References and Notes

1. E. Werle, A. Hevelke, K. Buthmann, *Biochem. Z.* **309**, 270 (1941); K. Semon, Ed. *Das Wehenproblem mit Besonderer Berücksichtigung des Oxytocin-Oxytocinase-Haushaltes* (Ferdinand Enke, Stuttgart, West Germany, 1960); A. Centaro, P. Periti, G. de Laurentiis, *Rev. Obstet. Gynecol.* **16**, 297 (1961); S. Melander, *Acta Endocrinol.* **49** (suppl. 96) (1965); G. Rydèn, *Acta Obstet. Gynecol. Scand.* **45** (Suppl.) (1966); M. A. Carballo, in *Oxytocin*, R. Caldeyro-Barcia and H. Heller, Eds. (Pergamon, New York, 1961), p. 343.
2. R. Walter, J. D. Glass, B. M. Dubois, M. Koida, I. L. Schwartz, in *Proceedings of the Second American Peptide Symposium*, S. Lande, Ed. (Gordon & Breach, London, in press).
3. J. D. Glass, B. M. Dubois, I. L. Schwartz, R. Walter, *Endocrinology* **87**, 730 (1970).
4. It is unknown whether this aminopeptidase activity is intrinsic to uterine tissue or whether it results from blood which has been trapped.
5. R. T. Havran, I. L. Schwartz, P. Eggena, R. Walter, *Proceedings of the Intra-Science Symposium on the Chemistry of Peptides*, N. Kharasch, Ed. (Intra-Science Research Foundation, Santa Monica, Calif., in press); R. Walter and R. T. Havran, *Experientia*, in press.
6. A third radioactive component was identified as glycinamide. This compound was present maximal.y at a concentration of 7 percent of the total radioactivity. Preliminary data reveal that the glycinamide can result from a direct cleavage of oxytocin as well as from a degradation of leucylglycinamide by another enzyme eluted in fractions 8 to 10.
7. M. Koida, J. D. Glass, I. L. Schwartz, R. Walter, *Endocrinology* **88**, 633 (1971).
8. J. D. Glass, I. L. Schwartz, R. Walter, *Proc. Nat. Acad. Sci. U.S.* **63**, 1426 (1969).
9. I. Rychlík, in *Oxytocin, Vasopressin and Their Structural Analogues*, J. Rudinger, Ed. (Pergamon, New York, 1964), p. 153.
10. I. Sjöholm, *Fed. Eur. Biochem. Soc. Lett.* **4**, 135 (1969); consult this paper for additional references.

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Human Fetal Cerebellar Cortex: Organization and Maturation of Cells in vitro

Abstract. *Human fetal cerebellar cortex was maintained up to 5 months in vitro. Important features included early migration of granule neurons followed by maturation of Purkinje and granule neurons. Unique areas of organization developed in which a rim of leptomeningeal cells surrounded an explant and its outgrowth zone; these areas subsequently grew as well-defined units.*

In this report we describe cell maturation and the development of a highly organized pattern of growth in organotypic cultures of human fetal cerebellar cortex. Using specimens removed surgically for the purpose of terminating pregnancy (*1*), we prepared explants from ten human fetuses, varying in gestational age from 10 to 19 weeks. Cerebellar cortex, with leptomeninges intact, was undercut and removed, deep cerebellar nuclei and brainstem structures being avoided. The tissue was sectioned into small serial blocks which were suspended in 15 ml of nutrient mixture F-12 (*2*). They were distributed among five to ten plastic petri dishes (Falcon), each dish containing several fragments. Explants were grown directly on the surface of the dish or on sterile glass cover slips previously introduced. Complete feeding medium consisted of 80 parts of nutrient mixture F-12, 20 parts of fetal calf serum, one part of fresh L-glutamine (200 mmole/liter), one part of nonessential amino acids (GIBCO), and one part of 50 percent glucose in water (which raised the total glucose content to 680 mg/100 ml). Collagen and plasma clot were not used, nor were antibiotics or embryo extract.

Observations were made on living cultures and on fixed cultures terminated after varied periods of growth. Phase, dark-field, bright-field, and polarizing optics were used to examine cultures stained with methylene blue, phosphotungstic acid-hematoxylin (PTAH), luxol fast blue, the trichrome method, or Bodian's silver protargol method. For electron microscopy, cultures were fixed in phosphate-buffered glutaraldehyde, treated with osmium tetroxide, dehydrated, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate. They were examined with an RCA EMU-3F electron microscope.

After a few days in culture, cells migrated from the explant in steadily increasing numbers. The nuclei of cells migrating earliest were pale and moderately large. The cytoplasm was broad and sheetlike, eventually giving rise to one or more processes of variable length and thickness. These cells were interpreted as immature astrocytes (*3*). By the end of the first week slender neuritic processes emerged from the explant, followed several days later by migration outward of the cell bodies from which they arose. These cells were small and migrated in groups. Their nuclear size (major diameter 7 to 10 μm), nuclear chromatin pattern, and scanty cytoplasm which gave rise to long, thin, unipolar or bipolar processes which stained strongly after silver reduction were characteristic of immature granule neurons (Fig. 1A). Ultrastructural appearance of a cell from a similar cluster of early migrating small cells is seen in Fig. 1B. At

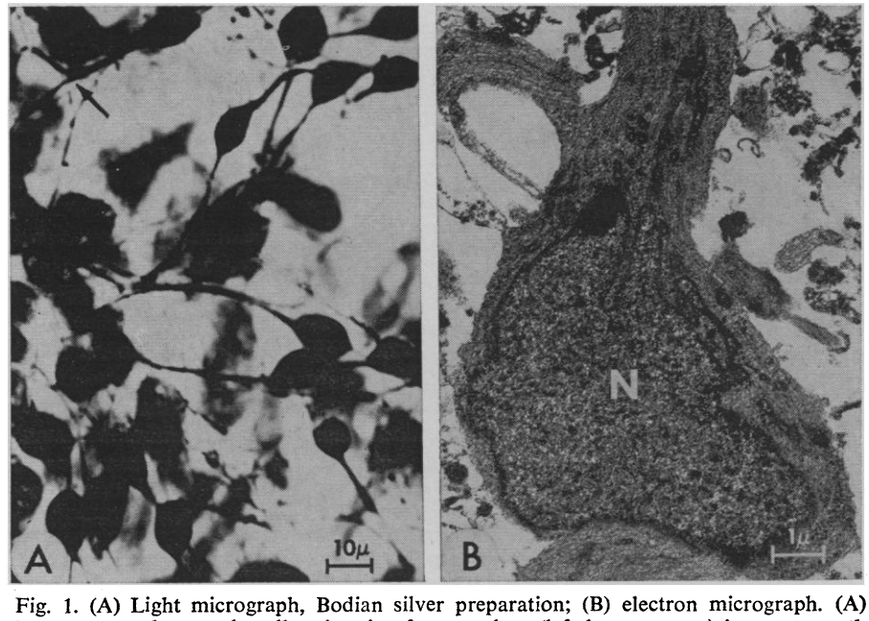

Fig. 1. (A) Light micrograph, Bodian silver preparation; (B) electron micrograph. (A) Numerous early granule cells migrating from explant (left lower corner) into outgrowth zone. Argyrophilic processes are prominent. Arrow (upper left) points to a bifurcating process. Culture of cerebellar cortex from a 16-week-old fetus after 39 days in vitro. (B) Cell from similar group of early migrating small neurons. A thin rim of cytoplasm surrounds the nucleus (*N*) and gives rise to a long process, only the initial part of which is shown. From 16-week-old fetus after 14 days in vitro.

higher magnification the cell processes were seen to contain microtubules and small, immature mitochondria. As judged from light and electron microscopy these cells resembled closely cells of the external granular layer in situ in promptly fixed (glutaraldehyde) blocks of cerebellar cortex removed from approximately 16-week-old fetuses for comparative observations. Their identification as immature granule cells was further strengthened by their similarity to early granule cells in explants of mouse cerebellum as described by others (*4–6*); these investigators have established that granule cells survive and can be identified in organotypic cultures and that they have the capacity to migrate away from the explant. We recognize that some of the small cells observed in our early cultures may be immature basket, stellate, or Golgi II neurons, but their large numbers indicate that they are predominantly precursors of granule neurons. It is interesting that during normal fetal growth, a distinct granular layer can be identified between the 16th and 21st weeks of gestation, according to studies of histologic preparations from human fetal cadavers (*7*).

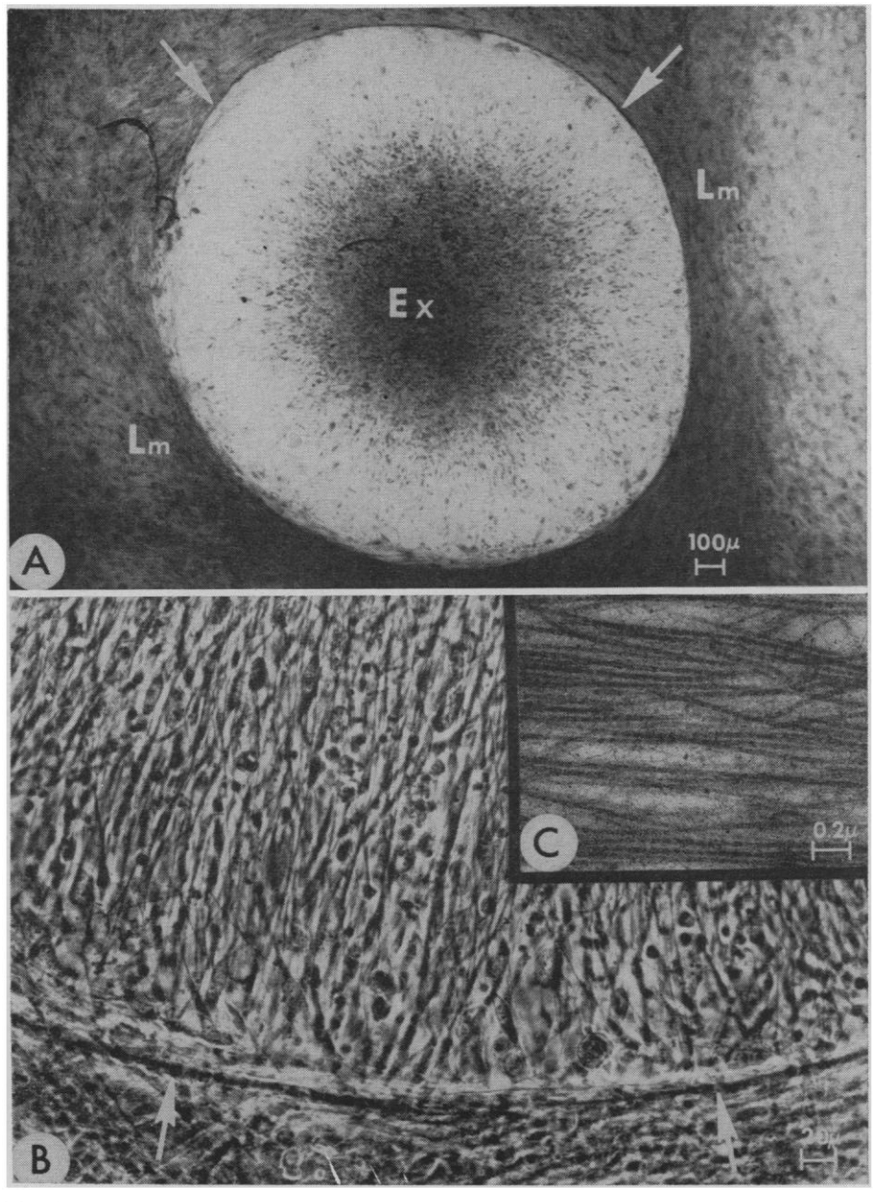

Fig. 2. (A) Bright-field micrograph, methylene blue stain; (B) phase micrograph; (C) electron micrograph. (A) Organized area in long-term culture of cerebellar cortex. The pale region surrounding the primary explant (*Ex*) is the outgrowth zone. The outgrowth zone is surrounded by a circular rim of cells. Along the inner aspect of the rim is a line (arrows), which is in sharp focus only at the top of micrograph. The circular rim of cells has no definable outer limit and fades into bundles of adjacent cells which are similar in appearance and are of leptomeningeal (*Lm*) origin. From 16-week-old fetus after 10 weeks in culture. (B) Portion of similar organized area from explant from 16-week-old fetus grown in vitro for 7 weeks, showing segment of curved line (arrows) at periphery of outgrowth zone, and part of ill-defined circular rim of cells outside. The processes of most cells located in the outgrowth zone are oriented perpendicularly to the line and surrounding rim and do not penetrate *into the rim*. Cells composing the circular rim likewise do not invade the outgrowth zone. (C) Fibers composing line having a periodicity varying between 400 and 600 Å.

An unexpected finding was the development of unique areas of organization usually within 3 to 6 weeks after explantation. These organized regions were a consistent development and occurred in cultures from all ten fetal specimens. The architectural pattern of these areas persisted (Fig. 2A). The explant was surrounded by a less cellular outgrowth zone. The latter, in turn, was enclosed by a circular rim of cells, frequently several cell layers in width. Within the circular rim or along its inner margin there was often a discrete line (Fig. 2, A and B). The substance forming this line consisted of collagen, as demonstrated with PTAH and trichrome stains and birefringence and electron microscopy (Fig. 2C). Examination of numerous cultures grown for many weeks led us to conclude that the collagenous line was produced by the circular rim of cells surrounding the outgrowth zone. From their appearance in light and electron microscopy, we judged the cells composing this rim to be fibroblasts. We also concluded that this multilayered rim of fibroblasts could not have arisen from cells migrating out from the explant, but must have originated from randomly growing leptomeningeal cells away from the organized areas.

Many of the neural and glial processes within the outgrowth zone were oriented perpendicular to the circular rim of fibroblasts (Fig. 2, A and B). Processes of some of the astrocytes arising from the explant curved at the outer margin of the outgrowth zone, following the contour of the surrounding rim. With rare exception, processes of cells in the outgrowth zone did not extend beyond the circular rim, and cells composing this rim did not penetrate and enter the region of the outgrowth zone, the rim in effect behaving like a leptomeningeal covering. After continued growth the diameters

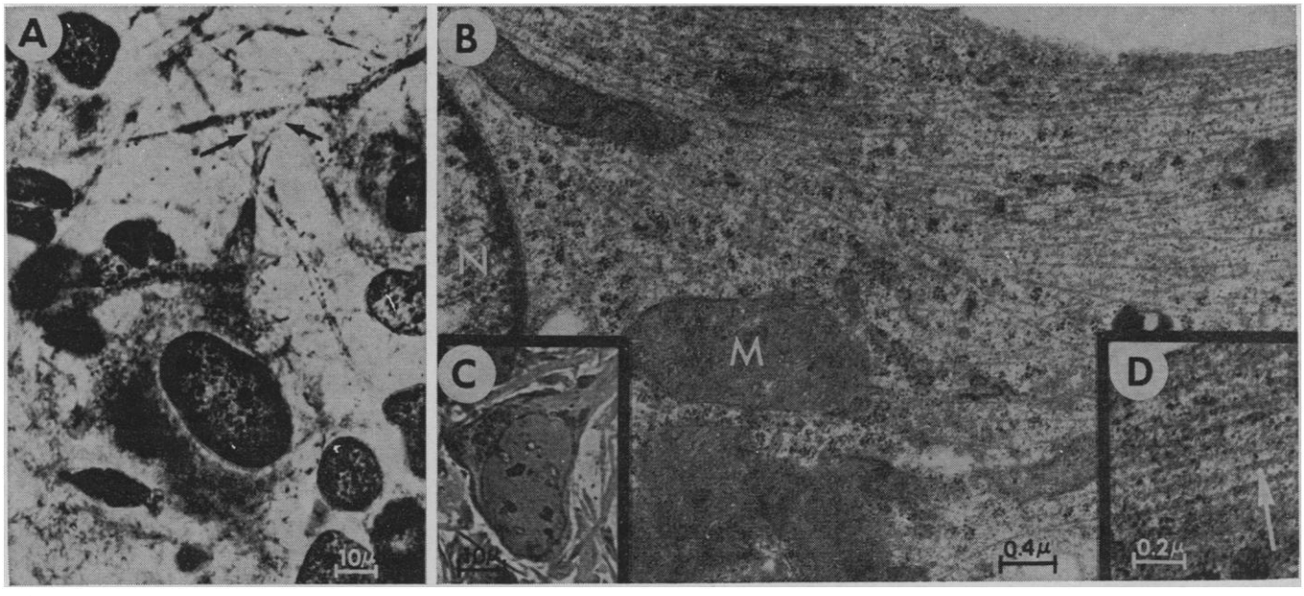

Fig. 3. (A) Bright-field micrograph. Bodian silver preparation; (B) electron micrograph; (C) 1-μm section embedded in Epon and stained with toluidine blue; (D) electron micrograph. (A) Purkinje neuron located centrally in micrograph. The nucleus is large, and the soma of cell gives rise to an argyrophilic process. Note branching of the process indicated by two arrows. From 18-week-old fetus after 103 days in vitro. (B) Electron micrograph of the initial segment of the process of the Purkinje neuron shown in (C). The process contains numerous microtubules which are approximately 250 Å in diameter and seen at higher magnification in (D). *N*, Nucleus; *M*, mitochondria. From 18-week-old fetus after 137 days in vitro.

across the circular rim of organized regions increased. At the time organization was first recognizable, diameters across the rim ranged between 0.5 and 4.0 mm. Within a month's time thereafter, diameters were one-fourth to one-third greater. We observed no significant zones of necrosis within explants or outgrowth zones even in those cultures surviving longest, although isolated necrosis of single cells was occasionally encountered. Toxic effects, such as described with the use of embryo extract (*6*), were never seen.

Large cells suggestive of early Purkinje neurons were first observed after 4 to 6 weeks in most cultures; they became conspicuous after longer periods of growth. They were located randomly within the explant proper, or at the interface between explant and outgrowth zone, where they sometimes formed a monolayer. Large cells were identified by light microscopy as presumptive early Purkinje neurons when they fulfilled these criteria: (i) possession of a large nucleus, nucleolus, and cytoplasm compared with other cells within the explant; (ii) presence of basophilic cytoplasmic material having the appearance of immature Nissl substance; and (iii) presence of an argyrophilic process, sometimes bifurcating (Fig. 3A), composed of neurofibrils. Cells of this type from our oldest cultures have been examined by electron microscopy. Their cytoplasmic fine structure revealed numerous free ribosomes, endoplasmic reticulum, and mitochondria. A tapering process (Fig. 3, B–D) extended from the cell soma and was filled with many long microtubules approximately 250 Å in diameter. Although astrocytes often contained ovoid or round nuclei similar in size to those identified as Purkinje neurons (between 25 and 40 μm in major diameter), their cytoplasm and processes, in contrast, were packed with filaments approximately 100 Å in diameter (Fig. 4, A and B). Astrocytes, unlike Purkinje neurons, were widely dispersed in the outgrowth zone as well as within the explant.

The primary explants surviving longest to date (4½ months and 5 months) were obtained from fetuses approximately 18 weeks old. The total period of growth of cells in these cultures (growth in utero combined with that in vitro) was, therefore, close to the normal 9-month gestational period for human fetuses. No myelin was observed in these older cultures, as studied with electron and phase contrast microscopy and myelin stains. Similarly, synapses were not seen in electron microscopic preparations, although cell contacts and vesicle-containing growth cones (*8*) were frequently observed at the tips of neuronal processes both in the explant proper and within the outgrowth zone. Granule cells, although numerous in the outgrowth zone during early stages after explantation, gradually disappeared from this region after extended growth in culture. They continued to be a prominent component within the explant proper, where they were often found in clusters (Fig. 5). The small nucleus and thin rim of cytoplasm of more mature granule cells (Fig. 5) are similar to the electron microscopic appearance of granule cells observed in newborn mouse cerebellum after 3 to 5 weeks in vitro (*4*, *9*).

The absence of myelin in those cultures surviving longest is compatible with existing timetables for the development of myelin in the cerebellar cortex of man as determined by light microscopy (*10*). We are not aware of studies documenting the onset of synaptogenesis in human cerebellar cortex. We note that synapses have not been observed until after birth in rat cerebellar cortex (*11*), although they have been described in the later stages of fetal development in *Macaca mulatta* (*12*). Thus far our observations suggest that further neuronal maturation in terms of cellular interaction in explants of human fetal cerebellar cortex is most likely to take place within or near the explant proper.

The unique architectural pattern developed in the organized areas we have

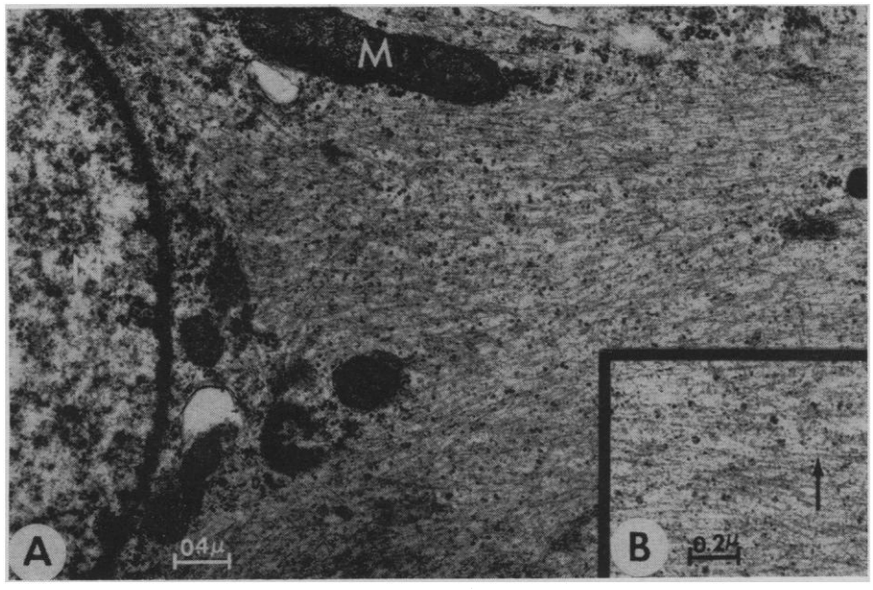

Fig. 4. (A and B) Electron micrographs. (A) Astrocyte containing closely packed filaments approximately 100 Å in diameter extending from the soma into the origin of the process. *N*, Nucleus; *M*, mitochondria. Details of filaments are seen in (B). From 18-week-old fetus after 137 days in vitro.

observed differed significantly from previous reports of organization described in long-term cultures of central nervous tissue from experimental animals (*5, 13*). Organization, as described in our cultures, was unique not only in the pattern of cells produced, but also in that each organized area constituted an integral, self-contained unit. These areas were unaltered by the disorganized monolayer of randomly growing cells outside, which was composed primarily of leptomeningeal elements. The presence of leptomeninges, thus, did not seem to interfere with the maturation and cytodifferentiation taking place in the explant and outgrowth zones.

How closely these developmental changes resemble those in the living human fetus in utero is unknown. The characteristics suggest, however, that regulatory mechanisms operate in vitro which influence not only developmental phenomena within cells but also those expressed in the organization of cells to form a tissue with definable architectural relationships. Long-term organotypic cultures may, therefore, offer an opportunity for investigation of some of the processes whereby regulation of growth and differentiation occur in the human fetus. Data from studies on experimental animals concerning histogenesis of cells in the cerebellar cortex, their morphology, synaptic connections, circuitry and biochemical-pharmacologic characteristics (*14*) add another dimension to the potential usefulness of cultures from human fetuses in the investigation of the control mechanisms essential for the expression of developmental phenomena in man.

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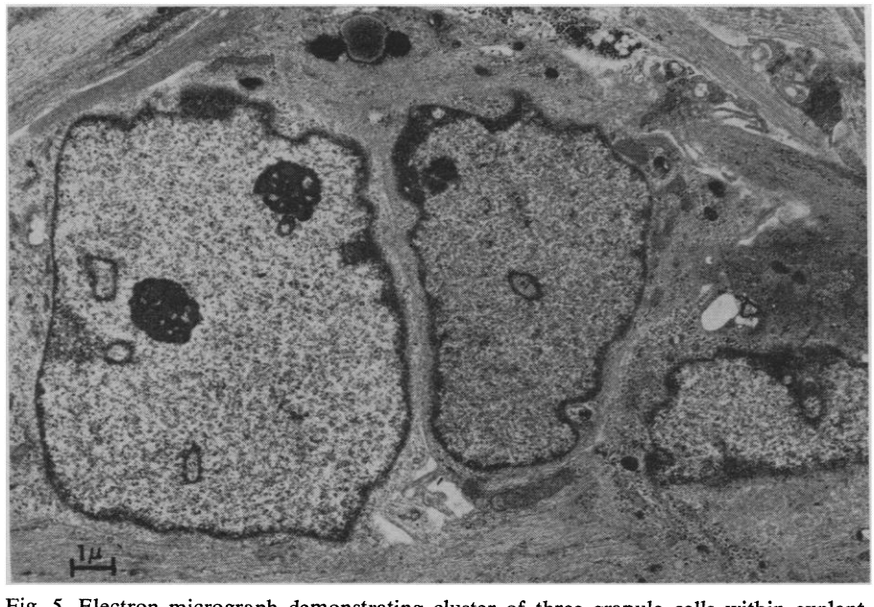

Fig. 5. Electron micrograph demonstrating cluster of three granule cells within explant proper from same culture as that in Figs. 3 and 4.

References and Notes

1. This study was done subsequent to changes in state law effective July 1970 and has been approved by the Clinical Investigation Committee of the University of Rochester, as required by the National Institutes of Health.
2. R. G. Ham, *Proc. Nat. Acad. Sci. U.S.* **53**, 288 (1965). This preparation was obtained from Grand Island Biological Company (GIBCO).
3. R. W. Guillery, H. M. Sobkowicz, G. L. Scott, *J. Comp. Neurol.* **140**, 1 (1970).
4. M. K. Wolf and M. Dubois-Dalcq, *ibid.*, p. 261; S. U. Kim, *Z. Zellforsch.* **107**, 454 (1970).
5. M. K. Wolf, *J. Comp. Neurol.* **140**, 281 (1970).
6. C. D. Allerand, *ibid.*, **142**, 167 (1971).
7. P. Rakic and R. L. Sidman, *ibid.* **139**, 473 (1970); L. B. Verbitskaya, in *Neurobiology of Cerebellar Evolution and Development*, R. Llinás, Ed. (American Medical Association, Chicago, 1969), p. 859.
8. M. P. Del Cerro and R. S. Snider, *J. Comp. Neurol.* **133**, 341 (1968); E. Kawana, C. Sandri, K. Akert, *Z. Zellforsch.* **115**, 284 (1971).
9. F. J. Seil and R. M. Herndon, *J. Cell Biol.* **45**, 212 (1970).
10. L. B. Rorke and H. E. Riggs, *Myelination of the Brain in the Newborn* (Lippincott, Philadelphia, 1969).
11. M. P. Del Cerro and R. S. Snider, *Int. J. Neurol.* **7**, 213 (1970).
12. S. E. Kornguth, J. W. Anderson, G. Scott, *J. Comp. Neurol.* **132**, 531 (1968).
13. C. M. Pomerat and I. Costera, *Amer. J. Anat.* **99**, 211 (1956); S. M. Crain and E. R. Peterson, *J. Cell. Comp. Physiol.* **64**, 1 (1964); M. R. Murray, in *Cells and Tissues in Culture*, E. N. Willmer, Ed. (Academic Press, New York, 1965), p. 373; H. M. Sobkowicz, R. W. Guillery, M. B. Bornstein, *J. Comp. Neurol.* **132**, 365 (1968); E. R. Peterson, S. M. Crain, M. R. Murray, *Z. Zellforsch.* **66**, 130 (1965).
14. J. C. Eccles, M. Ito, J. Szentágothai, *The Cerebellum as a Neuronal Machine* (Springer-Verlag, New York, 1967); C. A. Fox and R. S. Snider, Ed., *The Cerebellum, Progress in Brain Research* (Elsevier, Amsterdam, 1957), vol. 25; C. C. Bell and R. S. Dow, in *Neurosciences Research Symposium Summaries*, F. O. Schmitt, T. Melnechuk, G. C. Quarton, G. Adelman, Eds. (M.I.T. Press, Cambridge, Mass., 1967), vol. 2, p. 515; R. Llinás, Ed., *Neurobiology of Cerebellar Evolution and Development* (American Medical Association, Chicago, 1969).
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