Fine Structure of the Embryonal Granular Cells of the Chick Cerebellum

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ISHIDA, H. *Fine Structure of the Embryonal Granular Cells of the Chick Cerebellum.* Tohoku J. exp. Med., 1973, 109 (3), 261-280 — The fine structure, differentiation and destination of embryonal granular cells of the chick cerebellum were studied by means of electron microscopy. The embryonal granular layer (EGL) was divided into the external layer, the internal layer, and the transitional zone between the internal and molecular layers. Cells of the external layer showed active mitosis and appeared most primitive in shape and in development of cell organellae. Cells of the internal layer possessed long bipolar cytoplasmic processes parallel to the cerebellar surface as viewed in the frontal section. These processes were of two types: In some cells they were electron dense, with microtubules; in others, they were electron light, with microfilaments, and have some deeply folded rumples running longitudinally along the processes. In the transitional zone, the former cells (dense processes) obtained the characteristics of nerve cells, with developed rER and immature synapses; the latter cells (clear processes) the characteristics of glia cells. Those external layer cells with cytoplasmic end-feet in contact with the pia limiting membrane developed into Bergmann's astrocytes. Few degenerated cells were observed in the EGL, their number being far less than that of mitotic cells. Dense cored vesicles about 1,000 Å in diameter were observed in the EGL, but their nature is not known in the present study. ——— embryonal granular cell; development; cerebellum; chick; electron microscopy

Interest has been extant in the formation, development and disappearance of the cells of the cerebellar embryonal granular layer (EGL) since Cajal (1890) postulated that these cells differentiated into the nerve and glia cells which made up the molecular and granular layers. Shaper (1894) and Uryu (1939) suggested that the EGL cells developed from ependymal cells at the latero-caudal border of the cerebellar plate. According to the schematic drawing of EGL cells by Cajal (Fig. 1), the external layer is composed of uniform-sized ovoid cells with axes roughly perpendicular to the pia limiting membrane, while the internal layer is composed of spindle-shaped cells with axes roughly parallel to the membrane. Recent radio-autographic techniques applied to the EGL (Uzman 1961; Fujita *et al.* 1966) have revealed the migration of EGL cells from the surface to deeper portions of the cerebellum. This finding is in agreement with the hypotheses of Cajal and other classical neurologists, that the fast-labeled cells in the external

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layer migrate inward, making up the internal layer and, after 24 to 48 hours as internal layer cells, passing rapidly through the molecular layer and into the granular layer as granular and glia cells. This migration is a continuous process, and thus, if examinations are conducted at appropriate stages of development, the cytological differentiation can be noted sequentially from the outer to the inner layer of the EGL. Fig. 2 shows the changes in thickness of the EGL (I) and the increase in maximum perimeter of the cerebellar cortex (II), in sagittal section measurements. It indicates that the greatest mitotic activity of EGL cells occurs from the 12th to 14th embryonic day, and the greatest migrating activity from the 15th to 18th day.

The following study deals primarily with two morphological aspects: (1) the fine structural characteristics of cells in the external and internal layers of the EGL during the 14th to 16th embryonic day period, when mitotic and migrating activities are considerable, and (2) the morphogenesis observable in the external

Fig. 1. Schematic drawing of embryonic cerebellar cortex (Ramon y Cajal).

Fig. 2. Showing change in thickness of EGL (I) (μ) and increase in maximum perimeter of cerebellar cortex (II) (mm).

layer of the EGL from the 12th embryonic day through the 2nd day after hatching.

MATERIALS AND METHODS

Chicks from the 12th embryonic day through the 2nd day after hatching were used in this study. They were fixed by vascular perfusion with an acetate veronal buffered mixture at pH. 7.4 with 4% formalin and 4% glutaraldehyde. After perfusion, large blocks of the cerebellum were removed and postfixed for 2 to 3 hours in an acetate veronal buffered 2% OsO_4 solution, then dehydrated with a series of graded ethanol. From these blocks, embedded in Epon, the cerebellar vermis was removed, and thin sections were made on the Porter-Blum ultratome MT1. The specimens were stained with lead hydroxide and examined with a Hitachi Hu-11A or a Hitachi Hs-7 electron microscope, operating at 75 kV and 50 kV, respectively.

OBSERVATION

Fine structure of cells in the different layers of the EGL

1. Cells in external layer of EGL

As shown in Fig. 3, cells of the 14th to 16th embryonic day are round, oval or irregularly shaped, and about $10\ \mu$ in diameter, each having a centrally located nucleus about $7\ \mu$ in diameter.

a) Nucleus

The nuclei were round, oval or irregularly shaped (Fig. 3). Infolding of nuclear membrane was noted in some nuclei. Karyoplasm not having dense aggregations of chromatine contained 1 or 2 distinct nucleoli.

b) Cytoplasm

The cytoplasm of cells in the external layer occupied a narrow portion around the nucleus, and cytoplasmic processes were not fully developed. There were many free ribosomes in the cytoplasm, but cell organelles were rather poorly differentiated. Rough ER were rather small and few (Figs. 3 and 5, rER). Golgi apparatus consisted of 2 or 3 parallel lamellae, each approx. $3\ \mu$ in length (Figs. 3 and 6, GC). Many microtubules (Figs. 6 and 18, long arrow) and centrioles (Figs. 3 and 18, C) and some cilia having distinct basal bodies (Fig. 4) were observed. Also noted were dense cored vesicles having diameters of 1,000 to 1,200 Å in the cytoplasm (Fig. 3, arrows; Fig. 6, short arrow; Fig. 18, DCV) and in the inter-cellular space (Fig. 12, DCV).

c) Cell division and cell necrosis

Mitotic cells were very common in the external layer (Fig. 3, MC; Figs. 5 and 16). A typical mitotic cell is shown in Fig. 5, with chromosomes and spindle fibers. Some deteriorating nuclei suggestive of cell necrosis were also seen, as in Fig. 6, but their number was far less than that of mitotic cells.

2. Cells of internal layer of EGL

The frontal section of the cerebellum showed that each cell of the internal layer possessed two elongated processes roughly parallel to the cerebellar surface (Fig. 7).

a) Nucleus

The nucleus appeared long ovoid or spindle-shaped in the frontal section (Fig. 7, N₁ and N₂) and round in the sagittal section (Fig. 8).

b) Cytoplasm

The frontal section of the cerebellum (Fig. 9) revealed large numbers of processes running parallel and in close proximity to each other. The sagittal section (Figs. 8 and 10) showed numerous cross-sections of cells with round nuclei and narrow cytoplasm, and of processes, approx. 1 to 3 μ in diameter, which clustered among the cells. In deeper parts of the internal layer, well organized rough ER were observed in the cell cytoplasm, suggesting the development to Nissl body (Fig. 11, rER).

c) Cell processes

Two types of cell processes were distinguished: one with electron dense cytoplasm containing many microtubules of approx. 200 Å in diameter (Fig. 8, short arrow; Fig. 9, DP), and the other with relatively less dense cytoplasm containing a few filaments of approx. 70 Å in diameter instead of microtubules (Fig. 8, long arrow; Fig. 9, CP). In cross-sectional views (Figs. 8 and 10), it can be seen that the electron dense processes (short arrows and DP) are encircled by the electron clear processes (long arrows and CP). From these observations, it was concluded that clear processes are deeply folded around dense processes.

It should be noted that there were also dense cored vesicles in this layer. They were observed in the cytoplasm (Fig. 18, DCV), in the processes (Fig. 8, double arrow 3; Figs. 3 and 4), and in intercellular spaces (Fig. 8, double arrow 3; Fig. 12, DCV).

3. Cells in transitional zone

Fig. 13 is a low-power electron micrograph of the cerebellum in the sagittal section, which shows cells in the transitional zone between the EGL and the molecular layer.

a) Cytoplasm

The cytoplasm of cells in this zone was somewhat wider than that of cells of the external and internal layers.

B) Cell processes

Most of the cell processes in this zone contained many microtubules approx. 0.5 to 1 μ in diameter and were smaller than those of cells of the internal layer.

However, there were a few large processes approx. 2 to 3 μ in diameter with electron transparent cytoplasm containing only fine filaments approx. 70 Å in diameter or containing both microtubules and filaments.

c) Junctional apparatus and synapses

In the transitional zone, as in the external and internal layers, there were several junctional apparatus between processes, between processes and cells, and between cells (Fig. 12, D). An immature synapse was observed between a process containing microtubules and a cell perikaryon (Fig. 13, long arrow). A high-magnification electron micrograph of the synapse (Fig. 14) shows clear and cored vesicles on the pre-synaptic side.

Fine structural differentiation in cells of the external layer of the EGL

1. 12th embryonic day

Outermost cells of the external layer were located side by side with their end-feet attached to the pia limiting membrane (Fig. 15). The close apposition of cells can be seen (arrows). The pial surface of each cell process was covered by basement membrane. It should be noted that some cells in this stage of embryonic development had end-feet which were not fully differentiated and nuclei which were located close to the pia limiting membrane (Fig. 15, central portion).

2. 14th to 16th embryonic day

Cell processes elongated during this period as the nuclei migrated deeper into the cytoplasm, away from the pia limiting membrane. As a result, the outermost areas of the external layer became a mass of interwound processes (Fig. 16). Some elongated processes perpendicular to the cerebellar surface and possessing electron transparent matrices (Bergmann's fibers) were observed on the 16th embryonic day (Fig. 3, BF).

3. 2nd day of hatching

The outermost portion of the external layer (Fig. 17) was composed of processes with electron clear cytoplasm, namely the end-feet of Bergmann's astrocytes.

DISCUSSION

Wasano (1950) reported on changes in the number of rows of cells in the external and internal layers observed at various stages of development. In the following table are the observations considered significant in the present study:

Embryonic day	10th	12th	14th	16th	First day of hatching
Rows of cells in external layer	1-2	2-3	7-9	5-6	1-2
Rows of cells in internal layer	6-8	9-10	9-10	9-10	1-3

In the above table, absence of change in the internal layer from the 12th through 16th embryonic day is noted.

Uzman (1961) and others reported that activation of incorporated H^3 -thymidine was first observed in cells of the external layer.

From these two findings and the relationship noted between increase in cerebellar cortex perimeter and change in EGL thickness, it was concluded that in the development period of the cerebellum the cells of the external layer of the EGL have the greatest mitosing capacity from the 12th to the 14th embryonic day. The high level of mitotic activity occurs in cells of the two or three rows beneath the outermost row of this layer. These cells remain in a primitive stage of development, while those of the outermost row grow end-feet to the pia limiting membrane, indicating cell differentiation. In accordance with Wasano (1950), development of the internal layer of the EGL is not prominent between the 12th and 16th embryonic day. Cell mitosis and migration occur at approximately the same rate and result in no significant gain or loss. (Actually, mitotic activity is decreasing and migration increasing toward the 16th day.)

The fine structural characteristics of cells in the EGL, *e.g.*, large nuclei with light nucleoplasm, the occasional existence of centriols, and many free ribosomes in narrow cytoplasm, indicate that these cells are immature, as were found in a certain kind of malignant tumor (Dalton 1961) and undifferentiated cells such as primitive ependymal cells reported by Takeichi (1966) and Fujita *et al.* (1966). The existence of cilia in cells of the external layer (Fig. 4) appears to support Shaper's view (1894) that EGL cells develop from primitive ependymal cells.

Necrosis in the EGL, as reported by Retzius (1895) and Wasano (1950), was confirmed in this study (Fig. 6). However, very few necrosing cells were observed in comparison with the number of mitosing cells.

According to Uzman (1961), the incorporation of H^3 -thymidine into cells of the internal layer is apparently slower than that into external layer cells. It is suggested that cell differentiation is more advanced in the internal layer. Evidence of cell differentiation in the internal layer included the elongated cytoplasmic processes which develop bipolarly from the perikaryon surrounding the spindle-shaped nuclei, and the two types of processes into which they subsequently develop: the electron dense processes (DP) containing many microtubules and the electron clear processes (CP) containing a few filaments. Two types of processes (electron dense and clear) forecast the nature of eventual differentiation in the perikaryon. It is believed that cells with dense processes develop Nissl body and the processes themselves develop into parallel fibers, while cells with clear processes develop into glia cells.

In the transitional zone, several junctional apparatus and a few immature synapses were observed between processes and between processes and cells. This is in line with the view proposed by Hama (1960) and Kelley (1967) that synapses are junctions before they function as synapses.

The presence of axo-somatic synapses in this zone suggests that some cells

with dense processes develop into satellite cells of the molecular layer. Also noted in this zone was a gradual decrease in the diameter and increase in the number of cell processes as the molecular layer was approached. This gradual diminution in size may be due to the elongation of the processes, which would occur as the cell bodies migrated further into the molecular layer and into the granular layer.

In general, the cells of the external layer are the most undifferentiated in the EGL, mitosing actively, enlarging the cerebellar cortex and supplying cells for the internal layer.

However, the outermost cells of the external layer undergo special differentiation. The cytoplasm of each cell adjacent to the pia limiting membrane develops an end-foot, while the cell nucleus migrates toward the inner area. These cells, with the end-feet in close apposition and attached to the pia limiting membrane, elongate over a period of time and gradually become electron transparent.

Thus, the outermost cells are believed to develop into Bergmann's astrocytes.

Dense cored vesicles about 1,000 to 1,200 Å in diameter were observed in the EGL in almost all stages of development. They were found in cytoplasm and in intercellular space, without relation to the Golgi complex. In the cytoplasm, they appeared sporadically and the core was bounded by single membrane, while in the intercellular space, they showed double membrane structure and tended to cluster.

Kornguth (1963) reported the dense cored vesicles in the cerebellar EGL of *Macaca mulatta* and Uehara (personal communication 1969) observed them in the developing muscle spindle of mouse. Lentz (1967) insisted that the reorganization of newt limb depended greatly upon "membrane bounded vesicles."

Though the nature of the vesicles is not known in the present study, the intracellular cored vesicles may be considered to have some significance in the cerebellar development, while the extracellular ones are not ruled out as a kind of virus.

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Key to abbreviations used in plates

BF: Bermann's fiber. C: Centriol. CP: Electron clear cytoplasmic process. Cr.: Caryorrhexis. CV: Coated vesicle. D: Junctional apparatus. DCV: Dense cored vesicle. DP: Electron dense cytoplasmic process. GC: Golgi complex. MC: Mitosing cell. N: Nucleus (including N₁ & N₂). PLM: Pia limiting membrane. rER: Rough ER.

Fig. 3. External layer of EGL, 16th-day chick embryo (sagittal section, $\times 7,500$). Arrows point to dense cored vesicles.

Fig. 4. Cilium with basal body in an external layer cell ($\times 21,000$).

Fig. 5. Mitosing external layer cell ($\times 12,000$). Short arrow points to dense cored vesicles.

Fig. 6. Necrosing cell in EGL ($\times 22,000$). Long arrow points to microtubule; short arrow to dense cored vesicle.

Fig. 7. Internal layer of EGL, 16th-day embryo (frontal section, $\times 12,000$).

Fig. 8. Internal layer, 16th-day embryo (sagittal section, $\times 15,000$). Long arrows point to electron clear cytoplasmic processes; short arrows to electron dense cytoplasmic processes; double arrows 1 and 2 to intracellular dense cored vesicles; and double arrow 3 to intercellular dense cored vesicles.

Fig. 9. Internal layer, 15th-day embryo (frontal section, $\times 24,000$).

Fig. 10. Internal layer, 15th-day embryo (sagittal section, $\times 16,000$).

Fig. 11. Transitional zone of EGL, 16th-day embryo (sagittal section, $\times 12,000$).

Fig. 12. Junctional apparatus in transitional zone, 16th-day embryo ($\times 78,000$).

Fig. 13. transitional zone, 16th-day embryo (sagittal section, $\times 15,000$). Arrow points to immature synapse (axo-somatic).

Fig. 14. Magnification of immature synapse in Plate 13 ($\times 45,000$).

Fig. 15. Outermost cells of the external layer of EGL, 12th-day embryo ($\times 21,000$). Arrows point to close apposition.

Fig. 16. Outermost cells of external layer, 15th-day embryo ($\times 18,000$).

Fig. 17. Outermost cells of external layer, 2-day-old chick ($\times 31,000$).

Fig. 18. Internal layer, 16th-day embryo (sagittal section, $\times 38,000$). Arrow points to microtubules.