Microscope Study

FREDRICK J. SEIL and ROBERT M. HERNDON

From the Division of Neurology, Stanford University School of Medicine, Stanford, California 94305, and the Department of Neurology, Veterans Administration Hospital, Palo Alto, California 94304

ABSTRACT

The behavior of granule cells in mature cerebellar cultures derived from newborn mice was studied by light and electron microscopy. Many granule cells remained in the explants as an external granular layer. These cells were differentiated, as evidenced by formation of bundles of parallel fibers and by development of synapses between granule cell axons and Purkinje cell branchlet spines, and between Golgi cell axons and granule cell dendrites. Although the over-all architecture of the cerebellar explants after 18–33 days in vitro was similar to that of the newborn mouse, the evident differentiation of the granule cells suggested that interneuronal relationships resemble those of the mature cerebellum in vivo.

INTRODUCTION

Reports describing granule cells in cerebellar tissue cultures present varied interpretations with regard to the behavior and fate of these cells. In some studies (1, 6, 19, 21, 30), cerebellar granule cells in cultures derived from newborn or early postnatal animal sources were said to migrate away from the explant and into the outgrowth zone. Hild (13) found few cerebellar granule cells in his cultures, and speculated that granule cells degenerated in vitro because they were deafferentated at the time of explantation. Suyeoka and Okamoto (25) described numerous small cells with dense, round nuclei and scanty cytoplasm in their cerebellar explants. They considered at least some of these cells to be granule cells because their number was so great that they could not all be oligodendrocytes. Wolf (28) recognized few cerebellar granule cells in the explants and none in the outgrowth zone of his cultures in a histological study which relied on a modified Holmes silver-impregnation technique, but in a subsequent study employing the Golgi-Cox technique, Wolf, Holden, and Harlan (29) concluded that granule cells survived in considerable numbers in vitro, and described occasional bundles of parallel fibers. In electron microscope studies directed primarily at the ultrastructure of myelin in cerebellar cultures, neither granule cells nor synapses were investigated by Ross, Bornstein, and Lehrer (23), Perier and de Harven (20), and Field, Hughes, and Raine (4). Callas and Hild (3) described synapses in newborn rat brain stem and cerebellar cultures, but did not characterize the neuronal types on either side of the synaptic clefts. Hendelman (8) believed that cell stratification was lacking in cerebellar cultures derived from newborn mice, but identified granule cells on the basis of ultrastructural characteristics, and described synapses on dendritic spines (type not specified) and the rare presence of classic glomeruli, including mossy fiber terminals.

The present report is concerned with further analysis of the behavior of cerebellar granule cells in vitro, as determined by light microscope studies

based on a nuclear-Nissl stain and electron microscope studies of cerebellar explants derived from newborn mice.

MATERIALS AND METHODS

The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed during this study. Cerebellar cultures derived from newborn Swiss-Webster mice were prepared in accordance with previously described techniques (2, 24). Fragments 0.5–1.0 mm thick were cut in the parasagittal plane, explanted onto collagen-coated coverslips with a drop of nutrient medium, placed in Maximow assemblies, and incubated at 35.5°–36.0°C in the lying-drop position. The nutrient medium, which was changed twice weekly, consisted of two parts of three units/ml low-zinc insulin, one part of 20% dextrose, seven parts of bovine serum ultrafiltrate, seven parts of Simms' X-7 balanced salt solution, and twelve parts of human placental serum. Some of the cultures used in this study served as controls for a study of in vitro demyelinating activity of guinea pig sera (24), and were, therefore, exposed for periods ranging from ½ to 7 days to sera from guinea pigs injected with Freund's complete adjuvant. The guinea pig sera were present in a 25% concentration in normal nutrient medium.

Cultures were fixed after 18–33 days in vitro (DIV) for light and electron microscope examination. In the former case, the cultures were immersed in 80% ethanol at 4°C overnight and stained the following day with 0.5% acetic acid–thionine, after the method of Fletcher (5). In the latter case, specimens were fixed for 10–15 min in 1% osmium tetroxide, dehydrated, embedded in Epon, cut into fragments in a plane perpendicular to the plane of the culture, re-embedded in Epon, and sectioned. Thin sections were mounted on bare copper grids, stained with uranyl acetate (27) and lead citrate (26), and examined with a Siemens 1A electron microscope.

OBSERVATIONS

Thionine-stained whole-mount preparations revealed the presence of numerous small cells with dense round or slightly oval nuclei and little discernible cytoplasm. These cells were arranged in strata conforming to external and relatively thin internal granular layers in explants in which

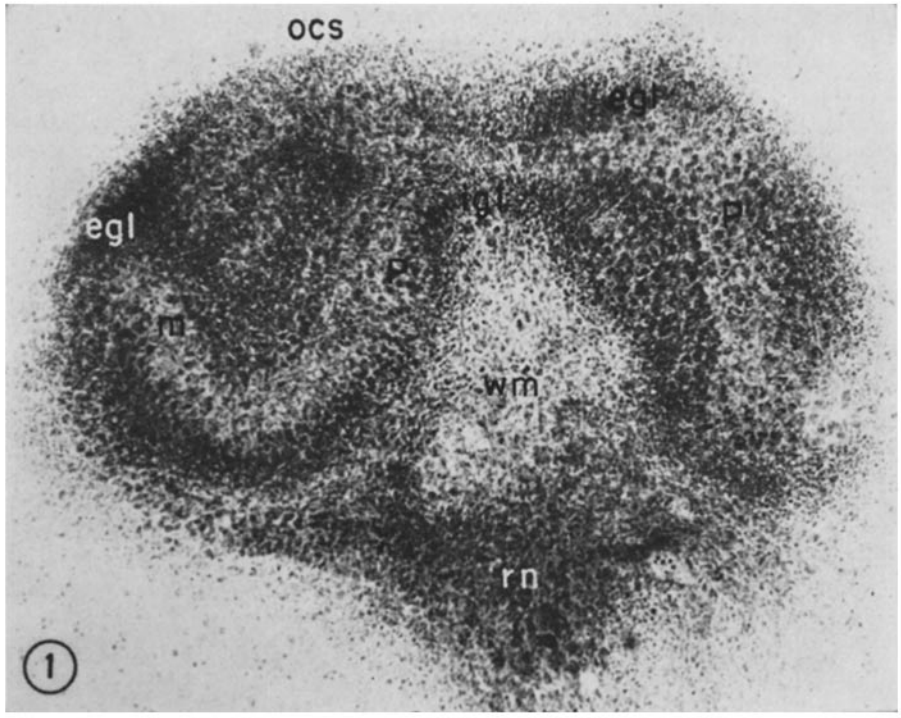


FIGURE 1 Cerebellar explant, 25 DIV, demonstrating the original cortical surface (*ocs*), an external granular layer (*egl*), a multilayered Purkinje cell zone (*P*), a trace of an internal granular layer (*igl*), a sometimes identifiable molecular layer (*m*), the white matter zone (*wm*) and a zone of roof nucleus neurons (*rn*). Thionine stain, whole-mount preparation (AFIP Neg. No. 68-7155). × 90.

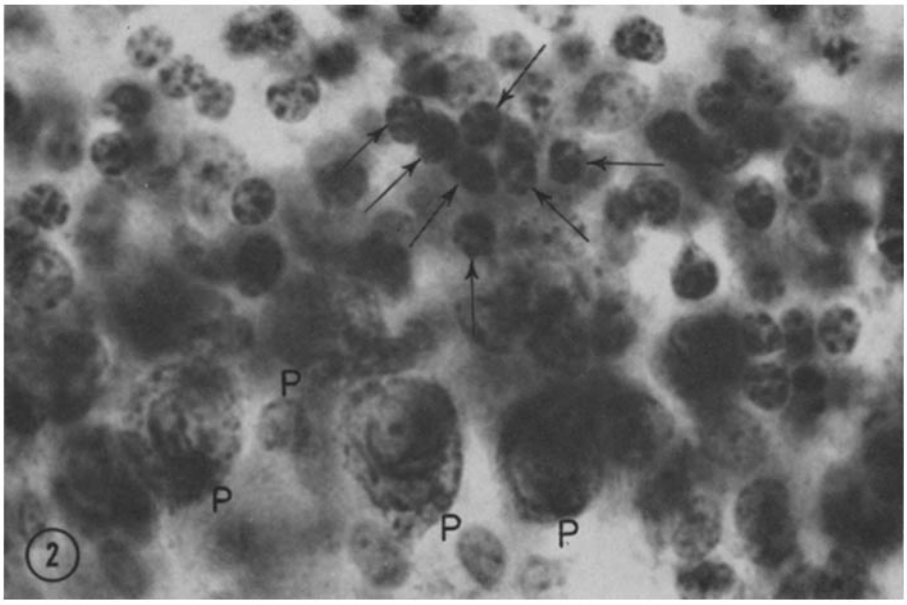


FIGURE 2 A higher magnification of the explant in the previous figure, demonstrating small round or oval densely staining nuclei of granule cells (arrows point to representative examples) in association with the much larger Purkinje cells (*P*), four of which are evident in the plane of focus of this micrograph. Thionine stain, whole-mount preparation (AFIP Neg. No. 68-7152). × 750.

the normal cerebellar architecture was fairly well preserved (Fig. 1). The strata of small cells were always contiguous with Purkinje cell zones (Fig. 2), which in culture were multilayered (Fig. 1) as they were in the animals at the time of explantation (28).

The cells most frequently encountered on electron microscope examination of the explants (outgrowth zones were not examined) were small neurons measuring 7–10 μ in diameter. These cells had a relatively large nucleus which consisted of homogeneously dispersed, finely granular chromatin interspersed with a smaller number of slightly larger and more dense granules. In favorable sections, a small area of heterochromatin could be seen at the periphery (Fig. 3) of the nucleus. The nucleus had a round or slightly ovoid shape, and occasionally a deep indentation could be seen arising near the Golgi region of the cell. The cytoplasm consisted of a thin rim ranging in thickness from perhaps 10 to 50 mμ, except in the Golgi region and at the base of cell processes (Fig. 3) where it was expanded considerably. A few mitochondria, free ribosomes, and an occasional element of granular endoplasmic reticulum were seen in this thin rim of cytoplasm. The Golgi apparatus was composed of vesicles and flattened cisternae and was restricted to a relatively small region of the cytoplasm. There were usually a number of mitochondria about the Golgi region along with an occasional multivesicular body and an occasional lysosome. Subsurface cisternae (9, 10, 22) were fairly commonly seen and usually appeared near the Golgi region of the cell.

The dendrites of the granule cells were only rarely seen in continuity with the granule cells (Fig. 3). They were very narrow, with a diameter of about 500 mμ, and contained irregular tubular structures measuring 30–50 mμ in diameter, along with an occasional mitochondrion. The axons had a similar structure, but the tubules had a more regular appearance with a diameter of about 50 mμ. Axodendritic synapses were seen fairly commonly on the granule dendrites (Fig. 4). The presynaptic structures appeared to be Golgi terminals, as judged by their size, their vesicle content, the absence of the central filamentous core usually present in the mossy fiber endings, and the appearance of their synaptic contact with granular cell dendrites (7).[1] These synapses occurred in re-

[1] R. M. Herndon. Unpublished observations.

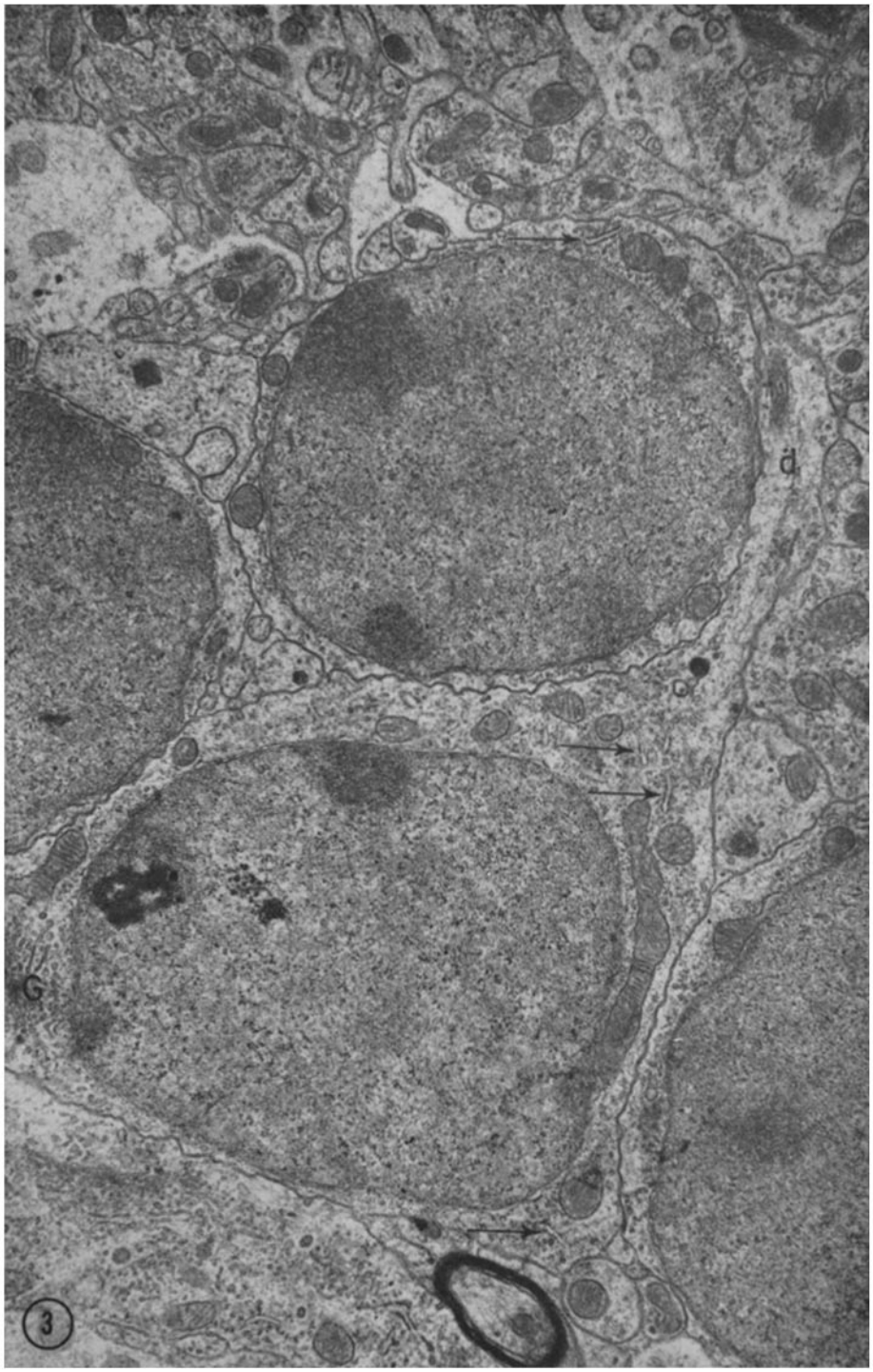
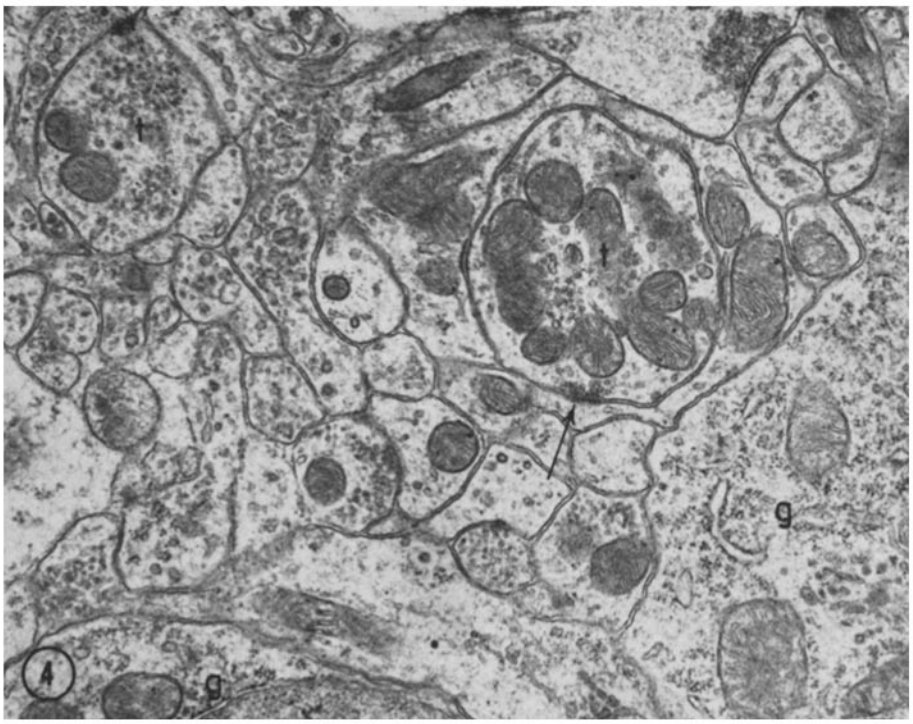


FIGURE 3 Portions of four granule cells surrounded by neuropil can be seen from a culture which was 26 DIV. The nuclei are rather homogeneous with a few areas of denser heterochromatin. Surrounding each nucleus is a thin rim of cytoplasm containing mitochondria, free ribosomes, and an occasional channel of endoplasmic reticulum (arrows). The Golgi apparatus (*G*) is seen in an area in which the cytoplasmic rim is expanded somewhat. A dendrite (*d*) is arising from the granule cell at lower center. × 11,500.



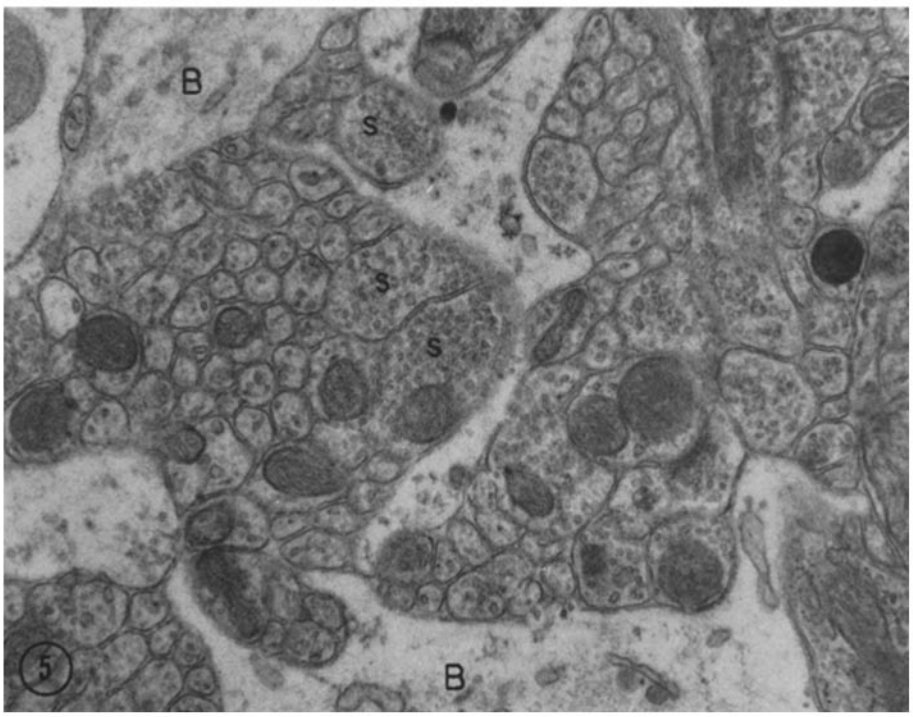


FIGURE 4 In this electron micrograph, two synaptic terminals (*t*), presumed on morphological grounds to be Golgi axon terminals, are seen. A synapse between the terminal and a granule cell dendrite is shown (arrow). Some of the smaller terminals containing synaptic vesicles are probably of Golgi cell origin also. Portions of two granule cells (*g*) are shown. From a culture 26 DIV. × 20,000.

FIGURE 5 This electron micrograph from a culture which was 26 DIV shows bundles of parallel fibers coursing through the molecular layer separated by hypertrophic processes of Bergmann astrocytes (*B*). A number of enlargements (*s*) containing synaptic vesicles are seen; however, points of synaptic contact are not well shown (see Fig. 6). × 26,000.

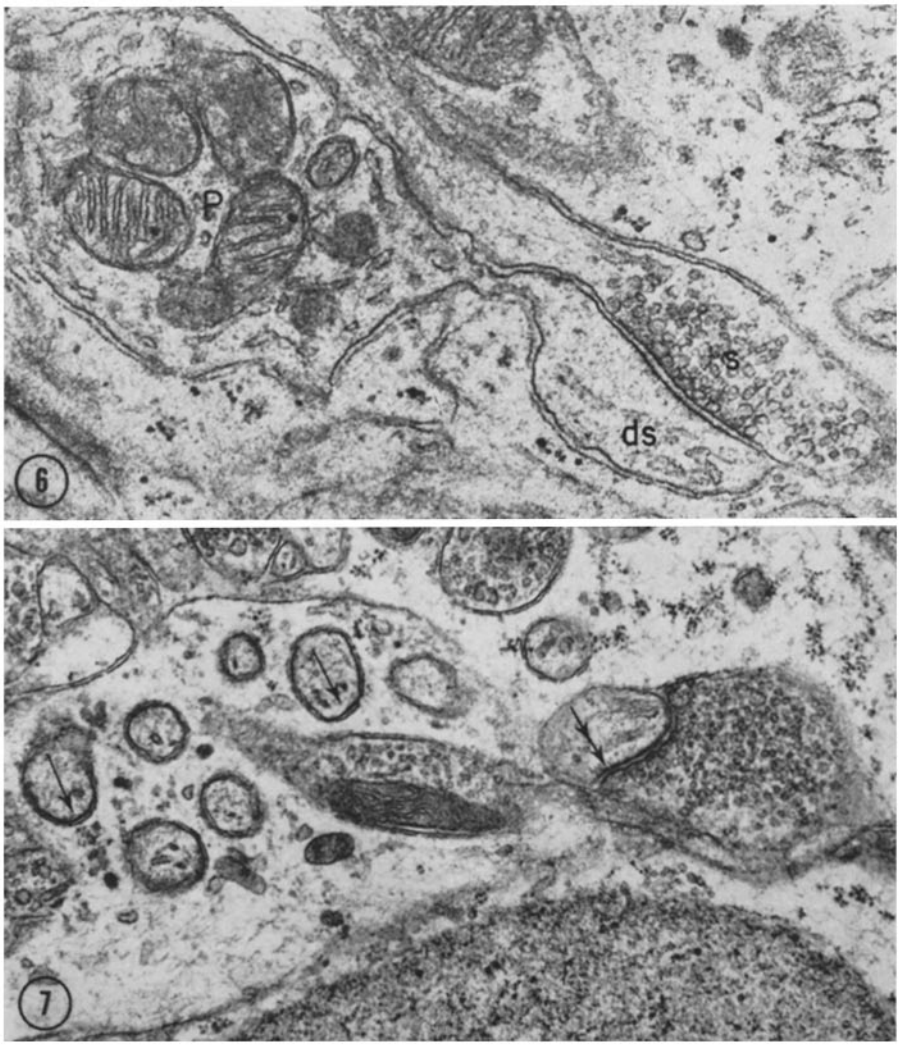


FIGURE 6 In this micrograph from a culture 33 DIV, a Purkinje cell dendrite (*P*) with a dendritic branchlet spine (*ds*) is shown in synaptic contact with the terminal enlargement of a granule cell axon (*s*). × 33,000.

FIGURE 7 Several Purkinje cell dendritic branchlet spines are seen in this micrograph. The one on the right is contacted by a granule cell axon terminal and shows the usual postsynaptic membrane thickening (double arrow). The remainder are enveloped by the process of a Bergmann astrocyte. Note the presence of the normal postsynaptic membrane thickening on these spines despite the absence of the presynaptic portion of the synapse (arrows). 26 DIV. × 25,000.

gions that resembled cerebellar glomeruli except that the central mossy fiber ending was absent. No true mossy fiber terminals were observed in this study.

In the molecular layer, between the Purkinje cell layer and external granular layer, there were numerous, highly oriented bundles of parallel fibers (Fig. 5) separated by processes of the Bergmann astrocytes. The bundles closely resembled those seen in normal mouse cerebellum (16). The astrocytic processes were larger and thicker than is the case in normal mouse cerebellum, however. Synaptic contacts between the parallel fibers and spines on the spiny branchlets of Purkinje cells

were commonly seen. These consisted of areas of dilatation along the course of a long, thin, parallel fiber. These enlargements contained mitochondria and numerous synaptic vesicles (Fig. 6) and conformed to the usual in vivo appearance of parallel fiber synapses on Purkinje cell branchlet spines (9, 15, 16). In some areas, Purkinje cell branchlet spines were seen from which the presynaptic terminals were missing. These spines were enveloped by processes of the Bergmann glia (Fig. 7). They retained their normal postsynaptic membrane thickening except that it appeared to be expanded somewhat, covering a larger proportion of the spine surface as compared to normally innervated spines (11, 12).

DISCUSSION

We interpret the cells described above as cerebellar granule cells for the following reasons: (*a*) They are the most numerous cell type encountered, and are associated consistently with Purkinje cells. (*b*) The nuclear and cytoplasmic morphology of these in vitro cells corresponds in detail with that of granule cells seen by light and electron microscopy in cerebellar sections from perfused animals. (*c*) The cells are neuronal in type, and can be distinguished on electron microscope examination from both oligodendrocytes and astrocytes. (*d*) The only cell processes which normally form synapses on the branchlet spines of Purkinje cells are granule cell axons (16), and synapses of normal appearance on branchlet spines are demonstrated in the in vitro preparations. The long branchlet spines are readily distinguishable from the low spines which are contacted by climbing fibers (16).

The results of this study demonstrate not only that the cerebellar granule cells survive in vitro, but that they also mature and differentiate, forming organized bundles of parallel fibers and synaptic relationships with other neural elements, in agreement with the conclusions of Wolf et al. (29) and Hendelman (8). The synapses formed are afferent as well as efferent, for although mossy fibers are not apparent in our cultures, the granule cell dendrites develop synaptic contacts with Golgi cell axons in similar fashion to that found in vivo (7). Granule cells in cerebellar cultures cannot, therefore, be said to be totally deafferentated.

Most of the surviving granule cells remain as an external granular layer. The retention of the fetal organization does not mean, however, that individual cells become arrested as embryonal granule cells, as Suyeoka and Okamoto believed (25). The evidence, on the contrary, points toward differentiation of the cells. A parallel situation is that of the Purkinje cells, which in culture retain their fetal multilayered organization, but which, as individual cells, progress in vitro toward maturation and differentiation, as demonstrated by others (1, 28).

We have not specifically approached the question of the migration of cerebellar granule cells into the outgrowth zone, but on the basis of observations of cultures in the living state and past observations of stained preparations of younger cultures, we consider it quite possible that a portion of the external granule cell population does migrate into the outgrowth zone, in concurrence with the reports of other investigators (1, 6, 19, 21, 30). Although most neurons do not exhibit migratory properties in vitro (18), we find it conceptually plausible that neurons poised for migration at the time of explantation, as is the case with external granule cells in the mouse at the time of birth (17), should retain some migratory capability after explantation. As the normal organization of the cerebellum is at least partially disrupted by the explantation procedure, and as mesenchymal and glial cell types do migrate into the outgrowth zone, it would seem reasonable that external granule cells might migrate away from the explant, as well as in their normal direction. The presence in our cultures of some Purkinje cell branchlet spines without synapses suggests that the full complement of granule cells is not present, and is consistent with either degeneration or outward migration of some of these cells. Similar deafferentated Purkinje cell spines have been seen in experimental granule cell degeneration (11) and in the malformed cerebellum produced by neonatal infection with the feline panleukopenia virus (12). With regard to the fate of outwardly migrating cells, the relative paucity of granule-like cells in the outgrowth zones of older cultures suggests that these cells, having lost the opportunity to interact with other neurons, either degenerate or develop along nonneuronal lines, as noted by others (13, 19, 25).

Of greater significance, however, is the fact that many granule cells remain as organized strata within the explants, and that these neurons mature and differentiate, with their axons and dendrites developing normal synaptic relationships with the dendritic and axonal processes of other neurons. The morphologically evident development of such

complex interneuronal relationships raises the hope that future functional studies of the cerebellum in vitro will provide evidence that it is a more faithful model of the cerebellum in vivo than past physiological investigations (14) have indicated.

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