Granule Cells of the Mouse Cerebellum Cultured in Vitro: Their Identification, Degeneration and Perikaryal Myelin

Osamu Suyeoka and Michio Okamoto

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Studies on the identification of cultured cells are painstaking, but by far important. Without such studies no valid conclusions could be drawn from any experiment made on cells in question. The differentiation of granule cells from oligodendrocytes has an important bearing on studies of myelin sheaths in vitro, because the latter have been considered to be possible myelin-supporting cells and show extraordinary similarities to the former in the living state.

Since the first description by Pomerat and Costero (1956) in living and stained preparations, several authors described cerebellar granule cells in culture and labeled them as such from the morphology of their nuclei (Mizuno et al. 1962), from their characteristic processes in stained cultures (Wolf 1964), or from their size (Hild 1966). Some discrepancies exist among cells identified by these workers as granule cells, but, curiously, few comments have been made on the differences of their interpretations.

Tissue culture is intended to deal with living tissues at cellular level and follow behaviors thereof by the serial observation of the very same cell. And it is this point, despite of some limitations, that makes the tissue culture technique so unique one among others which work on fixed tissues. It should be emphasized therefore that identification of cultured cells in stained preparations is of limited value.

This paper is primarily concerned with the identification of living granule cells, referring to the silver-impregnated cultures.

Materials and Methods

Albino mice of ICR strain, newborn to twenty days old, were used as materials. After decapitation, cerebellar cortices were cut sharply into fragments, about 1.5 mm in diameter. Following brief washing in Hanks’ balanced salt solution (BSS), a piece of these fragments was explanted onto each coverslip, 12×22 mm, coated with reconstituted rat-tail collagen* (Ehrmann and Gey 1956, Bornstein 1958). One coverslip was inserted into each roller-tube and 1 ml of fluid medium was added. Fluid medium was composed of 50% modified Hanks’ BSS (NaCl 720 mg%, glucose 600 mg%, sodium pyruvate 1 mg%)**, 45% inactivated calf serum and 5% embryo extract (from 9-day-old chicks). Hydrogen ion concentration was adjusted to 7.2 with isotonic 1.4% sodium bicarbonate solution. Incubation temperature was about 36°C and nutrient fluids were exchanged once weekly. Cultures were examined from

* This paper is dedicated to the memory of the late Prof. Masaji Seki, the founder of the Archivum histologicum japonicum.
time to time and photographed by a phase-contrast optics. Following observations in the living state, cultures were fixed and impregnated by Bodian’s method. Incubation time in protargol solution was considerably shortened to show fine processes of small cells.

For serial observation, some cultures were transferred into Maximow’s double-coverslip assemblies after three weeks in vitro. In these cases, culture procedures were similar to the method of BORNSTEIN and MURRAY (1958), with minor modifications.

Following results are based on the observations of more than 1000 cultures.

* In the case of roller-tube method, combined use of ‘classical’ plasma clot and collagen film greatly facilitates the culture handling, though not absolutely necessary; otherwise, cumbersome care should be taken to prevent drying or detaching of explanted tissues from the coverslips during the initial stage of cultivation (see, BORNSTEIN 1958). Plasma clots somewhat stimulate migration of mesenchymal elements, but essentially the same growth pattern is obtained either with or without plasma clot. Doubtlessly, the use of collagen is indispensable for better and longer maintenance of the cultures.

** Sodium pyruvate (1 mg%) was introduced into the BSS to stimulate the cellular migration, and thus flattening of the explanted tissues from older animals (SUYEOKA 1966).

**Observations**

1. Living cultures

   In the mouse cerebellum, more organized growth pattern is noted under the culture condition, and laminar organization with pallisade-like rows of large neurons is frequently retained. In healthy and flattened explants, thousands of small cells with scanty cytoplasm are seen to be closely packed in the vicinity of a group of large neurons. Diameter of their cell bodies measures 6—8 μ. Their nuclei are round or slightly oval, 5—7 μ in diameter, and several dense chromatin-like granules are seen scattered in a relatively dense nucleoplasm (Fig. 1).

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Fig. 1. Clusters of small cells. Note their scanty cytoplasm. A elongated nucleus in the center of the picture might be of microglial cell as judged from the nuclear morphology and its darker cytoplasm. 7-day-old mouse cerebellar cortex. 48 days in vitro (DIV). Living. Bar indicates 10 μ.
From their nuclear morphology, they are reminiscent of granule cells in the cerebellar granular layer. In the living state, however, it can be hardly determined whether they are granule cells, cells in the embryonal granular layer or oligodendrocytes, because their processes are not recognized in general and nuclei of these cell types closely resemble each other. In some parts of explants, where a group of these small cells are isolated from surrounding cells, basal portions of their processes are discerned to radiate from more or less rounded perikarya, but in no cases their fine processes can be traced over their whole length (Fig. 2).

They are seen in cultures from mice of all ages examined, including those from 20-day-old animals in which the embryonal granular layer has already disappeared from the surface of the cerebellar cortex.

In the earlier stage of cultivation, similar small cells with one or two fine processes migrate out into the framework of neuroglial cells, which encircle the flattening explant. However, they gradually fade away from the outgrowth zone as the culture ages (Fig. 7).

2. Silver-impregnated cultures

As described repeatedly (Pomerat and Costero 1956, Kim 1963, Wolf 1964, Kim 1965, Hild 1966), large neurons are clearly demonstrated by silver impregnation of cultures with their axons, axon collaterals and boutons terminaux. On the other hand, impregnation of these small cells is quite capricious; in most cases only their nuclei are well shown.

In some cultures, however, fine fibers are noted near a group of these small cells (Fig. 3). They intertwine one another, oriented irregularly or in bundles, among thick and intensely stained fibers of large neurons. Although it is difficult to trace them back to their parent cells, it is highly probable that these fine fibers are derived from the small cells. They are much easier to analyze in cultures from older mice, because in the cultures from newborn animals...
axon collaterals of large neurons are more numerous and as fine as these fibers.

In the well-organized portion of silver-impregnated cultures, rows of large neurons send out their axons to the one side among clusters of the small cells in question, while their branching apical dendrites, often overshadowed by amorphously stained masses, extend to the other side. In such occasions, these fine fibers pass among the perikarya of large neurons and turn their course to become bundles of fine fibers in the amorphous masses, parallel to the row of large neurons. They lie in a slightly different focal plane to the dendrites of large neurons and there are recognized no bouton-like structures between them (Fig. 4). Such a situation strongly sug-

Fig. 4. A more organized portion of a impregnated culture. Compare fine fibers with a thick and intensely-stained axon from a large neuron (N). Dendritic processes of the neuron are not discerned due to the difference of the focal plane. 7-day-old mouse cerebellar cortex. 27 DIV. Bodian. Bar indicates 30μ.

Fig. 5. A group of small cells with impregnated processes. 7-day-old mouse cerebellar cortex. 41 DIV. Bodian. Bar indicates 30μ.
suggests that these small cells near a group of large neurons are granule cells. These descriptions, however, never represent general situation of cultures, as there is a considerable variation from preparation to preparation and from portion to portion even in the same explant. Disorganized cultures are not infrequent, where large neurons are arranged in a more random or dispersed manner.

When somewhat isolated, processes of these cells become amenable to analysis (Fig. 5). They can be classified into following main types, though many intermediate or variant cells are seen.

Type I (Fig. 6, 7): Unipolar cells with round or oval nuclei. Their processes are short and relatively thick, but branching is rare. Sometimes another fine and short process is noted to extend from the opposite pole.

Type II (Fig. 8): Round or oval bipolar cells with bifurcating processes. One of the process is relatively thick and short, and bifurcates in a short distance from the cell body. Another non-branching process is fine and longer, and arises from the opposite pole.

Type III (Fig. 9): Bipolar cells with fine and long processes. Their nuclei and cell bodies are somewhat elongated. Branching of the process is rare.

Type IV (Fig. 10): Bipolar fusiform cells. One of the processes is short and thick, while another long and fine process bifurcates in a T- or Y-shape.

Type V (Fig. 11, 12): Round cells with long and fine bifurcating processes. They have several short and thick processes which end in a claw-like formation. In many occasions, however, they lack such short processes except for a single and long bifurcating process.

Type I and II are more frequently encountered in younger cultures from younger mice, while in older cultures from older animals cells of type IV and V prevail. A wide variety of these cells, including type I and II, is still recognizable in cultures from newborn mice even after 30 days' cultivation. On the other hand, when animals, older than 10 days, have been cultivated, practically all of type I and II cells disappear and type V cells predominate in population after 20 days in vitro. In these cultures a few type III or IV cells are still noted.

Besides large neurons and the preceding small cells, there are sometimes seen neurons of medium size, 9—11 µ in diameter (Fig. 13). Their thin axons are much shorter than those of large neurons and lack any apparent connections with other cell types. Although it is im-

Fig. 6. Type I cells in an impregnated culture. Newborn mouse cerebellum 28 DIV. Bodian

Fig. 7. A living type I cell which migrated out into the outgrowth zone. This cell has another fine and short process (arrow). 12-day-old mouse cerebellar cortex. 8 DIV. Bar indicates 10 µ
Fig. 8. A type II cell. Newborn mouse cerebellum. 20 DIV. Bodian.

Fig. 9. A type III cell. Newborn mouse cerebellum. 20 DIV. Bodian.

Fig. 10. A type IV cell. Its long bifurcating process is somewhat out of focus (arrow). Newborn mouse cerebellum. 19 DIV. Bodian. Bar indicates 20 μ

Fig. 11. A type V cell. Three thick and short processes radiate from the cell body, while a long process bifurcates in some distance from the soma. Newborn mouse cerebellum. 19 DIV. Bodian. Bar indicates 20 μ

Fig. 12. A type V cell. Note a characteristic digitiform process. A long bifurcating process (not fully illustrated) extend from the opposite pole. 7-day-old mouse cerebellar cortex. 23 DIV. Bodian. Bar indicates 10 μ

Fig. 13. A medium-sized neuron. They are slightly larger than the small cells and several longer dendritic processes radiate. Arrow indicates the axon. Newborn mouse cerebellum. 19 DIV. Bodian. Bar indicates 20 μ
possible to afford convincing evidence, they are tentatively identified from their size as stellate neurons of the molecular layer (basket or superficial stellate cells). So far, repeated attempts

Fig. 14. A localized swelling of the axon from a large neuron (N). These structures simulate granule cells in Golgi preparations. 10-day-old mouse cerebellar cortex. 16 DIV. Bodian. Bar indicates 50 μ

Fig. 15. Higher magnification of Fig. 14. The axonal swelling is connected with its parent cell (N) by a thin fiber. Bar indicates 30 μ
were futile to correlate possible cells in the living state with these impregnated medium-sized neurons.

Another relevant finding on the silver-impregnated cultures is the occurrence of circumscribed axonal swellings of large neurons (Fig. 14, 15). They are usually single in number, round, oval or fusiform, and measure 6—10 µ in diameter. Distance from the parent cell is relatively constant, 40—50 µ. They are more numerous in 'unhealthy' explants as well as in cultures from older mice, especially older than 12 days. Distal portion of these axons often bifurcates at some distance from the swelling. Hence, at lower magnification, some of these processes show striking resemblance to granule cells as revealed by Golgi method.

3. Degeneration of the small cells

These small cells appear to be highly vulnerable to certain unfavorable conditions. They are completely absent in some cultures, although large neurons and myelinated axons are maintained in a healthy state. In others, though extremely rare, many swollen cells with pyknotic nucleus and clear cytoplasm are recognized among intact neurons and myelinated axons (Fig. 16). These degenerating cells are soon necrosed and eventually replaced by the immigrating mesenchymal or glial cells in the following days, so that cultures resume apparently healthy appearance. When isolated from other cellular elements, they tend to round up and finally succumb to degeneration.

Although factors, which cause the necrosis of these small cells, have not yet been fully elucidated, some such factors should be pointed out from the technical standpoint. In the earlier stage of this study, two fragments, instead of one, have been explanted onto each coverslip. In the former case nutrient fluids become acidic after one week's incubation, as
judged from yellowish color of a pH indicator in the medium (0.001% phenol red), and these small cells are actually fewer in number. On the other hand, when only one explant is cultured per coverslip, color change of the medium is minimal even after one week.

Raised glucose concentration (600 mg%) makes the medium hypertonic. In order to adjust the tonicity, concentration of sodium chloride in the Hanks' BSS (800 mg%) was reduced to 720 mg% (Suyeoka 1966), which appears also to be favorable for better survival of these cells.

4. Small myelinated perikarya

Among these small cells, myelinated perikarya of similar size are recognized not infrequently. Some of them exist in an isolated position (Fig. 17) or in a group of two to six or more. More commonly, however, they are closely apposed to myelinated axons (Fig. 18). In the most favorable cases they become discernible as early as 10 days after initiation of the culture. Their occurrence is more frequent in a more 'healthy' explant in which large neurons and their myelinated axons are maintained in a healthy state. Furthermore, they remain intact in many aged cultures where once myelinated axons have become denuded.

Discussion

1. Identification of living granule cells

Although mouse cerebellar cultures retain a more organized structure in vitro, cytoarchitectonic arrangements of brain tissues are disorganized to a greater or lesser extent under the culture condition, which makes identification of neurons rather difficult. In order to identify neurons in culture definitely, a possible cell should be correlated with the same cell stained for Nissl substances or axons. In the case of smaller neurons, like cerebellar granule cells, this is almost impossible to perform, because these small neurons are devoid of recognizable Nissl substances and silver impregnation of their axons does not give consistent results. Hence other more indirect approach would be inevitable.

Granule cells are the most nume-
rous neurons in the cerebellar cortex and bear morphological similarities to oligodendrocytes except for their processes. In the cerebellar cultures from immature animals, another cell type of similar morphology should be differentiated, that is, cells in the embryonal granular layer.

According to Piper (1962), all the granule cells of all types degenerate under the culture condition. Hild (1966) also suggested that they undergo degeneration in vitro partly because of deafferentiation. From our observations, however, these interpretations do not seem justified, because there can be seen thousands of small cells with nuclei which are reminiscent of granule cells or oligodendrocytes as seen in the Nissl-stained cerebellum (Fig. 1). They are so numerous that all of them could not be oligodendrocytes. In addition, silver impregnation of the cultures depicts cells with processes, characteristic of the granule cells (Fig. 11, 12).

Differentiation from cells in the embryonal granular layer: In newborn mice, the embryonal granular layer covers the entire surface of the cerebellum. This layer increases in thickness during early postnatal days, then becomes thinner, and finally disappears around 19 days. During these stages, the internal granular layer gradually becomes thicker and attains to the adult level after 2 weeks (Suyeoka and Konishi, unpublished data).

The nuclei of small cells in these two layers are so alike that it is hardly possible to distinguish these two cell types in the living state, because in general their processes are not recognized. The stratified organization, sometimes retained in vitro, is not sufficient to adopt as general criteria, since it is not a regular finding.

Processes of these small cells are well impregnated in some occasions by Bodian's protargol method (Fig. 5). From the morphology of their processes, they are classified into several types, which may be correlated with the cells described by Cajal (1911) in the developing cerebellar cortex. Type I (Fig. 6, 7) may correspond to cells in the superficial layer of the embryonal granular layer, type II (Fig. 8) to cells in the deeper portion of the same layer, type III (Fig. 9) to 'cellules bipolaire horizontale' of the molecular layer, type IV (Fig. 10) to 'cellules bipolaire verticale' of the molecular layer, and type V (Fig. 11, 12) to granule cells, mature or immature, in the internal granular layer.

When newborn mice cerebella are cultivated, the cells in the embryonal granular layer are still observable in the silver-impregnated cultures even after one month in vitro. In the meanwhile, few such cells are encountered in cultures from animals, older than 10 days, after 3 weeks' cultivation, and most small cells with impregnated processes can be identified as immature or mature granule cells. Hence it is concluded that almost all small cells in question can be considered as granule cell neurons even if 10 days or more old mice are used as culture materials, and that cells in the embryonal granular layer, at least some of them, remain undifferentiated under the culture condition.

Migrating small cells in younger cultures are mostly cells in the embryonal granular layer, as identified by Mizuno et al. (1962) and confirmed also in this study (Fig. 7). As the cultures age, they either die away or transform into other cell types.

Differentiation from oligodendrocytes: It is well known that nuclei of oligodendrocytes are akin to those of granule cells. Since cultured oligodendrocytes and granule cells generally appear just like those in the Nissl-stained preparations (Fig. 1), individual cells cannot be identified with certainty also in the living state. Described differences between the two nuclei (Cammermeyer 1963) are too minimal for practical purposes. Isolated oligodendrocytes are extremely rare in the non-liquefying cultures on collagen film, especially in older cultures. Therefore, criteria, which were established for migrated or isolated oligodendrocytes in liquefied areas (Lumsden and Pomerat 1951, Pomerat and Costero 1956), cannot be applied to those
cells located in closely packed explants where myelinated axons are interwoven more abundantly. Considering great preponderance of the granule cell population in vivo, however, it seems justified and more practical to assume that these small cells in cultures from older animals are granule cells as a whole.

One important problem arises from this interpretation, concerning with studies on the myelin sheaths in vitro. There are some evidences that oligodendrocytes participate in the genesis and maintenance of central myelin sheaths. Since individual oligodendrocyte cannot be differentiated positively from granule cells in the cerebellar cultures, it is not feasible to describe whether deformations of myelin sheaths are entailed by changes of oligodendrocytes in the cultures treated with various experimental procedures. Further attempts are being made in this laboratory to identify oligodendrocytes in cerebellar explants.

Granule cells, identified by Pomerat and Costero (1956), have a Y-shaped process at one pole of the cell body and a non-branching process at the opposite side. They correspond to our type II cells (Fig. 8), which appear to be cells in the embryonal granular layer. Judging from his illustrations, it seems that Wolf (1964) misinterpreted the axonal swellings of large neurons (Fig. 14, 15) as granule cells. His description, that these structures are more frequent in cultures from older mice than these from newborn cerebellum, is also in good agreement with our finding on the axonal swellings. Cells, suggested by Hild (1966) as granule cells, fall into our category of medium-sized neurons (Fig. 13). They are a little larger and more argyrophilic than the granule cells. Mizuno et al. (1962) considered cells with granular appearance as possible granule cells in vitro, since their nuclei resemble to those of the granule cells observed in a squashed preparation of the cerebellum. As pointed out in their paper, they show degenerative tendency on the whole. And it seems to be these partly isolated and slightly degenerated cells that show more marked affinity for silver and enable one to trace their processes (Fig. 5).

Some of our type V cells are provided with several short processes which are characteristic of granule cells (Fig. 12). However, it is more frequent that similar cells with a long bifurcating process lack such digitiform processes. This may be a result of either partial degeneration or deafferentiation. The latter possibility seems to be more plausible, since Cajal (1911) suggested that, of many dendritic processes of immature granule cells, only those which have made contacts with incoming mossy fibers become digitiform dendrites, while other non-contacting processes are resorbed.

2. Degeneration of the cultured granule cells

Cultured granule cells appear to be more susceptible to certain unfavorable factors. One of these factors seems to be the altered physicochemical condition, in view of the fact that they are fewer in number when incubated in the acidic or hypertonic media. According to Yonezawa et al. (1962), a lower concentration of glucose (80–100 mg%) is favorable for better maintenance of their 'granule cells'. This may indicate that granule cells tend to deteriorate in vitro due to hypertonicity of the media, caused by raised glucose concentration (600 mg%), rather than "low tolerance of these cells for glucose".

Similar selective vulnerability of granule cells has been reported by many authors under various pathological conditions (cf.: Olsen 1959, Ikuta et al. 1963, Friebe 1963).

3. Perikaryal myelin

Hild (1963) found neurons with myelinated perikarya in cultures of rat cerebellum and midbrain. They were very rare and the smallest one in his illustrations measures over 10 μ in
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diameter. His findings were confirmed also in our mouse cultures. Besides these cells, however, similar myelinated perikarya of smaller size, 6—8\( \mu \) in diameter, are frequently noted among these 'granule cells' (Fig. 17, 18). They are often apposed to myelinated axons and seem to be identical with those which were found electron microscopically in the cerebellar granular layer of adult toads (ROSENBLUTH 1966) and adult mice (KONISHI and OKAMOTO, unpublished data). Although ROSENBLUTH labeled these small myelinated cells as granule cells from the paucity of neuroglial cells in the toad cerebellum, further analysis will be needed to decide their exact cell type.

From the available data in the mouse cultures, a few comments will be made here on the possible meanings of the 'redundant' myelin, conjectured by ROSENBLUTH so extensively. Aging process may be excluded, since they are seen even in younger cultures, as early as 10 days. Their more frequent occurrence in a more healthy culture suggests that this phenomenon is other than pathological.

It seems that these perikaryal myelin sheaths are more resistant to certain demyelinative factors, because they are still apparently intact in cultures where most axonal myelin became deformed or fragmented (Fig. 17).

Summary

Attempts were made to identify the living granule cells in the cultures of the mouse cerebellar cortex.

1. When 10 days or more old mice are used as culture materials, thousands of small cells with scanty cytoplasm can be identified as granule cells on the basis of a) their size, b) their nuclear morphology, c) their vast numbers in cultures, d) their characteristic processes depicted by the silver impregnation, e) absence of cells in the embryonal granular layer in impregnated cultures, and f) the fact that they are discernible even in cultures from 20 days old mice in which the embryonal granular layer has already disappeared.

2. When newborn mice are cultivated, granule cell neurons cannot be distinguished in the living state from cells in the embryonal granular layer.

3. Oligodendrocytes situated in the explant cannot generally be differentiated from granule cells in the living state.

4. These granule cells are more vulnerable to various unfavorable conditions, e. g. the acidic or hypertonic state of the culture media.

5. Small myelinated perikarya are frequently noted among these granule cells. They are often apposed to myelinated axons.

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組織培養による小脳顆粒細胞の研究——その同定、変性および
周細胞体ミエリン（内容自抄）

組織培養下の小脳顆粒細胞の同定を試み、次の結果を得た。

1. 生後10日以上のマウス小脳皮質を培養したさい出現する細胞質の乏しい小型細胞は、次の理由により顆粒細胞と同定する。すなわち、（1）培養下に多数認められること。（2）生殖内のもと酷似した核をもつこと。（3）鍍銀法により顆粒細胞に特有な突起が認められるが、胎生顆粒層の細胞が見られること、さらに（4）胎生顆粒層のすでに消
失した生後20日目のマウスを培養したさいにも同様な細胞が認められることなどである。
2. 新生仔を培養した場合、培養下では顆粒細胞と胎生顆粒層の細胞の鑑別は一般に不可能である。
3. 稀有起葲細胞は培養下においては顆粒細胞との鑑別が極めて困難である。
4. これら培養顆粒細胞は、ほかの神経細胞にくらべて種々の悪条件に対しとくに敏感に反応し、容易に変性、消滅する。
5. これら顆粒細胞中に、細胞体周囲にミエリンをもつものがしばしば認められる。

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