Z. Zellforsch. 107, 454—465 (1970)

Observations on Cerebellar Granule Cells in Tissue Culture

A Silver and Electron Microscopic Study*

SEUNG U. KIM**

Department of Anatomy, College of Medicine, University of Saskatchewan, Saskatoon, Canada

Received February 12, 1970

Summary. In long-term organized cultures of newborn mouse cerebellum, granule cell neurons were studied with silver impregnation and electron microscopy.

In silver impregnated cultures, small neurons are defined as granule cell neurons from their size, morphology and location. There are also occasional large nerve endings with the morphology typical of mossy fiber endings.

In correlative electron micrographs, granule cell neurons revealed a faithful reproduction of characteristic structures seen in vivo. The fine structural details of cerebellar glomeruli and myelinated granule cell bodies developed in vitro were also described.

Key-Words: Cerebellum — Neuron — Synapses — Myelin sheath — Tissue culture

Introduction

Several recent studies have shown that tissue culture of the nervous system can provide a simplified experimental model for morphological, pharmacological, physiological and pathological investigations (see reviews of Murray, 1965; Lumsden, 1968). Since the first descriptions of myelin formation in tissue culture of the central nervous system (Hild, 1957; Bornstein and Murray, 1958), the cerebellum has been a favorite material for many investigators providing a valuable tool for various studies (Bornstein and Appel, 1962, 1965; Yonezawa *et al.*, 1962; Hild and Tasaki, 1962; Kim, 1966, 1969, 1970; Lumsden, 1965; Silberberg and Schutta, 1967; Hoskin and Allerand, 1968; Kim *et al.*, 1970; and others). In this connection, the morphological features of cerebellum cultures have been reported by previous authors (Pomerat and Costero, 1956; Kim, 1963, 1965; Wolf, 1964; Hild, 1966; Lumsden, 1968). The morphological characteristics of the Purkinje cells and neurons of subcortical nuclei are well documented whereas little is known concerning the morphology of the small neurons including granule cells. Recent electron microscopic studies on cerebellum cultures have mainly been concerned with myelinogenesis and other structural details including synapses were left untouched (Ross *et al.*,1962; Field *et al.*, 1968). The present communication deals with the morphological characteristics of granule cells grown in tissue culture at both the light and electron microscopic levels.

* This work was initiated while the author was a fellow of National Multiple Sclerosis Society in the laboratory of Dr. Margaret R. Murray at the Department of Surgery, Columbia University, New York, U.S.A. This work was supported by the Medical Research Council of Canada.

** The author is greatly indebted to Dr. Asao Hirano, Laboratory Division, Montefiore Medical Center, New York, U.S.A., for his contribution in the preparation of certain electronmicrographs. The author wishes to thank Dr. S. Fedoroff for reading the manuscript.

Materials and Methods

The newborn mouse cerebellum was divided parasagittaly into 10—12 sections and two of these explants were placed in close proximity on a collagen coated coverslip, grown in Maximow's double coverslip assemblies (Bornstein and Murray, 1958; Allerand and Murray, 1968). The cultures, ranging in age from 14 to 170 days in vitro, were fixed and impregnated by a modified version of Bodian's protargol-copper method. Cultures were fixed in Ramon y Cajal's formol-ammonium bromide solution for 24 hours at room temperature and then immersed in 95 per cent ethanol for 2—3 days at 36° C. Cultures (numbering seven each time) were placed in a columbia staining dish containing 0.7 per cent protargol solution with copper shots (0.2 gram per 10 ml solution) at 36° C for 20—24 hours. Then cultures were transferred to a fresh 0.7 per cent protargol solution with pyridine (a drop of 20 per cent pyridine aqeous solution in 10 ml protargol solution) at 36° C for another 6—18 hours. Cultures were then processed in the reducing bath, 1 per cent gold chloride (without acetic acid added), 2 per cent oxalic acid and 5 per cent sodium thiosulfate solution for 5 minutes, 2 minutes, 2 minutes and 5 minutes, respectively. This modified method has proved to be an excellent stain for neuronal cell types especially granule cell neurons and for synaptic endings. Other cultures were fixed in 2 per cent veronal-acetate buffered osmic acid for 30 minutes, dehydrated and embedded in Epon. Ultrathin sections were cut with a diamond knife and were examined with Siemens 1 A and Phillips 200 electron microscopes.

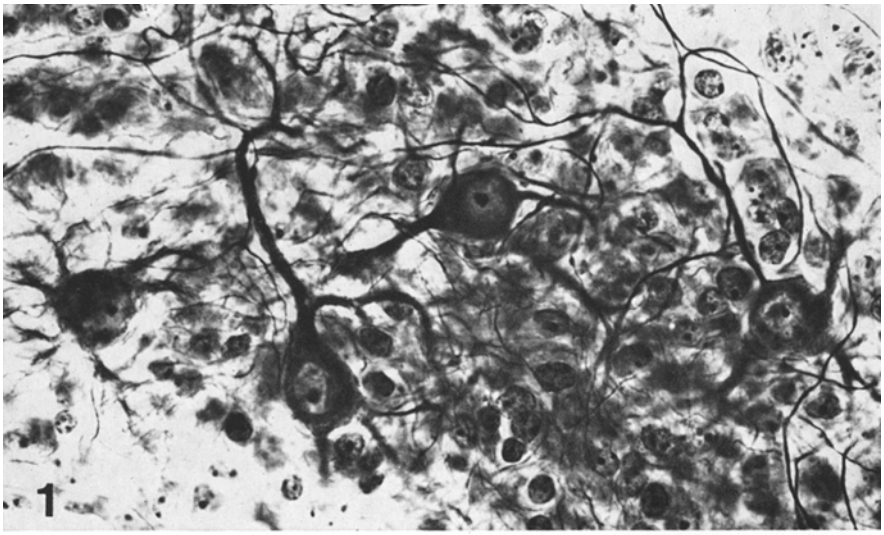
Results

Silver Study. In cultures of mouse cerebellum, after silver impregnation, large neurons (Fig. 1) with their axons, collaterals and synapses are clearly visible. The neurofibrils in the perikarya are delicate and extend to the dendrites. Besides large neurons, there are a fair number of small neurons ranging in diameter 5 to 8 μ. They are bipolar or stellate in shape and up to six fine processes were observed emerging from the cell bodies often to a length of 200 μ (Figs. 2—6). In some instances, there are suggestions of claw-like structures in the end of their processes (Figs. 5, 6) showing a remarkable resemblance to the in vivo morphology of granule cells shown by Golgi silver impregnation (Ramon y Cajal, 1911).

Sometimes these fine processes from different cells touched each other and revealed a rossette-like configuration (Figs. 3, 4). These small neurons are usually grouped together and located in the vicinity of the well-organized rows of large neurons which eventually identified as the Purkinje cells. Some small neurons are present frequently in the outgrowth zone indicating that they are capable of migrating out from the explant.

From their size, shape, and location, it is reasonable to identify these small neurons as granule cells. Since the starting material was newborn mouse brain, other small neurons in the cerebellar cortex, basket cells and small cortical cells of the molecular layer are undifferentiated; it is therefore possible that in tissue culture, these might appear morphologically similar to granule cell neurons.

In old cultures, there are some peculiar nerve endings other than normal ring-shaped terminal boutons (Kim, 1965) in silver impregnated cultures. These are grape-like groups of rings and bulbs connected with terminal fibers (Fig. 8) and are usually located in well preserved reminiscent granule cell layers. These structures are tentatively identified, from their morphology and location, as mossy fiber endings (rosettes).



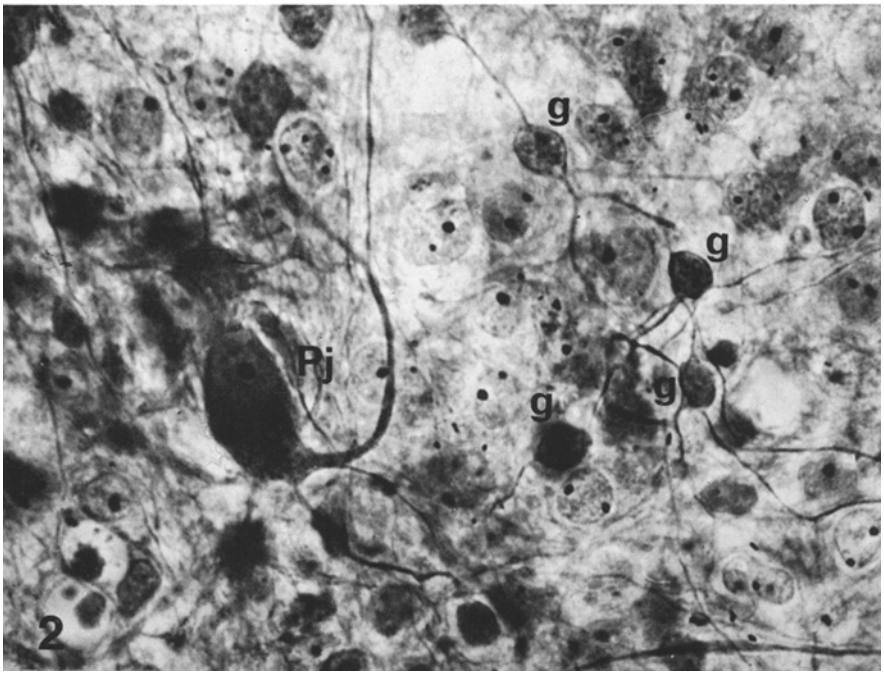


Fig. 1. Purkinje cells in various stages of their differentiation. 25-day-old culture. ×600
Fig. 2. A group of granule cells (*g*) located nearby a Purkinje cell (*Pj*). 28-day-old culture. ×600

Electron Microscopy. The profiles of granule cell nuclei are spheroid in shape and range in diameter from 5 μ to 8 μ. The chromatin is quite uniformly dispersed throughout the nucleus, though in instances a little accumulation of

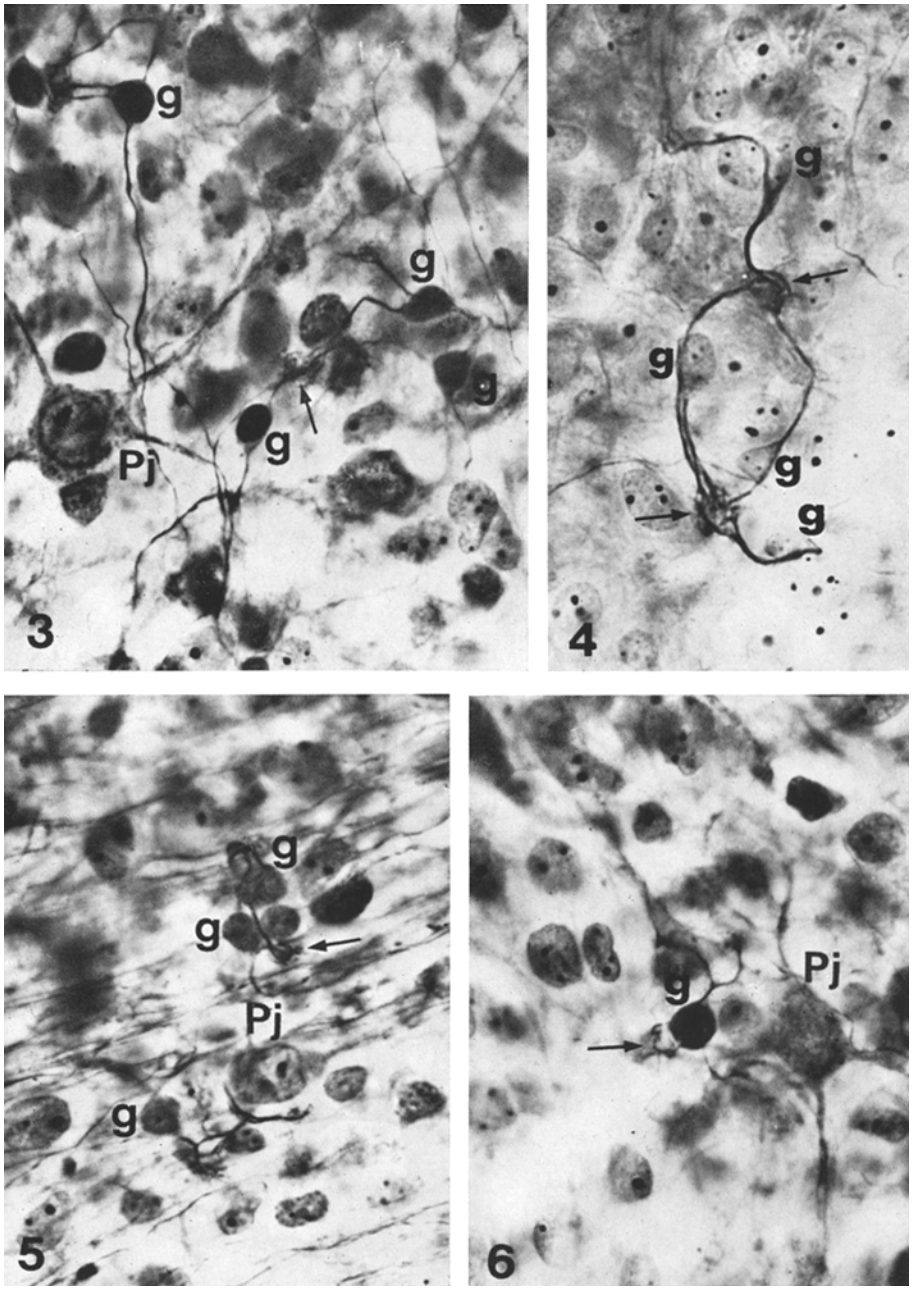
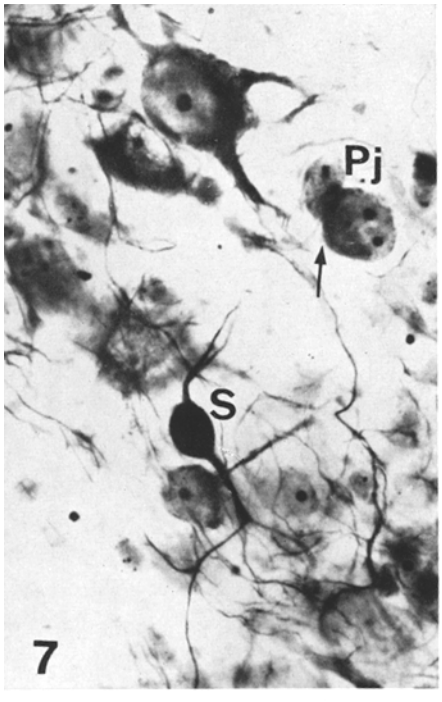
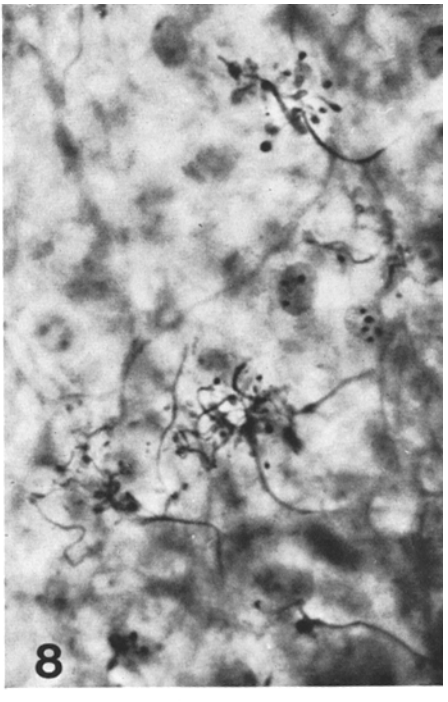


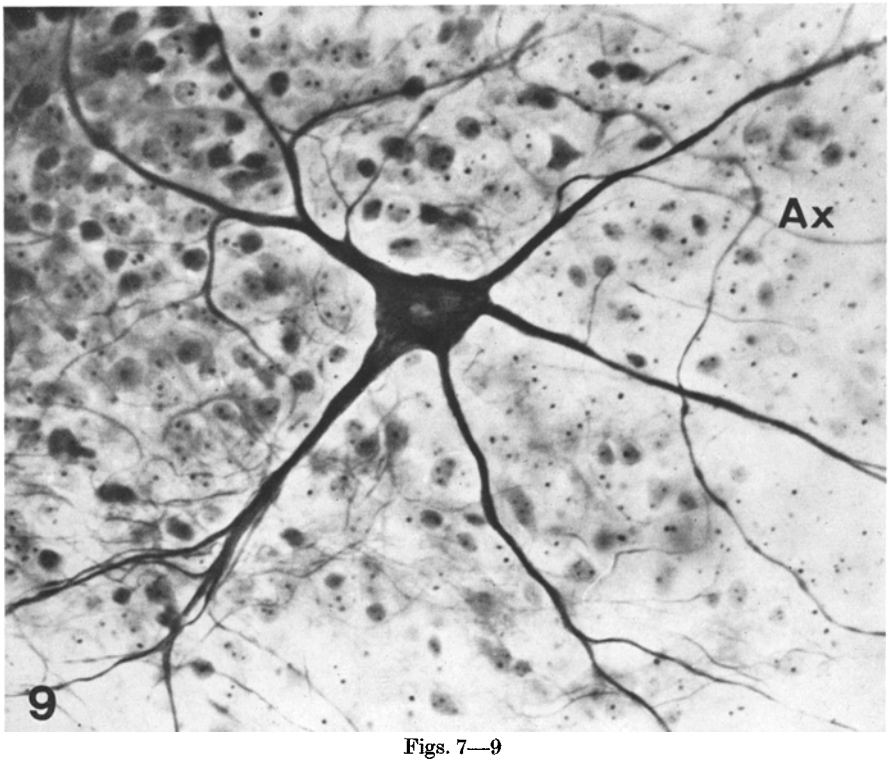
Fig. 3. A group of granule cells (*g*). There is a close contact of granule cell processes (arrow). Note a Purkinje cell (*Pj*) nearby. 32-day-old culture. ×600

Fig. 4. Two contacts of granule cell (*g*) processes are indicated by arrows. Each contact is made of three granule cell processes. 18-day-old culture. ×600

Figs. 5 and 6. Granule cells (*g*) with claw-like processes (arrows) morphologically typical of Golgi preparations. Note Purkinje cells (*Pj*) nearby. 24-day-old culture. ×600







Figs. 7—9

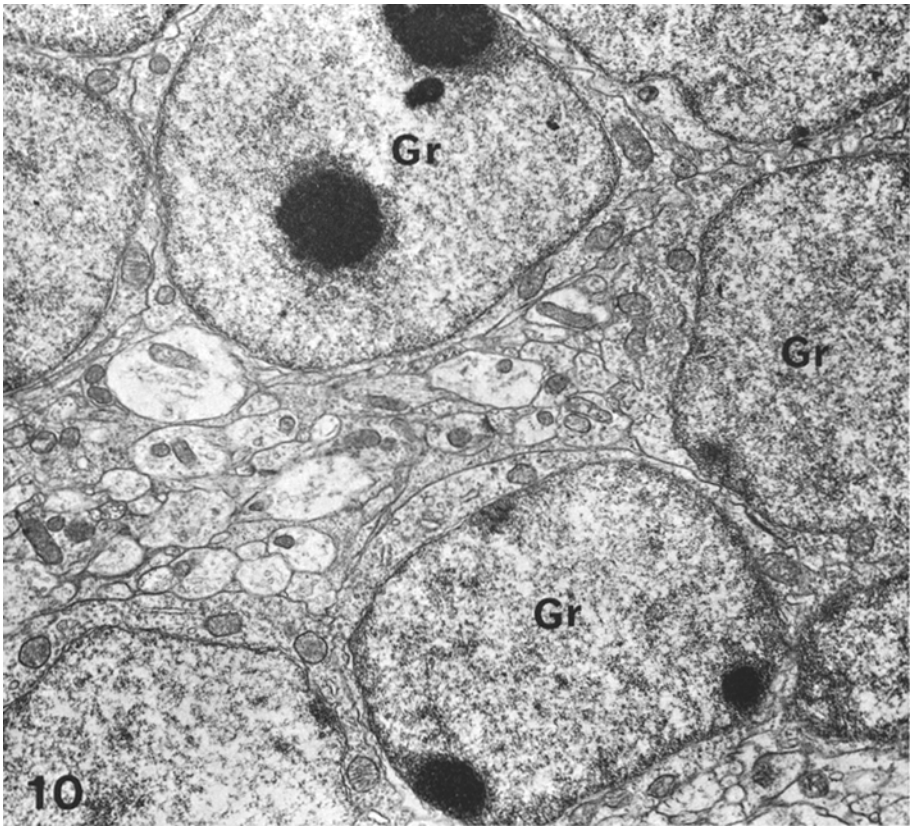


Fig. 10. A group of granule cells (*Gr*). Mitochondria, ribosomes and tubular as well as vesicular profiles of the endoplasmic reticulum are visible in their cytoplasm. 20-day-old culture. ×9,000

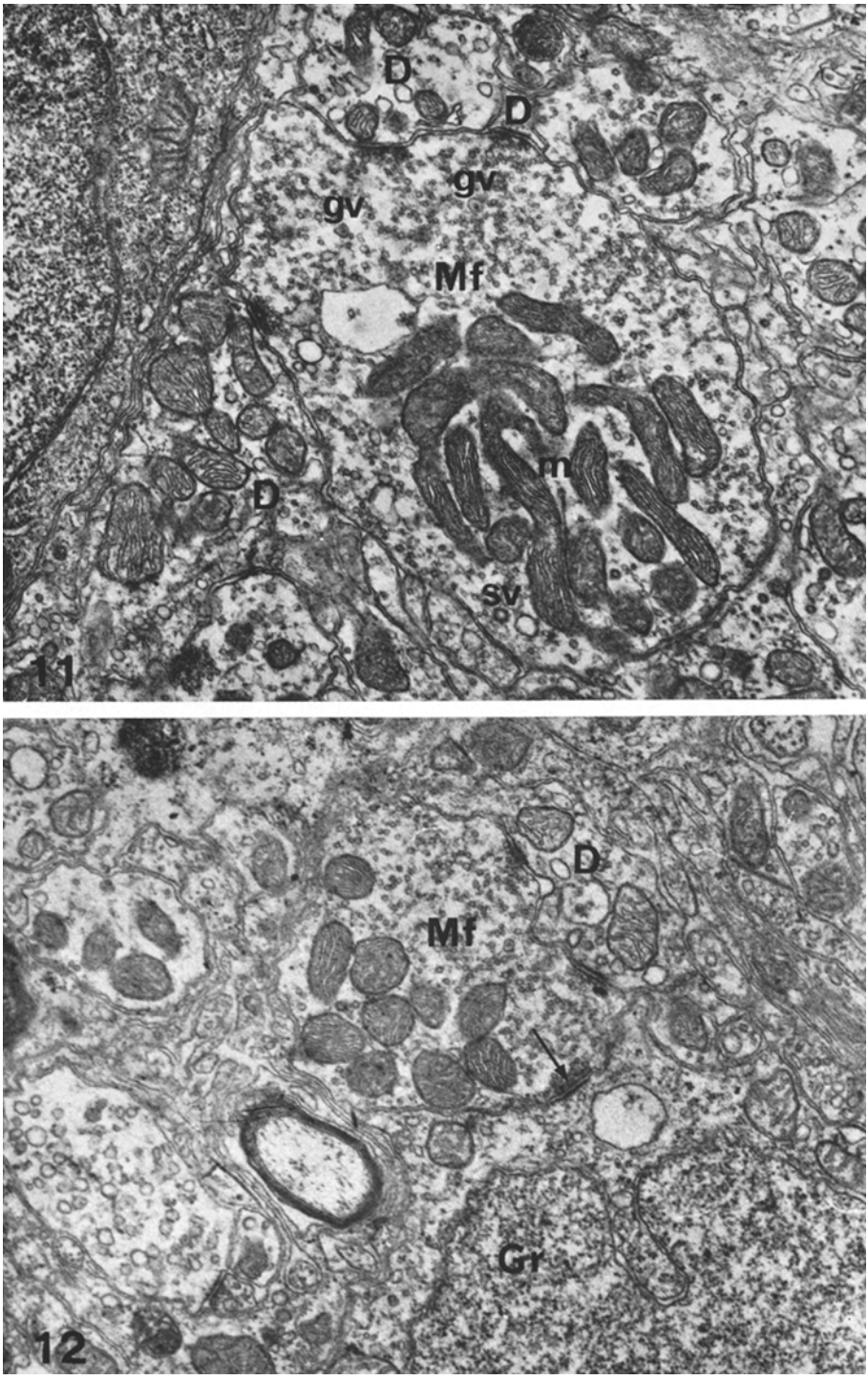
granules against nuclear membrane may be seen. A typical electron-dense nucleolus is visible, occasionally two, in a nucleus (Fig. 10). Nuclear indentations are rare and this is in contrast with in vivo observation of adult rat cerebellum by Herndon (1964) who reported frequent indentation of granule cell nuclei.

The granule cells possess thin rims of cytoplasm which vary from 300 Å to 0.5 μ (Gray, 1961; Fox *et al.*, 1967). The cytoplasm contains several mitochondria, a Golgi apparatus, membrane profiles of smooth surfaced and rough surfaced endoplasmic reticulum and free ribosomes scattered throughout the cytoplasm

Fig. 7. An axonal swelling (*s*) originating from a Purkinje cell (*Pj*). The origin of the axon is indicated by the arrow. 21-day-old culture. ×600

Fig. 8. Grape-like terminal endings which resemble mossy fiber endings of cerebellar glomeruli. 124-day-old culture. ×600

Fig. 9. A large multipolar neuron from vestibular (Deiter's) nucleus. 30-day-old culture. *Ax* Axon. ×600



Figs. 11 and 12

(Fig. 10). In addition, the cytoplasm presents an occasional dense body and multivesicular body. As described by Gray (1961), the cell bodies lie in groups and are in close contact with each other with two thin surface membranes apposed with 200 Å distance (Fig. 10).

In contrast to the observations of Gray (1961) and Herndon (1964), axosomatic synapses with mossy fiber endings can be seen (Fig. 12). A similar observation was made in late chick embryo cerebellum by Mugnaini and Forstrønen (1967).

Typical structures of cerebellar glomeruli consisting of a mossy fiber ending and granule cell dendrites were frequently encountered. Mossy fiber endings are large presynaptic bags containing numerous synaptic vesicles (300—500 Å), mitochondria, and membrane profiles of endoplasmic reticulum (Fig. 11). In addition to synaptic vesicles and mitochondria they often contain microtubules, granular dense-core vesicles and spiked (complex) vesicles (Gray, 1961; Fox *et al.*, 1967). Membrane thickenings are present at the contacts.

Dendro-dendritic attachment placques of granule cells (Gray, 1961; Fox *et al.*, 1967; Eccles *et al.*, 1967) were also seen occasionally.

Another important feature of granule cells is pericellular myelin formation (Fig. 13a) (Kim, Hirano and Murray, 1969).

These structures are seen as early as 8 days in vitro under a bright field microscope. The number of myelinated somata increases day by day and by the end of the third week in vitro all cultures eventually have such structures. In electron micrographs, it is evident that the lamellae of myelin sheaths enveloping granule cell bodies are in all respects indistinguishable from those observed in the central nervous system fixed in vivo (Fig. 13b).

Discussion

Previous authors have attempted unsuccessfully to identify granule cells in vitro either in living (Pomerat and Costero, 1956; Mizuno, Kim and Okamoto, 1962) or in silver impregnated cultures (Wolf, 1964; Hild, 1966). A possible exception might be a work of Suyeoka and Okamoto (1966) who demonstrated small neurons in mouse cerebellum cultures, and the descriptions of these cells correspond well with our observations on granule cells. The reason suggested by Hild (1966), in an effort to explain the scarcity of granule cells in his cultures, was massive degeneration partly because of their deafferentation. A similar view was expressed by Piper (1962) for cerebellum cultures. The suggestion that granule cells undergo a massive degeneration in culture is now in question, since there are, in our cultures, constant and numerous small neurons with all characteristic features of granule cells.

Fig. 11. A mossy fiber ending (*Mf*) forming synaptic contacts with granule cell dendrites (*D*). The ending contains numerous mitochondria (*m*) and synaptic vesicles. It also contains granular dense-core vesicles (*gv*) and a spiked vesicle (*sv*). 30-day-old culture. ×21,000

Fig. 12. A mossy fiber ending (*Mf*) in synaptic contact (at arrow) with a granule cell perikaryon (*Gr*), and with a dendrite (*D*). 30-day-old culture. ×19,000

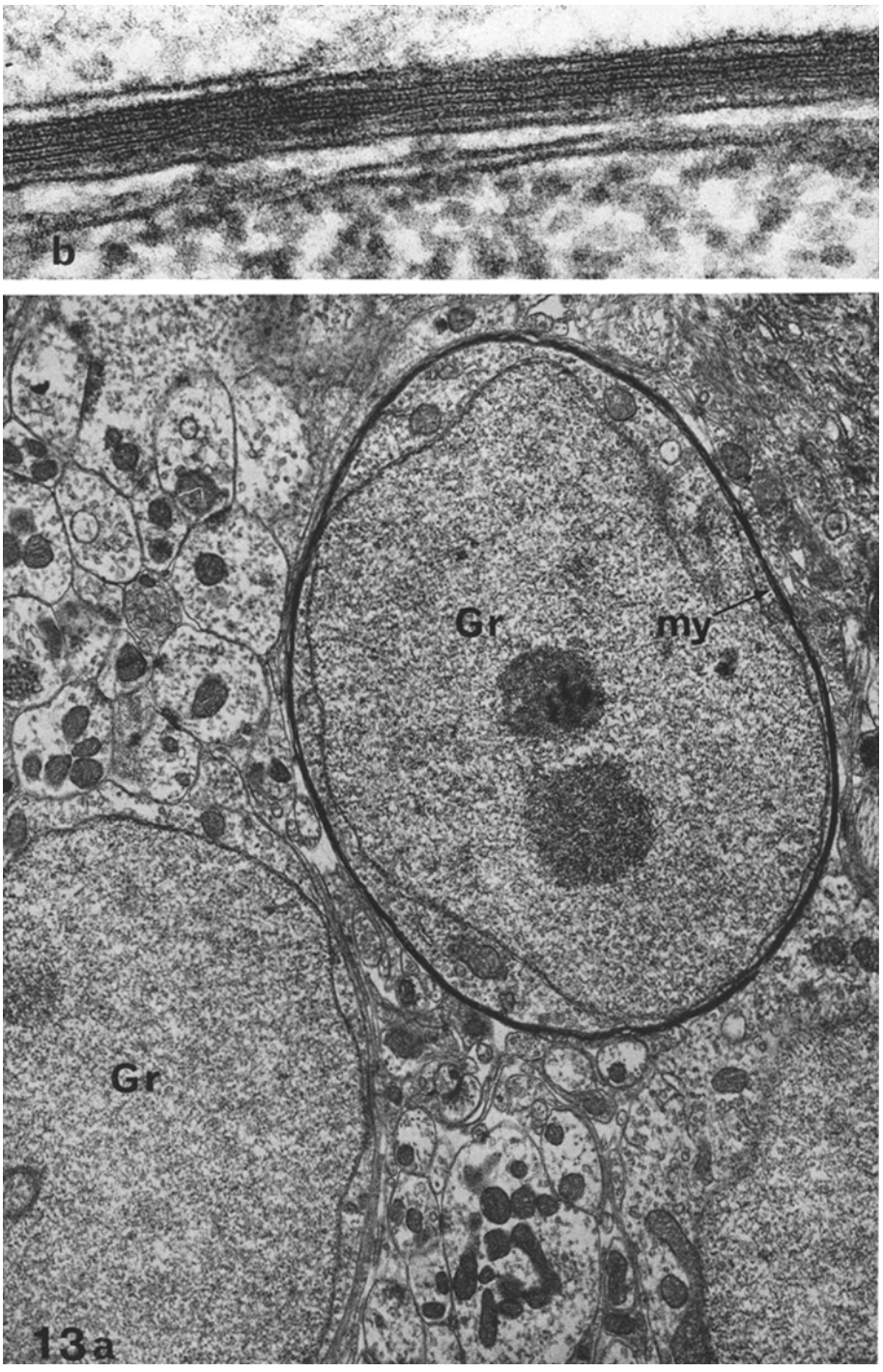


Fig. 13a. A granule cell (*Gr*) surrounded completely by myelin sheath (*my*). 20-day-old culture. ×10,000. b. Higher magnification of a part of the myelin sheath surrounding granule cell soma in Fig. 13a. Note the characteristic lamellar structure of myelin sheath. 20-day-old culture. ×192,000

The granule cells identified by Wolf (1964) are apparently axonal swellings of the Purkinje cells, since we have observed a massive occurrence of similar configurations which are parts of Purkinje cell axons (Fig. 7). Suyeoka and Okamoto (1966) have similarly questioned the validity of Wolf's findings. Ramon y Cajal (1959) has also shown such axonal swellings of Purkinje cells in his nerve regeneration study. These structures in vitro are, therefore, likely to be produced in the course of regeneration after damage occurring at the time of explantation.

It is clear from the present observations made by electron microscopy that virtually all ultrastructural characteristics of granule cells in vivo (Gray, 1961; Fox *et al.*, 1967; Eccles *et al.*, 1967; and Mugnaini and Forstrønen, 1967) could be demonstrated in mouse cerebellum cultures, including synaptic contacts between granule cell dendrites and mossy fiber endings.

It has been reported that synaptic connections occur in tissue cultures of central nervous system (Silver study: Kim, 1963, 1965; Wolf, 1964; Peterson *et al.*, 1965; Hild, 1966; Electron microscopy: Callas and Hild, 1964; Bunge *et al.*, 1965; Electrophysiology: Crain and Peterson, 1964; Crain, 1966) and that they can be newly formed in vitro (Bunge *et al.*, 1967; Stefanelli *et al.*, 1967). However, no observation has been made concerning the occurrence of cerebellar glomeruli in tissue culture.

The mossy fibers, which constitute most afferent fibers to the cerebellar cortex, are severed from their parent cells at the time of explantation and might be expected to degenerate. It was therefore surprising to see an intact glomerulus present in some of the cultures. As for a possible interpretation for the presence of these peculiar structures, it is reasonable to assume that some mossy fibers were left intact at the time of explantation and differentiated further under culture conditions, possibly from the vestibular (Deiter's) nucleus. This nucleus which is known to be one of the origins of the mossy fibers (Snider, 1936; Jansen and Brodal, 1954; Eccles *et al.*, 1967) is frequently explanted with cerebellum in culture because of its close anatomical relationship to the cerebellum.

It is not unusual to see large, stellate cells which may be defined as Deiter's neurons in cerebellum cultures (Fig. 9).

Myelinated neuronal somas in vitro have previously been reported by Hild (1963) and Suyeoka (1968) in cultures of mammalian midbrain and cerebellum. Recently Field *et al.* (1968) have described the fine structure of myelinated cells in rat cerebellum cultures, but they were not sure about the identification of the cells in question.

Myelinated granule cells have been found in a wide variety of mammals including man and are regarded as normal rather than pathological structures (Suyeoka, 1968).

We have extended these findings in observing that the cells ensheathed by myelin are actually granule cells and that their ultrastructure is identical with that in central nervous system fixed in vivo. It is reasonable to assume that the myelinated somata are much more widespread structures than might be thought from the scarce observations reported up to now. In fact, besides the cerebellum, we have observed them in neurons of cerebrum, olfactory bulb, brain stem, and spinal cord cultured in vitro.

References

Allerand, C. D., Murray, M. R.: Myelin formation in vitro. Endogenous influences on cultures of newborn mouse cerebellum. Arch. Neurol. (Chic.) **19**, 191—201 (1968).

Bornstein, M. B., Appel, S. H.: The application of tissue culture to the study of experimental allergic encephalomyelitis. 1. Patterns of demyelination. J. Neuropath. exp. Neurol. **20**, 141—157 (1962).

— — Tissue culture studies of demyelination. Ann. N.Y. Acad. Sci. **122**, 280—286 (1965).

— Murray, M. R.: Serial observations on patterns of growth, myelin formation, maintenance and degeneration in cultures of newborn rat and kitten cerebellum. J. biophys. biochem. Cytol. **5**, 499—504 (1958).

Bunge, M. B., Bunge, R. P., Peterson, E. R.: The onset of synapses formation in spinal cord cultures as studied by electron microscopy. Brain Res. **6**, 728—749 (1967).

Bunge, R. P., Bunge, M. B., Peterson, E. R.: An electron microscope study of cultured rat spinal cord. J. Cell Biol. **24**, 163—191 (1965).

Callas, G., Hild, W.: Electron microscopic observations of synaptic endings in cultures of mammalian central nervous tissue. Z. Zellforsch. **63**, 686—691 (1964).

Crain, S. M.: Development of 'organotypic' bioelectric activities in central nervous tissue during maturation in culture. In: Int. Rev. Neurobiol., vol. 9, p. 1—43, ed. by C. C. Pfeiffer and J. R. Smythies. New York: Academic Press 1966.

— Peterson, E. R.: Complex bioelectric activity in organized tissue cultures of spinal cord (human, rat and chicken). J. Cell comp. Physiol. **64**, 1—13 (1964).

Eccles, J. C., Ito, M., Szentágothai, J.: The cerebellum as a neuronal machine. Berlin-Heidelberg-New York: Springer 1967.

Field, E. J., Hughes, D., Raine, C. S.: Observations of the development of myelin in cultures of neonatal rat cerebellum. J. Neurol. Sci. 8, 49—60 (1968).

Fox, C. A., Hillman, D. E., Siegesmund, K. A., Dutta, C. R.: The primate cerebellar cortex: A Golgi and electron microscopic study. In: Progress in brain research, vol. 25, p. 174—225, ed. by C. A. Fox and R. S. Snider. Amsterdam: Elsevier 1967.

Gray, E. G.: The granule cells, mossy synapses and Purkinje spine synapses of the cerebellum: light and electron microscope observations. J. Anat. (Lond.) **95**, 345—356 (1961).

Herndon, R. M.: The fine structure of the rat cerebellum. II. The stellate neurons, granule cells, and glia. J. Cell Biol. **23**, 277—293 (1964).

Hild, W.: Myelinogenesis in cultures of mammalian central nervous tissue. Z. Zellforsch. **46**, 71—95 (1957).

— Myelin formation around central neurons in vitro. Tex. Rep. Biol. Med. **21**, 207—213 (1963).

— Cell types and neuronal connections in cultures of mammalian central nervous tissue. Z. Zellforsch. **69**, 155—188 (1966).

— Tasaki, I.: Morphological and physiological properties of neurons and glial cells in tissue culture. J. Neurophysiol. **25**, 277—304 (1962).

Hoskin, F. C. G., Allerand, C. D.: Metabolism of specifically labelled glucose by explants of newborn mouse cerebellum. J. Neurochem. **15**, 427—432 (1968).

Jansen, J., Brodal, A.: Aspects of cerebellar anatomy. Oslo: J. Grundt Tanum Forlag 1954.

Kim, S. U.: Neurons in the tissue culture. Arch. Histol. Jap. **23**, 401—429 (1963).

— Neurons in the tissue culture. Observations on terminal boutons in cultures of mammalian central nervous tissue. Arch. Histol. Jap. **25**, 371—381 (1965).

— Histochemical demonstration of oxidative enzymes associated with carbohydrate metabolism in cerebellar neurons cultured in vitro. Arch. Histol. Jap. **27**, 465—471 (1966).

— Response of neuronal lysosomes to anoxia in tissue culture of mammalian cerebellum. Experientia (Basel) **25**, 72—73 (1969).

— Cytochemical demonstration of 'marker' enzymes in nerve cells cultured in vitro. Experientia (Basel) **26**, 292—293 (1970).

— Hirano, A., Murray, M. R.: Neurons with myelinated soma in organized cultures of mouse cerebellum. J. Cell Biol. **43**, 68a (1969).

— Murray, M. R., Tourtellotte, W. W., Parker, J. A.: Demonstration in tissue culture of myelinotoxicity in cerebrospinal fluid and brain extracts from multiple sclerosis patients. J. Neuropath. exp. Neurol. (in press).

Lumsden, C. E.: The clinical neuropathology of multiple sclerosis. In: Multiple sclerosis. A reappraisal, p. 243—393, by D. McAlpine, C. E. Lumsden and E. D. Acheson. Baltimore: Williams & Wilkins 1965.

— Nervous tissue in culture. In: The structure and function of nervous tissue, vol. 1, p. 68—141, ed. by G. H. Bourne. New York: Academic Press 1968.

Mizuno, N., Kim, S. U., Okamoto, M.: Study on embryonal granule cells of the cerebellum. Identification in the tissue culture. Arch. Histol. Jap. **23**, 185—211 (1962).

Mugnaini, E., Forstrønen, F.: Ultrastructural studies on the cerebellar histogenesis. I. Differentiation of granule cells and development of glomeruli in the chick embryo. Z. Zellforsch. **77**, 115—143 (1967).

Murray, M. R.: Nervous tissues in vitro. In: Cells and tissues in culture. Method, biology and physiology, vol. 2, p. 373—455, ed. by E. N. Willmer. New York: Academic Press 1965.

Peterson, E. R., Crain, S. M., Murray, M. R.: Differentiation and prolonged maintenance of bioelectrically active spinal cord cultures (rat, chick and human). Z. Zellforsch. **66**, 130—154 (1965).

Piper, R.: Microtomy techniques and the problem of relation of cell outgrowth to events within explants in vitro. J. Med. Lab. Technol. **19**, 1—18 (1962).

Pomerat, C. M., Costero, I.: Tissue culture of cat cerebellum. Amer. J. Anat. **99**, 211—247 (1956).

Ramon y Cajal, S.: Histologie du système nerveux de l'homme et des vertébrés Tome 2. Translated by L. Azoulay. Paris: Maloine 1911.

— Degeneration and regeneration of the nervous system. Translated by R. M. May. New York: Hafner 1959.

Ross, L. L., Bornstein, M. B., Lehrer, G. M.: Electron microscopic observations of rat and mouse cerebellum in tissue culture. J. Cell Biol. **14**, 19—30 (1962).

Silberberg, D. H., Schutta, H. S.: The effect of unconjugated bilirubin and related pigments on cultures of rat cerebellum. J. Neuropath. exp. Neurol. **26**, 572—585 (1967).

Snider, R. S.: Alterations which occur in mossy terminals of the cerebellum following transsection of brachium pontis. J. comp. Neurol. **64**, 417—435 (1936).

Stefanelli, A., Zacchei, A. M., Caravita, S., Cataldi, A., Ieradi, L. A.: New-forming retinal synapses in vitro. Experientia (Basel) **23**, 199—200 (1967).

Suyeoka, O.: Small myelinated perikarya in the cerebellar granular layer of mammals including man. Experientia (Basel) **24**, 472—473 (1968).

— Okamoto, M.: Granule cells of the mouse cerebellum cultured in vitro: Their identification, degeneration and perikaryal myelin. Arch. Histol. Jap. **27**, 117—130 (1966).

Wolf, M. K.: Differentiation of neuronal types and synapses in myelinating cultures of mouse cerebellum. J. Cell Biol. **22**, 259—279 (1964).

Yonezawa, T., Bornstein, M. B., Peterson, E. R., Murray, M. R.: A histochemical study of oxidative enzymes in myelinating cultures of central and peripheral nervous tissue. J. Neuropathol. exp. Neurol. **21**, 479—487 (1962).

Seung U. Kim, M.D.
Department of Anatomy
College of Medicine
University of Saskatchewan
Saskatoon, Canada