Serial Observations on Patterns of Growth, Myelin Formation, Maintenance and Degeneration in Cultures of New-Born Rat and Kitten Cerebellum*

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ABSTRACT

New-born rat and kitten cerebellum may be maintained for prolonged periods (over 5 months) in the Maximow assembly if explanted on to a coverslip previously coated with a thin gel of reconstituted rat tail collagen and fed a glucose-enriched "natural" medium.

After a 2 week period of adjustment and early outgrowth, most cultures exhibit myelin formation. Axons located within the surrounding neuroglia sheet of the explant area myelinate. The sheaths are first evident as long, unsegmented, smooth, parallel, refractile lines. Simultaneously, neuronal nuclei tend to assume central positions and powdery granules of Nissl substance and lipoid materials begin to accumulate within the cytoplasm.

During prolonged maintenance, axons may increase in width, and the myelin may thicken. Some exhibit transient irregularities and swellings. Degeneration of some axons occurs manifested either by (a) progressive swellings and distortions of the myelin sheath and thinning of intervening portions of the axons which finally yield, leaving the swellings as myelin bodies, or by (b) small aneurysm-like distortions of myelin sheaths on thinning axons which become dull, irregular, and thread-like filaments beaded by the former herniations.

The observations are compared with previous studies of in vitro and in vivo myelin formation with particular reference to neuronal-neuroglial relationships.

The Maximow double coverslip assembly (1) permits repeated high power microscopic observations of cultured tissue at will during its entire in vitro existence. Dynamic morphological relationships thus visualized may be profitably compared with structural patterns as observed with other techniques such as electron microscopy.

Peterson and Murray (2) maintained chick embryo dorsal root ganglia in this manner and first described in vitro myelin sheath formation. Pursuing the studies of Pomerat and Costero (3) on growth patterns of kitten cerebellum cultured on coverslips enclosed in roller tubes, Hild (4) first observed in vitro myelin formation in tissue derived from the mammalian central nervous system. The latter method, however, allows but a limited high power examination period of the cultured material.

The purpose of this communication is to describe in detail a method of culturing new-born rat and kitten cerebellum with the Maximow technique and to present long term, serial observations on the patterns of growth, myelin formation, maintenance and degeneration (5).

Material and Methods

One- to 3-day-old rats and kittens were used. After ether anesthesia and occipital craniotomy, the entire cerebellum was removed to a sterile dish containing a few drops of Simms' balanced salt solution (BSS) at

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pH 6.9-7.1. The tissue was stripped of its meningeal coverings, cut into fragments 0.5 to 1.5 mm. in diameter, and explanted on to gold seal No. 1, 22 mm. round coverslips previously coated with a thin film of reconstituted rat tail collagen (6). A single drop of nutrient medium was added to the 2 to 4 fragments on each coverslip which was then incorporated into a Maximow assembly and sealed with paraffin. The nutrient medium was composed of equal parts of Simms' BSS supplemented with glucose to produce a final concentration of 300 mg. per cent, human placental cord serum (1), bovine serum ultrafiltrate (Microbiological Associates), and embryonic extract from 9-day-old chicks.

Cultures were incubated in the lying drop position at approximately 36°C, except for their daily removal to room temperatures for microscopic examination and occasional photographic recording. Twice weekly, the cultures were washed in Simms' BSS at pH 6.9-7.1 and fed a fresh drop of nutrient medium. No patching was necessary since the collagen gel was not liquefied by the cultured tissue for periods up to 177 days.

Observations of tissues with polarized light as well as fixation in 10 per cent formalin or mercuric chloride-10 per cent formalin and staining as whole mounts with cresyl violet, Sudan black B and Bodian's silver impregnation were the same as described by Peterson and Murray (2). Cultures were also stained with luxol fast blue MBS (DuPont) and counterstained with either cresyl violet (7) or neutral red (8). Control tissues from new-born, 8-day-old, and adult rats were fixed in 10 per cent formalin, embedded in paraffin, sectioned and stained with luxol fast blue MBS, and cresyl violet.

RESULTS

Fixed and stained tissue obtained from new-born rat cerebellum possesses no visible myelin. Similarly treated adult rat reveals the characteristic myelinated appearance.

The results are derived from over 1400 cultures of which about 70 per cent were from the rat and 30 per cent from the kitten.

Kitten cerebellum was the first tissue studied in the Maximow slide. When this tissue was explanted into a classical plasma clot or directly on to glass, healthful prolonged maintenance and myelin formation were not obtained. After the described technique had been developed and its success demonstrated, it was extended to the new-born rat cerebellum, a more easily available source of material.

Since most of the experimental material was derived from the rat, the descriptions will emphasize observations of these tissues. The patterns of development are similar, although the kitten's schedule tends to lag behind the rat's by 1 to 2 weeks.

For purposes of presentation, the growth patterns may be considered in 3 periods: (a) early outgrowth and adjustment to in vitro conditions, (b) myelin formation, and (c) maintenance of the developed state.

(a) Early Outgrowth and Adjustment.—The explanted fragments adhere to the surface of the collagen gel immediately. Rarely, a fragment becomes loosened during the manipulations of sealing the slide. It reattaches, however, when the slide is returned to the lying drop position.

Within 24 hours, neuroglial cells appear about the margin of the fragment. By migration from the explant as well as mitotic divisions in the surrounding area, they gradually extend their domain to form a cellular network completely surrounding the flattening fragment. Simultaneously, single or corded neurites penetrate the glial collar and may, within 5 to 7 days, reach 1 to 2 mm. beyond its spreading periphery. In the outgrowth, some of the dispersed neuroglia may occasionally be seen closely applied to portions of an extended nerve fiber. Within the explant area, however, the neuronal extensions are completely surrounded by a sheet of neuroglial cells which obscures them. Neuroglia compose the majority of cells in the explant area. Since the sheet of cells remains unbroken in most fragments, the individual cell boundaries cannot be easily distinguished. Some explants, however, spread to a considerably greater degree than others. In these, the neuroglial cells are revealed to be about 10 to 15 microns in diameter, excluding their processes, and to contain a relatively small nucleus and nucleolus.

The neuron cell bodies become distinguishable during the 2nd week in vitro. They have never been observed to undergo cell division or to migrate into the outgrowth zone, although by chance positioning of the explant, some neurons may lie along the periphery of a fragment. The cells appear larger (20 to 30 microns in diameter) than the surrounding neuroglia. At this time, their scanty cytoplasm—less abundant in the rat than in the kitten—appears remarkably clear, high lighting a prominent and large eccentric nucleus and nucleolus.

After 3 to 5 days in vitro (DIV), the ependymal cells and their beating cilia are easily identified. They appear either as monacellular sheets or as formations resembling pseudo-columnar epithelium bordering bizarre shaped cavities, as described by Hild (9). In the vicinity of rat ependyma, there
frequently appear intercellular cystic formations filled with an aqueous substance (Fig. 14). These may increase in number and size and, in some cultures, almost entirely exclude cellular elements from relatively large areas.

Within 24 hours after explanation, microglia cluster over the explant area. A small number, rounded and granular, move freely over the outgrowth zone. After from 10 to 15 DIV, they migrate in vast numbers into the outgrowth zone where they may fall into the fluid medium or settle down to a sedentary existence. This migration leaves the thinning fragment relatively open to observation and may be thought to mark the end of the period of adjustment to in vitro existence.

(b) Myelin Formation.—In the rat, myelin has become visible as early as the 12th DIV, although usually the first signs appear during the 3rd week. Myelin sheath formation characteristically occurs about those axons which lie within the surrounding neuroglial sheet of the flattened explant. An en-sheathed axon may extend into the glial network of the outgrowth zone, but its myelin disappears within 50 to 100 microns from its departure from the neuroglial concentration of the explant. Myelin formation does not occur about the neurites extending through and beyond the glial collar. Should neurons be located at the periphery of an explant, their extensions toward the explant, i.e., through neuroglial sheets, may myelinate whereas those directed outward, contacting only occasional neuroglial cells, do not. Lastly, although apparently healthy neurons may be present within fragments which have flattened excessively, so that only a network (not a sheet) of neuroglial cells exist within the explant areas, myelinated axons have not been found.

The highly refractile myelin sheath becomes visible abruptly as a pair of uninterrupted, uniform, almost perfectly smooth parallel lines (Fig. 1). Frequently, the myelin sheath is manifest simultaneously over long portions of axon—1 mm. or more. That this image represents myelinated axons has been demonstrated by polarized light studies (Fig. 2)—the birefringence being negative with respect to the long axis of the fiber (10)—and by staining with Sudan black B (Fig. 4) and with luxol fast blue MBS (DuPont). Preliminary observations with the electron microscope (11) reveal patterns of lamellae resembling those described by Luse (12) as representing myelin sheaths in central nerve tissue of rats and mice.

During the 10 to 14 days following the first appearance of myelin, other axons become visibly ensheathed with equal abruptness (Fig. 3). In addition, the myelin extends to include longer portions of axons without any evidence of a segmental distribution. At this time, the axons are of relatively uniform diameter, varying from 1.5 to 2.5 microns in the rat and from 2.0 to 3.0 in the kitten.

There is no second period of myelin formation.

At the end of the period of myelin formation, the number of myelinated axons in different fragments varies considerably. About 10 per cent of the cultures possess innumerable myelinated axons winding throughout the explant area (Fig. 3). Approximately 30 to 40 per cent have over 20 myelinated axons; a further 30 to 40 per cent less than 20. The remaining 10 to 30 per cent of the fragments never demonstrate myelinated axons.

During myelin formation, many neuronal nuclei tend to assume a more central location (Fig. 5). A few, however, remain at the periphery of the cell during their entire in vitro existence. Small particles begin to dot the cytoplasm. The previously distinct nuclear outlines become gradually clouded. Staining with cresyl violet and Sudan black B reveals some of the particles to be basophilic—Nissl substance—(Fig. 9) and others to be lipid material (Fig. 7). The particles are frequently distributed evenly throughout the cytoplasm although the Nissl substance may have a tendency to concentrate near the cell membrane or as a nuclear cap.

(c) Maintenance of Developed State and Degeneration.—During the period of maintenance, the gradual accumulation of cytoplasmic granules progressively masks the neuronal nuclei. The cell is finally camouflaged against the neuroglial background (Figs. 6 and 8). When stained, however, the neurons are again easily distinguishable.

After about the 30th DIV, the cultures stabilize. Although no further axons myelinate, the increasing refractility of those already ensheathed suggests an increase in the thickness of the myelin. In addition, the axons themselves may increase in diameter so that by the 100th DIV they may vary from about 2 to 5 microns (Fig. 15).

Although most axons and their sheaths retain a smooth, regular appearance, changes occur in some. At first, they may be disturbed by slight irregularities or expansions (Figs. 3, 15 and 16), which may shift in position along the axon and
may sometimes disappear. Swellings may, however, progress to more profound alterations, precursors of degeneration. There then develop increasingly marked, grotesque swellings connected by thinning portions of axon (Figs. 10 and 11). The thread-like axon finally yields, leaving the swellings as myelin figures—the last stage of this type of degeneration. These events have been observed to occur over a 2 to 3 week period in an otherwise healthy culture. However, by exposing a culture to a noxious influence, such as a constant strong light, or by alterations of the nutrient medium (13), one may produce this type of degeneration as an acute process requiring but a few hours or days.

This first pattern of degeneration most frequently occurs in young cultures. A second type is more often seen in those maintained for over 100 days, although it, too, may be observed earlier. Moreover, once begun, its course seems to be inexorable: a reversal of the process has not been observed. At first, small, round or oval aneurysm-like dilatations appear along the course of the myelin sheath (Fig. 12). The axon itself loses its sharp refractility, becomes somewhat dull and homogenous and undergoes a generalized narrowing. The process continues until there remains but a thin, irregular, tortuous, poorly defined thread of myelin beaded with small, refractile bodies, the relics of the earlier herniations (Fig. 13).

Although some axons degenerate, a large number may be maintained in a healthy state for a considerable time (Figs. 15 to 18). Some cultures have been observed for 177 days. Many healthy myelinated axons were still present, although a few were marked with slight irregularities as evidence of their age, daily exposure to room temperatures, microscopic examinations, and periodic photographic sessions.

**DISCUSSION**

Serial observations of cultured new-born rat and kitten cerebellum have revealed characteristic patterns of cellular organization, myelin formation, and maintenance which may be compared with the observations of other investigators (2, 4).

The major patterns of adjustment to *in vitro* existence and the early growth of kitten cerebellum have been described by Pomerat and Costero (3) and Hild (4). Those of the new-born rat and kitten maintained in the Maximow slide do not differ from the established patterns and serve to confirm the previous studies.

The presence of cystic formations in areas populated by ependymal cells, however, was not mentioned in Hild's (9) recent presentation of his observations of ependyma in cultures of kitten cerebellum, nor were they seen in cultures of kitten tissue in this series. In the rat, however, they repeatedly occur. Their early appearance and later increase in number and size suggests that they represent collections of material produced by either the ependymal cells or closely associated neuroglia. The nature of their aqueous content is being examined with various staining techniques.

The patterns of myelin formation in these tissues, however, differ significantly from those previously reported.

In their description of myelin sheath formation in cultures of avian dorsal spinal ganglia (peripheral nerve tissue), Peterson and Murray (2) demonstrated a regular, interrelated sequence of developmental changes involving neuron somas, axons, and Schwann cells. Should the development of any of these elements be suppressed, myelinization would not occur (14, 15). The cellular requirement for myelinization seemed to be fulfilled by a single Schwann cell closely applied to an axon, whether the association happened to occur within the area of the original explant or in the outgrowth zone. The segmental process, however, might be repeated along a single axon. Thus, myelin first became apparent in the form of isolated segments, related to individual Schwann cells, and might progress along the fiber in either direction. Where successive segments were developed, the interruption between segments suggested nodes of Ranvier.

In the new-born rat and kitten cerebellum (central nerve tissue) as observed in these cultures, the pattern of maturation of the neuronal cell bodies is different from that described for the spinal ganglia. At the time of active myelin formation, very little Nissl substance is observed in either the living cultures or in fixed and stained preparations and, although many nuclei tend to assume central locations, some maintain their peripheral positions. This same pattern is evident during *in vivo* myelin formation as determined by histological preparations of tissue obtained from new-born, young, and adult rat cerebellum. In addition, the area in which myelinization characteristically occurs is limited to sites where axons are intimately surrounded by a high concentration of neuroglial cells; *i.e.*, within the explant. Single contacts between neuroglia and axon, as may be
found in the outgrowth zone or in excessively thinned fragments, did not result in myelin sheath formations visible with the ordinary light microscope. The abrupt, simultaneous, non-segmented appearance of myelin along relatively long portions of myelinating axons also differs from the pattern described for peripheral nerves. Obviously, the process of myelin formation has been continuing for some time at a number of points along the axon until the moment when the sheath attains a thickness which permits visualization with ordinary or polarized light. Thereafter, the sheath continues to thicken.

Although these experiments do not offer direct proof of a functional relationship between neuroglial cells and myelinating axons, they do permit a working hypothesis that not only are neuroglial cells significantly present about myelinating axons, but also that a degree of neuroglial density is necessary for the development of myelin sheaths of sufficient magnitude to be visible by ordinary light microscopy.

This hypothesis, based on cultural patterns, agrees with that derived from Luse's (12) electron microscope observations of myelin formation in the central nervous system of rats and mice. She demonstrated the closely arranged lamellae of the myelin sheath to be formed by membranes of more than one glial process and possibly more than one neuroglial cell. In later stages, multiple connections of myelin lamellae with plasma membranes of adjacent glial processes were prominent.

To place the sole or major responsibility for myelin sheath formation on the axon proper because of a paucity of recognizable oligodendrogial cells in the vicinity (4) does not appear justified for two reasons. Utilizing classical histological methods, Alpers and Haymaker (16) demonstrated that both astroglial and oligodendroglial cells participate in myelin formation in the prenatal human brain, a fact recently confirmed by Luse's electron microscopic observations of rats and mice (12). Secondly, certain identification of neuroglial cells as either astrocytes or oligodendroglia is at least as difficult in tissue culture as it is in fixed and stained material when viewed either through the light or the electron microscope (17). The difficulty is not with cells which are morphologically similar to those described in classical anatomy, but, rather, with the many intermediate forms present. Moreover, the possibility of modulations should be considered. This is strongly suggested by Luse's observation that, at the time of myelin formation, the origin of the glial process is largely oligodendroglial, with no clear indication, however, that they are all so derived. Later, it is the astrocytic folded membranes which closely invest most central myelinated fibers. Although she suggests that the characteristic structures of the two cell types may be an expression of functional differences, she does not propose the possibility that they may represent the same cell under different functional conditions. Serial observations of living tissue during months of in vitro development suggest the latter hypothesis.

A last point of discussion arises from Hild's (4) statement that cuff-like swellings along axons represent areas of myelin formation. Serial observations of either kitten or rat cerebellum maintained in the Maximow slide assembly have not demonstrated any kind of swelling as characteristic of myelin formation. Rather, the myelin sheaths appear abruptly as long, smooth, unsegmented formations resembling railroad tracks in their uniformity. Irregularities, swellings and distortions of various kinds, however, do appear later as alterations of previously formed myelin sheaths. They may represent normal variations of axon-neuroglial relationships. They may, however, be manifestations of more or less serious dysfunctions and can progress to more marked alterations as axons degenerate. Studies of the early alterations and the obvious degenerations are being pursued in an effort to determine the possible roles played by the neuron cell body, the axon and the satellite cells in their production.

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Bibliography

EXPLANATION OF PLATES

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FIG. 1. Rat cerebellum (R 11—9/8) 19 DIV. Living, unstained culture. Portions of newly myelinated axons and surrounding neuroglia. The smooth, non-segmented sheaths had appeared abruptly along a considerable length (over 1 mm.) of the lower axon. Note absence of swellings. X 1050. Scale = 50 microns.

FIG. 2. Rat cerebellum (R 2—1) 19 DIV. Living, unstained culture. Polarized light. Several axons demonstrating birefringence. X 1150. Scale = 45 microns.


FIG. 4. Rat cerebellum (R 6—655) Fixed in 10 per cent formalin after 34 DIV. Stained with Sudan black B. Myelinated axons and surrounding neuroglia. X 1050. Scale = 50 microns.
Bornstein and Murray: Patterns of growth and myelin formation.
Fig. 5. Rat cerebellum (R 6--3S5) 20 DIV. Living, unstained culture. Neurons and myelinated axon. Nuclei tend to assume central positions. Cytoplasm contains sprinkling of fine particles. Neuroglial cells (arrow). X 1050. Scale = 50 microns.

Fig. 6. Rat cerebellum (R 8--6/3) 38 DIV. Living, unstained culture. Neurons in which cytoplasmic granules begin to cloud nuclear outlines. X 1050. Scale = 50 microns.

Fig. 7. Kitten cerebellum (K 3--3) Fixed in 10 per cent formalin after 28 DIV. Sudan black B stain demonstrating lipid nature of cytoplasmic granules in neuronal soma. X 1050. Scale = 50 microns.

Fig. 8. Rat cerebellum (R 4--SG6) 84 DIV. Living, unstained culture. A neuron whose nucleus is almost entirely obscured by cytoplasmic granules. X 1050. Scale = 50 microns.

Fig. 9. Rat cerebellum (R 8--12/2) Fixed in 10 per cent formalin after 34 DIV. Cresyl violet stain for Nissl substance. X 1050. Scale = 50 microns.
(Bornstein and Murray: Patterns of growth and myelin formation)
Fig. 10. Rat cerebellum (R 4—SG6) 47 DIV. Living, unstained culture. Enlarged swellings and distortions of myelinated axons. X 1050. Scale = 50 microns.

Fig. 11. Rat cerebellum (R 11—9/5) 46 DIV. Living, unstained culture. Enlarged swellings with thinning intervening portions of axons. X 1050. Scale = 50 microns.

Fig. 12. Rat cerebellum (R 4—SG6) 47 DIV. Living, unstained culture. Aneurysmal dilatations (arrow) on a normal appearing axon. X 1050. Scale = 50 microns.

Fig. 13. Rat cerebellum (R 4—SG6) 126 DIV. Living, unstained culture. Advanced degeneration of second type. Thread-like axon beaded with aneurysmal dilatations. X 1050. Scale = 50 microns.

Fig. 14. Rat cerebellum (R 10—6/12) 31 DIV. Living, unstained culture. Cystic formations against background of ependyma at a different plane of focus. X 525. Scale = 100 microns.
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FIG. 15. Rat cerebellum (R 4—SG6) 84 DIV. Living, unstained culture. Myelinated axons and surrounding neuroglia. Axons vary in diameter and are slightly irregular. X 1050. Scale = 50 microns.

(Bornstein and Murray: Patterns of growth and myelin formation)
Fig. 17. Rat cerebellum (R 4—SG6) 126 DIV. Living, unstained culture. Healthy, myelinated axons. X 1050. Scale = 50 microns.

Fig. 18. Rat cerebellum (R 4—SG6) 143 DIV. Living, unstained culture. Healthy, myelinated axons. X 1050. Scale = 50 microns.
(Bornstein and Murray: Patterns of growth and myelin formation)