A MORPHOLOGICAL STUDY OF THE EXTERNAL GRANULAR LAYER OF THE POSTNATAL RAT CEREBELLUM

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by

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1. ABSTRACT

The external granular layer (EGL), a transitory layer of cells covering the surface of the developing vertebrate cerebellum, produces cells which populate the definitive cerebellar cortex, principally the internal granular layer (IGL). To study the nature and fate of the cells of the EGL, a variety of morphological techniques were applied to the cerebellum of the rat in the first three postnatal weeks. Transmission and scanning electron microscopy were used to investigate the morphology and relationships of the constituents of the EGL. Stereological analyses of EGL cells and IGL neurons showed that although the latter were larger overall, the degree of maturity of the two cell populations was similar at any given age. Both EGL cells and IGL neurons appeared to mature in situ at a similar rate. Stereological comparison between cells of the outer and the inner zones of the EGL revealed no maturational differences between the two regions. The lack of clear distinction between the two zones was further shown by a quantitative analysis of the pattern of proliferation in the EGL using light microscopy and autoradiography. The vasculature of the EGL was studied, revealing that with the disappearance of this layer there was a reduction in the density of the overlying capillary bed, and an increase in intrinsic vessels. Immunocytochemical localisation of glial- and neuronal-specific proteins showed an absence of perikaryal staining for astrocytes and oligodendrocytes in the EGL. This reinforces the view that this layer does not produce neuroglia. Cell death in the EGL was examined morphologically and an experimental system for simulating physiological cell death was established. Cell death was found to occur in the EGL throughout its

postnatal existence, and was quantitated. Calculations of rates of cell death suggested that this process does not significantly regulate cell production from the postnatal EGL.

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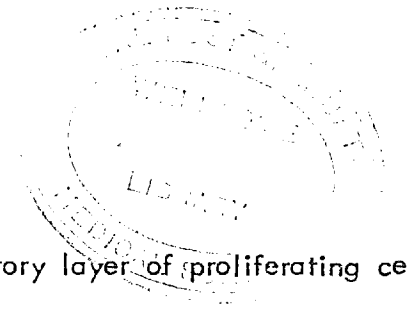

5. INTRODUCTION

The external granular layer (EGL) is a transitory layer of proliferating cells covering the cerebellum of young vertebrates. It has been studied in fish (Schaper, 1894; Pouwels, 1978a - e), amphibia (Gona, 1976), birds (Mugnaini and Forströnen, 1967), and many mammals, including mice (Miale and Sidman, 1961), rats (Altman, 1972a), monkeys (Rakic, 1971) and man (Raaf and Kernohan, 1944).

In mammals, the EGL appears during later intrauterine life and persists for some time after birth. It is responsible for the production of the bulk of the microneurons of the cerebellar cortex. Granule cells of the internal granular layer (IGL) are its principal product, though the EGL is also the source of basket and stellate cells (Altman, 1969). Whether or not the EGL produces glial cells has been the subject of controversy for nearly a century.

Though there is no agreement as to whether the EGL generates glia, the neurogenic function of the EGL is undisputed. The postnatal timing of cerebellar microneurogenesis makes the EGL an exceptional structure in the central nervous system, where postnatal neurogenesis is confined to a very small number of sites. Apart from the EGL, it is believed to be significant only in the mammalian olfactory bulb (Hinds, 1968a; 1968b), in the hippocampus (Altman, 1966; Altman and Das, 1965b) and possibly the cochlear nuclei (Taber Pierce, 1967). The other feature of the EGL which makes it unique is the path of migration of its constituent cells. Neurons appear invariably to be formed at sites removed from its final destination

(Rakic and Sidman, 1973). They proliferate in one region and then migrate to a new location where they become established as functional nerve cells. However, the cells of the EGL undergo two separate migrations. They are believed to originate from a region of the margin of the fourth ventricle known variously as the rhombic lip (Miale and Sidman, 1961) or the germinal trigone (Altman and Bayer, 1978). By proliferation and/or migration the EGL cells cover the cerebellar surface. At this site they continue to proliferate so as to produce a layer several cells deep. From here a second migration occurs, this time centripetally into the cerebellar cortex. Granule cells are produced during the entire existence of the EGL. In the rat this period extends over the first three postnatal weeks. Basket cell production is maximal at the end of the first postnatal week. The peak of stellate cell production occurs a week later (Altman, 1969).

The EGL is an important structure because it is responsible for the generation of large number of neurons (10 billion in man, 1.7 billion in the rhesus monkey (Blinkov and Glezer, 1968). Despite its importance, however, many aspects of the origin and fate of the constituent cells of the EGL remain in some degree of confusion. EGL cells may originate in the rhombic lip and migrate out and over the cerebellar surface (Miale and Sidman, 1961). They may equally originate in the subependymal zone of the fourth ventricle and reach the cerebellar surface by a centrifugal, radial migration (Hanaway, 1967). Alternatively the EGL may be a product of the fusion of the roof-plate epithelial cells with the cerebellum (Woodard, 1960).

The early studies of the developing cerebellum (see below) involved the light microscopic investigation of thin sections. The Golgi impregnation

technique proved especially useful. However, the limitations of light microscopy meant that the answers to certain questions had to await the development of new techniques. Autoradiography and electron microscopy were first used to examine the EGL in the early 1960s. While autoradiography yielded much information on migration patterns and cell-cycle kinetics, there were still problems to be solved. Since in the postnatal rat cerebellum cell proliferation is occurring in several sites (such as the EGL, the internal granular layer and the subependymal zone of the fourth ventricle), it is not easy to determine the site of origin of a labelled cell - hence the difficulty in determining the migration path of the EGL cells from their source to the cerebellar surface, and also the failure of autoradiography to solve the controversy of whether the EGL produces glial cells. Electron microscopy has been included by some authors in their investigations of the EGL (e.g. Smith, 1962; Shofer et al, 1964; Meller and Glees, 1969; Rakic, 1971; Altman, 1972a;Gona, 1978), but detailed descriptions of the morphological changes in the rat EGL in the course of development are rare (see del Cerro and Snider, 1972a). No data have been published on the quantitative electron microscopy of the EGL, and despite the fact that stereology has been criticised by a leading developmental neurobiologist (Jacobson, 1978), it is the only way of extracting objective information on ultrastructural developmental changes.

This thesis sets out to examine some of the changes in the postnatal rat EGL. It looks at the ultrastructure of the EGL cells and describes the changes that occur in them and in their relationships with each other during the course of development, using both scanning and transmission

electron microscopy. Some of these changes have been quantified, and then compared with the changes occurring in the progeny of the EGL cells - the granule neurons of the internal granular layer.

During its existence the EGL becomes subdivided into two adjoining zones. These can be most clearly seen in coronal sections in which there appears to be an outer zone consisting of round cells, and an inner zone composed of spindle-shaped cells. A currently popular schema of EGL anatomy (Altman, 1972a)makes an absolute functional distinction between these zones. The outer zone has been described as a "proliferative" zone comprising primitive, actively dividing cells, whereas the inner zone has been considered to consist entirely of postmitotic differentiating neurons. Cajal (1911) observed mitotic figures at all levels of the EGL, though he was of the opinion that mitoses in the depths of the EGL were infrequent. There is thus need to re-examine the EGL using a more critical approach than has sometimes been applied recently. Stereological analyses of the zones of rounded and elongated cells were made in order to ascertain whether there were any measurable differences between the cells of the two populations. Patterns of cell proliferation in the EGL were studied. Mitotic figures and labelled nuclei after [^{3}H] thymidine injection were counted, and their position within the EGL plotted.

The mechanisms controlling the onset, rates and cessation of cell division, differentiation and migration within the EGL are, as yet, not known. The signals which initiate such changes may come from cells within the EGL; there is some evidence that neurotransmitters may play a role as initiators of developmental events, and the function of both serotonin and dopamine

have been studied in this context (Toneby, 1977; Lauder and Krebs, 1978). However, there is greater understanding of the effects of substances synthesised and released at sites removed from the central nervous system. The most extensively studied of these substances is thyroid hormone. In both hyperthyroid and hypothyroid animals, profound changes in the cerebellar morphology, and alterations in cell acquisition rates in the EGL, have been noted (Nicholson and Altman, 1972; Lauder et al, 1974; Lewis et al, 1976; Lauder, 1979; Patel et al, 1979). Hydrocortisone is another substance that has been similarly implicated (Schapiro et al, 1973; Bohn and Lauder, 1978), and irreversible changes in neuronal morphology have been caused by alterations in the neonatal levels of androgens and oestrogens (Raisman and Field, 1973; Matsumoto and Arai, 1976). If circulating factors such as hormones play a significant role in the development and disappearance of the EGL it would seem that an understanding of the vascular supply to this layer would be of great importance. However, there have been only a few studies of the development of the intrinsic vasculature of the cerebellum. As part of this thesis the literature pertaining to this field has been reviewed, and the development of and changes in the blood supply to the rat EGL have been studied in the first three postnatal weeks.

The lack of agreement over whether the EGL is a source of glia has already been mentioned. Schaper (1894) introduced the concept of the "indifferente Zelle", a cell which has the potential to become either a nerve cell or a glial cell. Cajal (1911) never accepted this theory, and

evidence has accumulated supporting the strictly neuronal nature of the EGL cells (notably Swarz and del Cerro, 1977). However, the concept of the bipotential nature of the EGL cells has been given wide currency, particularly in the field of neuropathology. This is because the EGL is believed to be the source of medulloblastoma, a cerebellar tumour principally of children. Medulloblastomas may be composed of both neuronal and glial cell types; if the neoplastic glial cells are not derived from reactive, non-tumour elements, then it is not unreasonable to assume that the parent cells of the tumour, i.e. the EGL cells, are bipotential (Rubinstein, 1975). In view of this controversy, one of the aims of this thesis is to examine the cells of the EGL for neuronal- and glial-specific proteins, using immunocytochemical markers.

Cell death is recognised to be an important modulating factor in the developing organism (Glücksmann, 1951). Its occurrence in the EGL of the rat has been noted (del Cerro and Snider, 1972a) and partially quantified (Lewis, 1975). Since no detailed observations of regional and temporal differences in rates of cell death have been made, this thesis includes a study on the changes in distribution of cell death with time. In the subependymal layer of adult mice, metaphases were present in equal numbers to pyknotic nuclei, suggesting that newly formed cells died within the layer instead of migrating (Smart, 1961). To determine whether cell death is a mechanism for limiting cell acquisition in the EGL it is necessary to calculate the clearance rate of dead cells from the EGL. In order to do this, an experimental model for producing physiological cell death has to be established. The present thesis describes such a model, which allows a value for the mean duration of pyknosis to be calculated.

6A. HISTORICAL BACKGROUND

The presence of a layer of cells on the surface of the immature cerebellum and its absence in adult animals was first described by Hess in 1858.

Obersteiner made extensive studies of this transient layer in man and other animals (1869). Later he described the division of two strata within the EGL (which he mistakenly stated was composed of "gleia granules") (1888, 1890). As a result of his observations the EGL was known as Obersteiner's layer for many years. Lahousse (1888) and Bellonci and Stefani (1889) documented the EGL in the developing cerebellum of chickens and pigeons.

With the establishment of the basic knowledge of this layer, attention began to focus on the origin of its constituent cells. Vignal (1888, 1889) thought that the EGL cells were lymphocytes which had migrated from pial vessels infiltrating the molecular layer. Herrick (1891) proposed that the proliferating ventricular cells at the lateral caudal margins of the guinea pig cerebellum gave rise to the EGL. Schaper (1894), in morphological and histological studies of the cerebellum of teleosts (salmon and trout), concluded that newly formed cells extended out from an ependymal wedge, which was situated at the point of transition from the posterior margin of the cerebellar lamella, into the posterior medullary velum. These cells then migrated by moving medially and dorsally over the surface to cover the cerebellum. However, further speculation about the origin of the EGL had to await the development of techniques which could trace the progress of migrating cells.

Since the EGL is absent in adult animals the other focus of interest of nineteenth century workers was the fate and function of its constituent cells. Hess (1858) was of the opinion that the cells of the EGL were destroyed, and the material thus liberated formed the molecular substance. Schwalbe (1881) thought that the EGL was responsible for the formation of the radial fibres of the reticular matter (the molecular layer) of the adult cerebellum. Obersteiner suggested the superficial EGL cells might be responsible for the secretion of the basement membrane covering the surface of the cerebellum, while the deeper ones moved into the substance of the molecular layer (1888, 1890). These ideas subsequently received little attention, but an earlier proposal of Obersteiner (1883) initiated a controversy that remains as yet not fully resolved. From his observations of the two zones of the EGL he concluded that the outer zone of the EGL either became glial cells or atrophied, while the inner zone of cells migrated through the molecular layer into the internal granular layer.

Bellonci and Stefani (1889) described mitotic activity, the zoning and the disappearance of the external granular layer. They did not speculate on the origin of the molecular substance, nor did they propose a source of the neurons and glia which they observed to be disseminated throughout it. Schaper (1894) also noticed the intense mitotic activity of the EGL cells, and that it continued after the ventricular germ cells (now known as the subependymal cells) had ceased to divide. He also was aware of the presence of neurons and glia in the molecular substance, and concluded that the function of the EGL was twofold. It was responsible for building up the molecular layer,

and it produced cells which he called "indifferente Zelle" (indifferent or undifferentiated cells). Using this term he was expressing his belief that the cells of the EGL were bipotential, capable of maturing into either neurons or glia. Their final destination, Schaper (1894) proposed, was the cerebellar cortex.

Lugaro (1894a) did not wholeheartedly support this view and postulated that all the cells from the EGL were destined for the internal granular layer. However, in a second paper (1894b) he claimed to be able to identify stages in the formation of both neurons and glia from the EGL cells. This finding was independently reported by Popoff (1895, 1896) and Schaper accepted these confirmations of his "indifferente Zelle" theory (1895).

In a series of publications Cajal, (e.g. 1890a, 1890b, 1911 and in translation 1960), using the Golgi impregnation technique, gave a very thorough description of the dog, rat and rabbit EGL. He observed that it was composed of two distinct layers or strata; a superficial layer consisting of epithelioid cells and a deep layer of horizontal bipolar elements. The cells in the superficial layer rarely showed Golgi impregnation and when they did they were often irregularly stained. Some had vertically aligned projections which were short and stout, sometimes reaching to the surface of the cerebellum. Haematoxylin or carmine staining of thin sections of the cerebellum revealed the deep zone to be distinguished by a definite longitudinal striation and by a similar longitudinal orientation of the nuclei. Examining Golgi impregnated specimens Cajal described these cells as being

bipolar, with an oval, elongated body and projecting a long protoplasmic extension from each pole parallel to the surface. This description held true for young birds and mammals, and the younger the animal the thicker was the superficial layer relative to the deep layer.

Athias (1897) applied the rapid Golgi impregnation technique in order to examine the cerebellum of new born cats, rabbits, dogs, guinea pigs and mice. He confirmed Cajal's description of the EGL and was inclined to support Cajal in his opinion that all the EGL cells were destined to become neurons. In his summary of his studies on mammalian cerebellar development Cajal (1911) rejected Schaper's (1894) indifferente Zellen theory, taking the view that all the cerebellar glia were epithelial cells derived from the subependymal layer of the fourth ventricle. He examined the mitotic activity within the EGL - already documented by Herrick (1894) and Schaper (1895) - and, referring to a section of newborn rabbit cerebellum, stated that "mitoses were almost exclusively found in the most external of the peripheral strata of the peripheral germinal layer (i.e. EGL) and that the deepest cells were already a little differentiated and in consequence incapable of division henceforth". He drew particular attention to the two-stage migration of these cells : first, from the neighbourhood of the ependyma (i.e. the subependyma) to the surface of the cerebellum, where they continued to proliferate and created in consequence, new contingents of neurons, and secondly, an inward movement of germinal cells, once transformed into neuroblasts, one after another, and up to a relatively late stage, into the internal (granular) layer where they metamorphosed into adult neurons.

Little was added to Cajal's extensive descriptions of the morphology and development of the EGL until the advent and application of new techniques in the early 1960s. However, in the intervening years, interest in the EGL was re-awakened when Bailey and Cushing (1925, 1926) analysed a group of medulloblastomas, tumours which characteristically occur in the midline of the cerebellum of children. They found little evidence of differentiation, but occasionally detected groups of neuroblasts in some areas and groups of spongioblasts (glial precursor cells) and astrocytes in other areas. They formed the opinion that both types of cell were derived from the basic tumour cells, which they named medulloblasts, and thus concluded that the medulloblast was bipotential and therefore analogous to the "indifferent cell" described by Schaper (1894). In a study of human embryos Kershman (1938) referred to Bailey and Cushing's work, and also to the studies on the cerebellum of Jacob (1928) who supported the bipotential theory of Schaper - although with some reservation. Kershman was convinced however that the cells of the EGL were capable of giving rise to both neuroblasts (forming granule and basket cells) and spongioblasts (forming Bergmann cells - fibrous astrocytes - and Fañanás cells or protoplasmic astrocytes). He thus equated the medulloblast of Bailey and Cushing with the EGL cell.

It will be evident from this brief review of studies antedating World War II that the examination of stained sections of the developing cerebellum by light microscopy did not make clear the origin and fate of the EGL. The posterior tip of the root of the fourth ventricle was considered its most likely source, but the nature of the two layers within the EGL remained

unconfirmed, as did the fate of the cells. The latter was particularly significant since the majority of opinions favoured Schaper's theory of the bipotential nature of the cells, and this had not been reconciled with Cajal's emphatic assertion that the EGL only generated neurons. In the early 1960s this area of study received renewed attention through the application of two novel investigative tools : autoradiography and electron microscopy. The former enabled workers to track the pathway of migrating cells and to examine patterns of proliferation, while the latter permitted a far more accurate assessment than hitherto of the morphology and consequently the identification of the various cell types.

The application of autoradiography to tracing migration makes use of the fact that thymidine, a specific precursor of DNA, is incorporated into the nucleus of cells synthesising DNA prior to division (Amano et al, 1959; Wimber, 1963). If radioactive (usually tritiated) thymidine is injected into an animal it remains available for incorporation for a only limited period of time; in the mouse and rat this is between 30 minutes and 1 hour (Cronkite et al, 1959; Nygaard and Potter, 1959). Thus, only the cells which are replicating their DNA at the time of injection and for up to one hour afterwards will incorporate the labelled thymidine into their nucleus and become radioactive. Once the nucleus has been labelled it will remain so, and can be followed through subsequent divisions and migrations (Altman, 1964).

Uzman (1960a, b) was the first to apply this technique to the developing mouse cerebellum. He concluded that Purkinje and Golgi II cells originated in the germinal zone, migrated to the EGL where they matured and differentiated and then migrated to the Purkinje and internal granular layers

respectively. The EGL, Uzman stated, contributed the small stellate neurons of the outer two thirds of the molecular layer and he was unable to find any evidence for the granule cells having their origin in the EGL. His final conclusion was that the EGL was both neurogenic and gliogenic, the glial cells produced by the EGL having their final destination in the molecular layer (Uzman, 1960b).

Woodard (1960) examined human foetal material in order to determine the origin of the EGL. He could not use autoradiographic techniques but relied on light microscopy of postmortem material. He concluded that the EGL was not the product of cell migration but was formed by the fusion of roof-plate epithelial cells with the cerebellum. This accounted for the ability of EGL cells to form both neurons and glia, since epithelial cells are bipotential. However, this theory did not find any support. Examination of Woodard's illustrations reveals poorly preserved specimens and it is probable that what he interprets as a sheet of epithelial cells just prior to fusion with the cerebellum is in fact a damaged EGL which has peeled off from the rest of the cerebellar cortex. Miale and Sidman (1961) employing the same autoradiographic methodology as Uzman (1960a, b) and examining the cerebellum of the same animal (the mouse), reached different conclusions. While they found that Purkinje and Golgi II cells originated from the primitive ependyma they never found them occupying positions in the EGL. They stated that the EGL arose by migration from the thickened ependyma in the lateral part of the fourth ventricle out and over the surface of the cerebellum, but found no evidence to confirm Uzman's (1960b) hypothesis that the sole function of the EGL was to contribute the majority

the microneurones of the molecular layer. They argued that this was not an adequate explanation for the fate of the EGL cells since they greatly outnumbered the stellate and basket neurons of the molecular layer; thus the EGL cells would have to be otherwise accounted for as the EGL itself disappeared. Further, the number of cells in the IGL and the deep white matter, and the frequency of their mitoses, were too low to account for the large number of mature IGL cells. Their implication was that the EGL was mainly involved in contributing microneurons to the IGL. Miale and Sidman (1961) also disagreed with Uzman (1960b) over the mitotic frequency of the EGL cells. They felt that Uzman's estimate of one division every two hours was too high (in fact this frequency is unknown in any other population of vertebrate cells) and gave a more conservative estimate of one division every 24 hours with the interval between S phase and mitosis averaging five hours. They were also unable to find evidence that the EGL was gliogenic. Rather, their observations showed the EGL cells to be heavily labelled for the first two postnatal weeks and then slowly disappearing during the third postnatal week, these cells reappearing in the molecular layer and the IGL. This pattern of migration was much the same as that described by Cajal (1911), with the cells destined for the IGL passing through the molecular and Purkinje cells layers. The cells that they saw labelled in the IGL and which subsequently migrated to the Purkinje cell layer they considered to be glial cells.

This study was duplicated by Fujita et al (1966) and Fujita (1967). Miale and Sidman's (1961) observations were essentially confirmed. A comment was made on the two strata in the EGL, first described by Obersteiner (1888, 1890) and in detail by Cajal (1890); the proliferating cells in the outer zone were given the name of external matrix

cells while the inner zone was called the external mantle layer. Fujita gave a more detailed analysis of the mitotic cycle of the germinative cells in the EGL, calculating a generation time of 19 hr (G_1 8.5 hr, S 8 hr, G_2 2 hr and M 0.5 hr). Fujita and his co-workers held that their observations revealed that the external matrix cells were multipotential germinative cells. They claimed that they sequentially produced more external matrix cells, then basket and other stellate cells, neuroblasts of IGL cells and finally glial cell precursors. This was in direct contradiction to Miale and Sidman (1961) and once again opened the question of the potentiality of the EGL cells.

The EGL of animals other than the mouse began to receive attention; Altman and Das (1966) and Altman (1966) examined the rat; Hanaway (1967) the chick and Phemister and Young (1968) the dog. Altman (1966) noted that the EGL increased in thickness for several days after birth and then declined over the next three weeks until it had completely disappeared. While acknowledging that this might be a fortuitous coincidence with birth, he suggested that there might in fact be a functional relationship, with birth and a changed pattern of activity triggering the tissue changes.

Hanaway (1967) would not have agreed with this view since he found that the EGL had almost disappeared at the time of hatching, with most of the inward migration of EGL cells taking place from day 15 post-fertilisation. He was also unable to support the view that a dorsomedial migration of cells occurred along the cerebellar surface from the ventro-lateral angle of the fourth ventricle. He proposed that the EGL cells originated from the neuroepithelium bordering the ventricular surface of the cerebellar plate

and migrated from this position centrifugally to the cerebellar surface. He also added support to the theory of the bipotential nature of the EGL cells as a result of finding DNA-sythesising cells among the Purkinje and Golgi II cells. While he felt that some of these were endothelial cells, he identified the majority as glioblasts, and thus concluded that, although most of the EGL cells differentiated into neurons of the IGL, some became glial elements.

Phemister and Young (1968) made a study of the cerebellar cortical layers of the dog, and compared their findings to those previously reported. They emphasised that not only their histogenetic observations, but also their actual measurements were similar to those made in other species. These included those of Ellis (1920) and Raaf and Kernohan (1944), who examined the human cerebellum, Addison (1911), who studied the rat cerebellum, and Friede (1963), who made measurements on the cerebellum of 18 different species of vertebrate.

The developing cerebellum received the attention of the electron microscopists for the first time when Mugnaini and Forströnen (1967) gave evidence to support Cajal's (1911) observations that the EGL cells migrate into the deeper layers and that at least a number of adult IGL cells arise this way. Examining the cerebellum of chick embryos of 17 - 20 days incubation they showed a continuum of transitional forms between the cells in the EGL and the cells in the IGL. They were unable to confirm the presence of astrocytes and mitotic astroblasts in the molecular layer, thus supporting Miale and Sidman (1961). However they pointed out that their study only covered the pre-hatch period and thus were unable to exclude the possibility that the

EGL might generate glial cells in the post-hatch period. Del Cerro and Snider (1972a) in a comprehensive study of the ultrastructure of the EGL of rats also reported a number of transitional forms of cell : germinal cells, bipolar neuroblasts, and triangular cells which are bipolar neuroblasts that have formed a descending process, before the nucleus commences its downward migration. They were unable to identify apolar neuroblasts, nor could they support the claim of Meller and Glees (1969) that many glial cells can be seen migrating from the EGL. This discrepancy is ascribed to a difference in the interpretation of what appear, in the absence of any published electron microscopic evidence (Meller and Glees, 1969), to be the same data.

The many studies on the cerebellar external granular layer have left several questions still unanswered ; the origin of the constituent cells, the histogenesis of the layer, and the fate of the disappearing cells, have all remained, to some extent, subjects of controversy. The theory of Vignal (1888) that the EGL cells were supplied by blood vessels from the pia appears to have received no further consideration. Woodard's (1960) theory has been disputed by Forströnen (1963) and Phemister et al (1969b). Hanaway's (1967) theory has received little attention although Swarz and Oster-Granite (1978) have published electron micrographs showing the apparent migration of EGL - like neuroblasts from the subependyma of the fourth ventricle in apposition to radially aligned Bergmann glial fibres. Miale and Sidman's (1961) widely held view is that the EGL cells arose by migration in a mediodorsal direction from the lateral caudal cerebellar surface lining the fourth ventricle. This view is supported by Sidman and Rakic (1973) who use the term of His (1891), the rhombic lip, to describe the source of the EGL cells, and

Altman and Bayer (1978) who prefer the term germinal trigone. The actual nature of the cells in the EGL has never been convincingly established, although the consensus of opinion is that the outer zone consists of primitive germinal cells while the inner zone consists of maturing neuroblasts (Mugnaini and Forströnen, 1967, del Cerro and Snider, 1972a; Altman, 1972a; Mareš et al, 1970).

There have been several theories about the eventual fate of the EGL cells. These cells have to help to generate neurons of the internal granular layer (Athias, 1897; Cajal, 1911; Miale and Sidman, 1961; Swarz and del Cerro, 1977). They may contribute neurons and glia to the molecular layer (Uzman, 1960a, b), or they may be bipotential (Schaper, 1894; Kershman, 1938) contributing both neurons and glia to the internal granular layer (Fujita et al, 1966; Fujita, 1967; Hanaway, 1967; Meller and Glees, 1969; Privat, 1973 and 1975) (see Table 1).

Table I Does the EGL produce neuroglia?

YES	NO
1894 Schaper: EGL gave rise to glia and nerve cells	1897 Athias: EGL generated neurons for the IGL
1920 Ellis: EGL gave rise to glia and nerve cells (in the human)	1911 Cajal : confirmed Athias 1897
1938 Kershmann: the human EGL cell was a medulloblast	
1960 Uzman: EGL produced nerve cells and glia in the molecular layer (in the mouse)	1961 Miale and Sidman: EGL did not produce glia (in the mouse)
1966 Fujita: EGL produced mainly nerve cells but also a few glia (in the mouse)	
1967 Hanaway: EGL produced nerve cells and glia (in the chick)	
1969 Meller and Glees: claimed ultrastructural evidence of gliogenesis in EGL	
1973 Privat: EGL gave rise to oligodendrocytes	1972 del Cerro and Snider: EGL only produced neurons (in the rat)
1975 Privat: supported Fujita (1966)	
	1977 Swarz and del Cerro: EGL did not produce glia (in the mouse)

7. MORPHOLOGY

The experimental work presented in this thesis pertains to the EGL in rats from birth to the end of the third postnatal week. In order to put this in a wider context the following section reviews the components of the adult cerebellum (that is, the progeny of the EGL and the cells they interact with), the prenatal development of the cerebellum, including the formation of the cerebellum and EGL, and the postnatal development of the cerebellum.

A. Structure of the adult mammalian cerebellum

The adult mammalian cerebellum has four distinct layers. Starting from the pial surface and working inwards they are : molecular layer, Purkinje cell layer, internal granular layer and medullary layer (or deep white matter).

These layers are made up of six distinct types of neuron (Purkinje, basket, stellate, Lugaro, granule and Golgi cells) (Fig. 1) and four types of glial cell (Bergmann cells, smooth protoplasmic astrocytes, oligodendrocytes and microglia). The structure, function and organisation of these cells have been meticulously documented and for a detailed account the reader is referred to Palay and Chan-Palay (1974) . What follows is a summary of some of the salient features of the cerebellar constituents.

i. Purkinje cells

Palay and Chan-Palay (1974) described the Purkinje cells as being one of the most spectacular nerve cells known. They are the largest cells of the

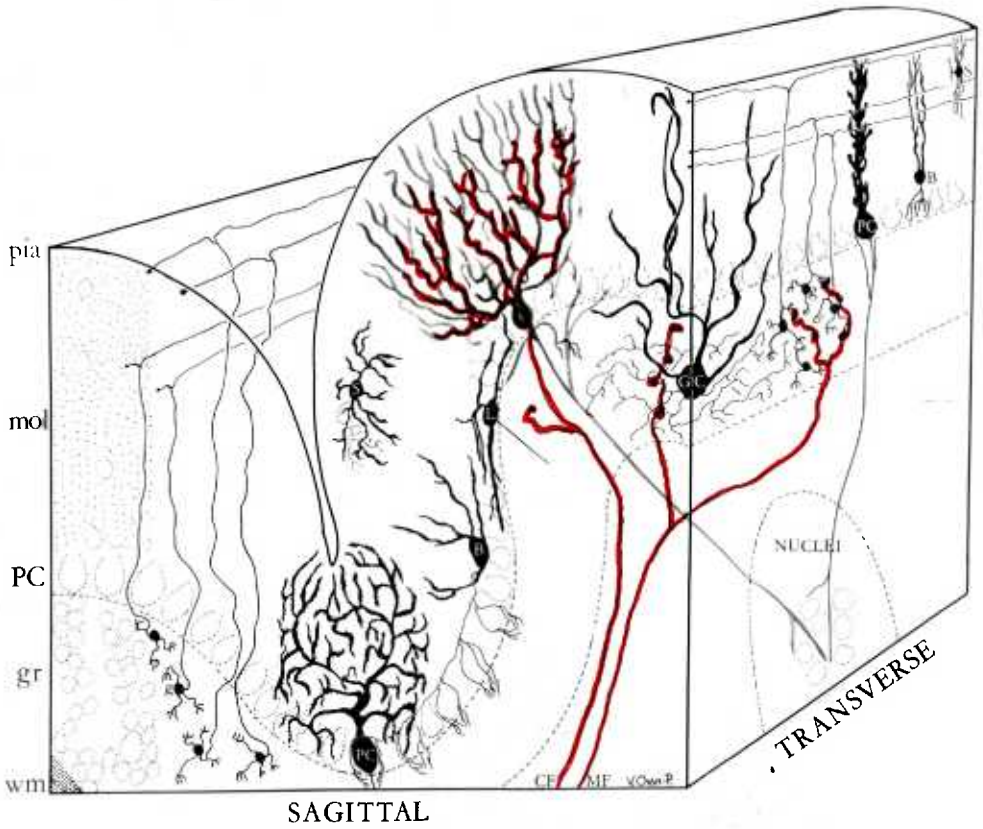

Fig. 5. The neurons of the cerebellar cortex. Beginning from the left, the boundaries and layers of the cortex are indicated: the pial surface (*pia*), molecular layer (*mol*), Purkinje cell layer (*PC*), granular layer (*gr*), and white matter (*wm*). Four granule cells (g) are shown with their ascending axons bifurcating like a *T*. The resultant branches travel parallel with the long axis of the folium in the transverse plane. Two Purkinje cells are shown in the sagittal plane (black, *PC*, and gray). A basket cell (*B*) with its axon running in the sagittal plane gives off collaterals to the Purkinje cell body. A stellate cell (*S*) and a Lugaro cell (*L*) are also shown. The second Purkinje cell (gray) is shown with the terminal arborization of a single climbing fiber (*CF*, red). The Golgi cell (*GC*) occupying the corner position in this slab of cortex has a three-dimensional axonal and dendritic plexus. The axons of the Golgi cell participate with the mossy fiber (*MF*, red) and the dendrites of granule cells in the construction of glomeruli. Three neurons, a Purkinje cell (*PC*), a basket cell (*B*), and a stellate cell (*S*) display their characteristic profiles in the transverse plane. The axons of two Purkinje cells are shown extending to the cerebellar nuclei

Fig. 1 Neurons of the cerebellar cortex from Palay and Chan-Palay (1974)

cerebellar cortex, having a diameter of 25 - 40 μm (Herndon, 1963) and are situated between the molecular layer and the IGL. They contribute the sole efferent pathway of the cerebellar cortex. In the rat there are an estimated number of 3.2×10^5 Purkinje cells (Armstrong and Schild, 1970). Golgi impregnations reveal the Purkinje cell to have an extensive dendritic tree arising from the large primary dendrite that issues from the apical pole of the soma (Cajal, 1911, Addison, 1911). This primary dendrite divides to give two or more secondary dendrites which in turn divide to yield tertiary dendrites. The resultant ramification extends vertically in two dimensions in the sagittal plane of the molecular layer (Fig. 1). It has been the subject of detailed network analyses (Berry et al, 1975; Berry, 1976; Berry and Bradley, 1976). All the dendrites give off small branches and the branches are covered with distinctive thorns or spines. It has been calculated that a single Purkinje cell of the rat may have as many as 1.8×10^4 spines on its dendrites (Palay and Chan-Palay, 1974). Through the dendritic tree of the Purkinje cell, at right angles to its plane of ramification, pass the parallel fibres of the granule cells, as many as 3×10^5 running through a single dendritic tree (Fox, 1962). Known intracortical afferent synaptic contacts with Purkinje cells are granule cells, basket cells, stellate cells, Purkinje cell axons and climbing fibres.

The axon of the Purkinje cell is myelinated. It runs out from the basal pole of the cell body, through the IGL into the white matter. The axon has recurrent collaterals, also myelinated, which extend into the IGL and the molecular layer. In the rat most of the recurrent collaterals' synapses are made on Purkinje cell somata and dendrites, with a few synaptic contacts also made with Lugaro cells (Palay and Chan-Palay, 1974). Known intra-

cortical efferent synaptic contacts of Purkinje cells are Lugaro cells, Purkinje cells, Golgi cells and granule cells.

The main ultrastructural features of the Purkinje cell are its large pale nucleus with prominent nucleolus, and organelle-rich cytoplasm. Prominent among the organelles are mitochondria, multivesicular bodies, agranular reticulum, Golgi apparatus, subsurface cisternae, lysosomes, microtubules and neurofilaments (Herndon, 1963; Palay and Chan-Palay, 1974).

The main neurotransmitter of the Purkinje cell is GABA (Curtin and Johnston, 1974; Chan-Palay et al, 1979). The Purkinje cell is also reported to have β adrenoceptors on its surface (Melamed et al, 1976; Atlas et al, 1977) although this work is now under question (Hess, 1979; Barnes et al, 1980).

ii. Basket cells

These neurons, which lie in the lower third of the molecular layer (Fig. 1) have a pyramidal or ovoid cell body about 10 μm long. They produce a fan-shaped field of four to six dendrites, spreading out vertically in the sagittal plane. The dendrites are branched and the branches are sparsely invested with spines. The afferent synaptic connections of basket cells are with granule cells, climbing fibres, basket cells and stellate cells.

The basket cell axon arises from the perikaryon or one of the major dendrites and runs horizontally in the sagittal plane through the dendritic trees of the Purkinje cells. Collaterals of the axon form a basket around the Purkinje cell soma. The efferent synaptic connections of basket cells are with Purkinje cells, basket and stellate cells.

The ultrastructural features of the basket cell are a large oval nucleus with a small nucleolus which often shows marked infolding. There is a thin rim of cytoplasm, but the eccentricity of the nucleus and the thick bases of some dendrites provide space for the organelles which include Golgi apparatus, lysosomes, vesicles, centrioles, free ribosomes and rough endoplasmic reticulum (Palay and Chan-Palay, 1974). The main neurotransmitter of basket cells may be GABA (Lasher, 1974; Burry and Lasher, 1978a, b).

iii. Stellate cells

Stellate cells are small (cell body 5 - 9 μm) polymorphous neurons lying in the outer two thirds of the molecular layer (Fig. 1). Cajal (1911) proposed a classification for stellate cells which was summarised by Palay and Chan-Palay (1974) thus :

1a) dwarf cells with spindle-shaped cell bodies, few dendrites and a thin, short axon which ramifies into a simple arborisation;

1b) larger cells with spindle-shaped stellate or ovoid cell bodies, numerous dendrites and a long axon which ramifies into an extensive network of ascending and descending collaterals. This network sometimes reaches as far as the basket cells.

2) larger cells still, with similar shaped cell bodies to 1b, long dendrites and a long axon running transversely across the folium generating a few short collaterals which end in the outer molecular layer; the main axon ends in the outer half of the molecular layer in a sparse ramification.

The afferent synaptic contacts of stellate cells are granule cells, climbing fibres, basket cells and stellate cells while the efferent synaptic contacts are Purkinje cells, basket cells and stellate cells.

Ultrastructurally stellate cells are similar to basket cells; the main differences are that the stellate cell nucleus almost fills the entire soma and that there are fewer ribosomes and rough endoplasmic reticulum cisternae in the stellate cell.

The main transmitter of the stellate cell is also probably GABA (Lasher, 1974; Burry and Lasher, 1978a, b).

iv. Lugaro cells

These are ellipsoidally shaped neurons lying just below the Purkinje cell layer (Fig. 1). Cajal (1911) suggested that this type of neuron was a variant of the Golgi cell. The cell body is about 10 μm in diameter and up to 30 μm long. Extremely long dendrites extend from the poles of the cell and follow the curve of the cerebellar folium entering the molecular and internal granular layers. The afferent synaptic contacts of the Lugaro cell axon arises from the cell body or a large dendrite and passes into the molecular layer. The efferent synaptic contacts of the Lugaro cells are not known.

Lugaro cells have the typical ultrastructure shown by large neurons. The lobulated nucleus almost completely fills the perikaryon, and possesses a prominent nucleolus. The cytoplasm, greater in volume at the poles, contains all the organelles typically found in neurons. Both the cell

body and the dendrites are partially invested with a neuroglial envelope.

V Granule cells

These are the smallest neurons of the cerebellar cortex (with a cell body of 5-9 μm), and also the most numerous with a density of 3.2×10^6 granule cells per mm^3 in the rat (Smolyaninov, 1971). The granule cells are located in the granule cell layer, below the Purkinje cell layer (Fig. 1). Granule cell dendrites are short and twisted, and terminate with branches producing a knobby inflorescence, several granule cell dendrites curving around, and synapsing with, a single mossy fibre terminal to form part of a cerebellar glomerulus. The afferent synaptic connections of the granule cells are mossy and climbing fibres, Golgi cells and Purkinje cells. The granule cell axon arises from the cell body or a vertically oriented dendrite and ascends into the molecular layer where it makes a 'T' shaped branch. The two horizontal bars of the 'T' run parallel to the long axis of the folia in the transverse (coronal) plane. These components of the granule cell axon are termed parallel fibres (Cajal, 1911). In the cat, parallel fibres average 6 mm in length, and may attain a maximum length of 7 mm (Brand et al., 1976). The efferent synaptic contacts of the granule cells are with Purkinje, basket, stellate, Lugaro and Golgi cells.

The ultrastructural appearance of the granule cell perikaryon is that of a

cell body almost filled by its nucleus which has chromatin unevenly distributed in a clockface or chessboard pattern. The rim of cytoplasm may be as thin as 0.2 - 0.3 μm (Herndon, 1964). The cytoplasm, however, possesses a full complement of organelles including mitochondria, Golgi apparatus, rough and smooth endoplasmic reticulum, lysosomes and centrioles (Herndon, 1964; del Cerro and Snider, 1972a).

The probable neurotransmitter of the granule cell is glutamate (Sandoval and Cotman, 1978).

vi. Ectopic granule cells

In the normal mammalian cerebellum, foci of granule cells have also been found at the pial surface (Chan-Palay, 1972; Lemkey-Johnson and Larramendi, 1968a; Sosa, et al, 1971; Mugnaini, 1972). These cells resemble internal granule cells and have been observed to make some normal contacts, for example with mossy fibres (Lemkey-Johnson and Larramendi, 1968a; Mugnaini, 1972). Ectopic granule cells can also be produced experimentally (Ebels, 1972; Ebels and Peters, 1974; Ebels et al, 1975; Hicks and D'Amato, 1966; Phemister et al, 1969a; Phemister et al, 1969b; Shimada and Langman, 1970; Nathanson et al, 1968). The presence of ectopic granule cells may be due to a desynchronised morphogenetic processes (Ebels, 1972) or an arrest in the migration of the last cells to descend from the EGL (Chan-Palay, 1972).

Granule cells have also been detected in cerebellar fissures of normal and experimentally treated rats (del Cerro et al, 1976; Stoughton et al, 1978).

These ectopic granule cells are well differentiated, forming synaptic contacts, and are accompanied by parallel fibres, Bergmann glia, capillaries and pericytes (Stoughton et al, 1978).

vii. Golgi cells

There are two kinds of Golgi cell : large cells (up to 24 μm long) in the upper half of the internal granular layer and small cells (as small as 10 μm long) in the lower half of the internal granular layer (Palay and Chan-Palay, 1974). The large Golgi cells have two to four main dendrites which ramify in the molecular layer to form a sparse, three dimensional dendritic tree terminating at the pial surface. The afferent synaptic contacts of the Golgi cell are mossy and climbing fibres, Purkinje, granule, stellate and basket cells. The Golgi cell axon is very distinctive. From one to three axons arise from the cell body, ramifying in the molecular layer to form part of a cerebellar glomerulus. Efferent synaptic contacts are made only with granule cells. Small Golgi cells have a similar configuration to large Golgi cells but their dendritic tree arises from a trunk radiating out from the cell body and branching only a few times. The unique feature of the small Golgi cell is the synapse en marron found on them. This is a morphologically distinct synapse made between climbing and mossy fibres and the somata of the small Golgi cells. Ultrastructurally large Golgi cells have large clear centrally-placed nuclei with numerous infoldings. A prominent nucleolus is present. The cytoplasm contains a comprehensive range of organelles. The small Golgi cells are distinguished from the large Golgi cells at the ultrastructural level by the small amount of free ribosomes

(Nissl substance), their many small mitochondria and their less elaborate Golgi apparatus.

viii. Bergmann cells

Bergmann glial cells, also known as Golgi epithelial cells, are a specialised form of astrocyte, whose soma lies between, or just below the Purkinje cell layer. Each cell projects a minimum of two processes into the molecular layer. These processes are known as Bergmann fibres. The fibres terminate with club-like swellings, known as end-feet, at the pial surface. The bundles of Bergmann fibres are usually accompanied by various ascending neural processes : granule cell axons, climbing fibres, the collateral axons of basket cells and the dendrites of Golgi and basket cells. Lamellae from these processes invest the Purkinje cell soma.

In the electron microscope a vertically oriented elongated nucleus can be seen. It is often indented but never creased. The chromatin is evenly distributed and pale. The cytoplasm is also pale and contains rough endoplasmic reticulum, glycogen granules, microtubules and filaments, free ribosomes, multivesicular bodies, mitochondria and lysosomes (Herndon, 1964). The Bergmann fibres are electron-lucent and contain only few organelles : ribosomes, mitochondria, numerous filaments, a few tubules and glycogen granules (del Cerro and Snider, 1972a).

ix. Fanañas cells

These cells, which are a sub-class of Bergmann cells, are said to appear

only in gold-sublimate preparations (Jacob, 1928). They differ from Bergmann cells only in that their cell bodies lie in the inner half of the molecular layer and in that their ascending processes do not quite reach the pial surface. Palay and Chan-Palay (1974) hold the view that the necessity for distinguishing these cells from Bergmann glial is "somewhat forced".

x. Velate protoplasmic astrocytes

These cells are found in the internal granular layer, and more rarely in the molecular layer. They have a bean-shaped nucleus in a cell body the same size as, or a little larger than the cell body of the granule cell. The processes radiate out in all directions from the cell body and are blade-like or sheet-like. They enmesh single or clustered granule cells, and often terminate on blood vessels. As distinct from the granule cell nucleus, the oval nucleus of the velate protoplasmic astrocyte has a diffuse, homogeneous chromatin distribution. Other ultrastructural features are rough endoplasmic reticulum, free ribosomes, dense bodies, a small Golgi apparatus, some mitochondria and occasionally a multivesicular body (Herndon, 1964).

xi. Smooth protoplasmic astrocytes

The two distinctions between velate and smooth astrocytes are :

1) that the smooth astrocytes are found as frequently in the molecular layer as in the internal granular layer, and

2) that their processes are not lamellar, but smooth and long. They

are similar to the fibrous astrocytes of the white matter.

Palay and Chan-Palay (1974) state that in their experience smooth protoplasmic astrocytes are much less common than the velate form.

xii. Oligodendrocytes

These glial cells are confined to the internal granular layer and, rarely, the inner third of the molecular layer. They are most common, however in the depths of the internal granular layer, near the white matter. They are small (5 - 6 μm diameter) rounded cells with a small number of slender branched processes. In the electron microscope they are easily distinguished from other cells; their nucleus is round and dense with marginated chromatin. The nucleolus is not always prominent. The cytoplasmic matrix is dark, a result of its containing fine granules (Peters, Palay and Webster, 1976). The cytoplasm contains small mitrochondria, small Golgi apparatus, free ribosomes, rough endoplasmic reticulum, lysosomes and microtubules (Herndon, 1964).

xiii. Microglia

Microglia are rarely observed in the cerebellar cortex of the normal adult rat. Cells resembling resting macrophages are found outside the brain tissue (between the basal lamina of the capillary endothelium and neural tissue). Such cells increase in numbers in the internal granular layer after a remote lesion. The ultrastructure of the cerebellar microglia is characterised by an elongated nucleus with clumped chromatin and dense cytoplasm containing

rough endoplasmic reticulum, free ribosomes, mitochrondria, dense bodies and a large Golgi apparatus (Herndon, 1964; Privat, 1975).

xiv. Mossy fibres

Mossy fibres represent one of the two fibre inputs of the cerebellar cortex, originating in the spino-cerebellar tracts and the ponto-cerebellar and vestibulo-cerebellar fibres. They are large and myelinated and enter the granular layer from the white matter in the centre of each folium. As they travel through the white matter they give off as many as 30 collaterals to the internal granular layer. The mossy fibre owes its name to the characteristic swellings that represent the terminals of the arborizations which occur along the course of the fibre in the internal granular layer, at branching points and at the terminals themselves (Cajal, 1911; Palay and Chan-Palay, 1974). These terminal knobs are the synaptic contact sites.

In the electron microscope the mossy fibre terminals appear as large (up to 5 μm diameter) presynaptic endings containing many synaptic vesicles and mitochondria, and neurofilaments. The synaptic contacts made with the granule cell dendrites that surround the mossy fibre terminals comprise the cerebellar glomerulus (Palay and Chan-Palay, 1974). The mossy fibre terminals also synapse with axons and descending dendrites and the soma of Golgi cells (Hamori and Szenthagothai, 1966b).

xv. Climbing fibres

Climbing fibres represent the second fibre input of the cerebellar cortex.

In the rat, all olivo-cerebellar fibres end as climbing fibres in the cerebellum (Desclin, 1974) while in the cat another group of fibres, originating outside the olive, interdigitate with the olivo-cerebellar fibres (Courville, 1975). Climbing fibres are thick, myelinated fibres. They give off fine collaterals (which enter the internal granular layer) as they pass through the white matter. The main stem penetrates the internal granule cell layer to reach the Purkinje cell layer. Here it loses its myelin sheath and forms a terminal arbour reaching into the molecular layer, the branches of which are intimately associated with the dendritic tree of the Purkinje cell (Fig. 1). Synaptic contacts are made between the climbing fibre and the Purkinje cell dendritic spines (Hamori and Szentagothai, 1966a). In its passage through the internal granular layer the climbing fibre produces two kinds of collateral. The first consists of varicosities connected by a fine thread; these synapse on the bodies of Golgi cells, the shafts of Golgi cell dendrites and on granule cell dendrites. The second type is a branch, as thick as the main stem which ends in a large spray of fine branches. These collaterals are surrounded by granule cell dendrites with which occasional synaptic contacts are made, forming climbing fibre glomeruli (Palay and Chan-Palay, 1974).

Ultrastructurally, the climbing fibre stem contains microtubules, long slender mitochondria and sparse smooth endoplasmic reticulum. The terminals contain synaptic vesicles, mitochondria and microtubules (Hamori and Szentagothai, 1966a).

B. Prenatal development of the cerebellum

i. Morphology

The neural tube develops into the various parts of the brain by forming thickenings followed by pocket-like invaginations. Shallow constrictions form around the neural tube to isolate three main "brain vesicles". The first (i.e. most anterior) is the prosencephalon, the antecedent of the telecephalon and diencephalon. The second is the mesencephalon, which later develops into the midbrain. The third, the rhombencephalon, gives rise to the metencephalon (the cerebellum) and the myelencephalon (the medulla oblongata) (Balinsky, 1975). The earliest stage of development of the cerebellum from the metencephalon occurs as the dorsal part of the alar plate on each side expands as the rhombic lip. The cranial part of each rhombic lip thickens, forming the cerebellar anlage (Hamilton, Boyd and Mossman, 1972). In the rat, the cerebellar anlage is first detected at embryonic day (E) 13 (Altman and Bayer, 1978). The rhombic lips then begin to project over the roof plate of the fourth ventricle, eventually approaching each other at the midline where they fuse, producing the cerebellar plate. This forms into a small central portion (the vermis), on either side of which a larger lateral portion (the cerebellar hemispheres) develops.

At this stage fissures begin to appear, the first being the posterolateral fissure, which separates the flocculonodular lobe from the corpus cerebelli and is visible in the 24 mm long embryo of the white rat. When the

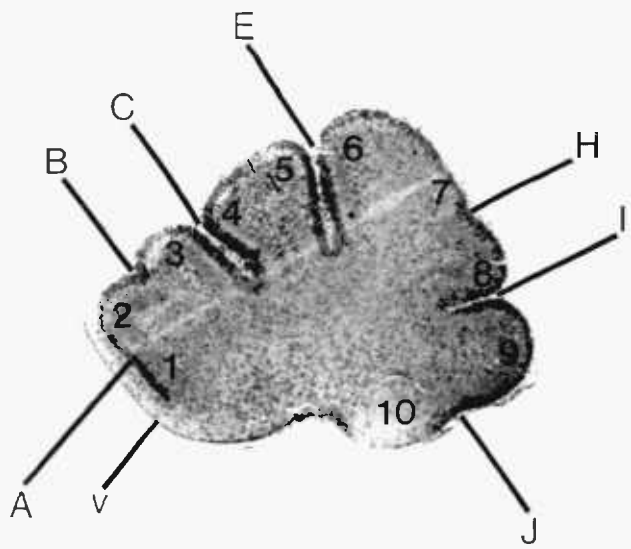

Fig. 2 The cerebellum of the newborn rat.
Mid-sagittal section Vermian lobules.
Toluidine blue stained 1 μm resin section (x 37)
(for key see Fig. 4)

embryo reaches 28 - 30 mm in length, transverse folds develop in the medial part of the corpus cerebelli. The preculminata and prima fissura develop from these furrows (Aciron, 1950) (Fig. 2). According to Komeliussen (1968) the first fissure to develop is the sulcus anonymus, while the posterolateral fissure develops later. The human vermis begins to undergo foliation by 13 - 14 weeks of estimated gestational age, and the major fissures isolating the lobules appear about a week later (Loeser et al, 1972).

ii. Histogenesis

In order to clarify the nomenclature of the different layers and cells of the developing nervous system, the following zones have been distinguished :

1) The ventricular zone, which lines the ventricular system during early development and contains the nuclei of proliferating neuroepithelial cells; these show inward and outward movement.

2) The marginal zone, which lies external to the ventricular zone, and contains cell nuclei which have ceased movement.

3) The intermediate zone, which develops between the ventricular and marginal zones and contains the soma of postmitotic cells.

4) The subventricular zone, which develops between the ventricular and intermediate zones and contains proliferating cells. The nuclei of these cells, unlike those of the ventricular zone do not move during their mitotic cycle (Boulder Committee, 1970; Sidman, 1970).

This terminology relates primarily to the cerebral cortex and the cerebellum deviates from this schema in two important aspects : its has no marginal zone, but possesses a second germinal layer, similar to the subventricular zones, which is termed the external granular layer.

At embryonic day (E) 13 the cerebellar anlage of the rat may be seen as composed of primitive cells, those nearest the lumen showing mitotic activity. X-irradiation, by supposedly destroying proliferating and migrating cells, permits the identification of three zones; a zone of radio-resistant cells, an intermediate zone of pyknotic cells and a superficial zone of radio-resistant cells (Altman and Bayer, 1978). The Purkinje cells are formed mainly on days E14 and E15 (contrasting with Miale and Sidman's (1961) observations that in the mouse the bulk of the Purkinje cells are generated between days E11 and E13 and migrate out from the ventricular layer to the cerebellar parenchyma). Golgi cell differentiation commences on E19 and continues slowly until after birth (Altman and Bayer, 1978).

The external granular layer (EGL) arises from the subventricular layer. It is the future source of basket, stellate and above all internal granule cells (Cajal, 1911; Mugnaini and Forströnen, 1967; Altman, 1969). The path by which the cells which will constitute the EGL reach the surface of the cerebellar surface has been subject of some dispute. Woodard (1960), from his studies on human foetal material, proposed the hypothesis that the EGL was formed by the apposition of the roof plate epithelial cells to those of the intraventricular cerebellar surfaces, followed by the occlusion of the intraventricular space between them. This view has been rejected by

Forströnen (1963) and Phemister et al (1969 a and b). Miale and Sidman (1961) proposed that in the mouse the EGL cells arose from the proliferation of subventricular cells in the lateral caudal portion of the cerebellar surface lining the fourth ventricle. Beginning at E13 these cells migrated over the cerebellar surface, which became entirely covered by E15. Altman and Bayer (1978) were essentially in agreement with this view. They observed that in the wedge-shaped posteroventral margin and the lateral protuberance of the cerebellum the neuroepithelium had three prongs. The inferior prong produced the choroid plexus, the horizontal prong was the regressive ventral portion of the cerebellar neuroepithelium and the superior prong gave rise to the EGL. The point of origin of the three prongs they called the germinal trigone, which (they wrote) might be identical with the rhombic lip. They pointed out that if that were the case, their observations do not support the classic hypothesis that all the neurons of the cerebellum derived from the rhombic lip, since the "rhombic lip" (their germinal trigone) formed on E17, which is after the production of both the Purkinje cells and the deep nuclear neurons. The EGL, they observed, arose on E17 and had completely covered the cerebellar surface by E20. That this does not agree exactly with the observations of Miale and Sidman (1961) may be due to species-specific differences. The proposed pattern of migration of the EGL cells from the germinal trigone is that the cells which are situated laterally move medially over the cerebellar hemispheres, while the caudally situated cells move in a rostral direction over the future vermis (Altman and Bayer, 1978). How this migration takes place is not yet known. The two major facts contradicting this line of

argument are that no tangentially oriented cellular guideshave ever been described and that the migrating EGL cells are round, and do not exhibit the usual fusiform morphology of migrating cells. However, the migration process may be one in which the daughter cells are passively pushed over the cerebellar surface as new cells are produced (Sidman and Rakic, 1973).

The third possibility was one put forward by Hanaway (1967), who examined the chick cerebellum. He suggested a radial migration of neuroepithelial cells from the intermediate layer directly to the cerebellar surface. This theory has not received much support in the literature but recent observations by Swarz and Oster-Granite (1978) might be interpreted as confirmation of a radial migration. They describe glial cells, the perikarya of which lie in the ventricular and subventricular zones of the embryonic mouse cerebellum. These glial cells have radial processes which extend to the pial surface. Juxtaposed along the length of the glial processes and sometimes attached to them by punctata adhaerentia are unidentified cells which they suggest might be neuroblasts. At the ages examined (E13 - E15) the EGL is only in the process of formation, and there is little possibility that these cells could be descending granule cells. However, these cells could be subventricular cells ascending to form the EGL. Hanaway's (1967) arguments for the origin of the EGL have however been emphatically refuted by Altman and Bayer (1978) and Gona (1976), who observed that in the developing bullfrog cerebellum, the EGL cells orginated and migrated from the ependyma of the free edge of the cerebellar plate and the medial regions, where the two lobes of the plate are fused.

In the matter of prenatal gliogenesis in the cerebellum there is also some conflict of opinion. Ishida (1973) found electron-lucent processes in the embryonic chick EGL with features characteristic of Bergmann glial fibres. He suggested that the Bergmann astrocytes of the chick cerebellum were derived from the outermost EGL cells. Prenatal gliogenesis has been described in primates (Rakic, 1971; Antanitus et al, 1976), chickens (Ishida, 1973), man (Choi and Lapham, 1978) and rodents (del Cerro and Swarz, 1976). However, Das and his colleagues (Das et al, 1974; Das, 1976, 1977) claim that gliogenesis in the cerebellum only occurs postnatally. In their study of the embryonic rat cerbellum Altman and Bayer (1978) suggested that the prenatal cerebellar glial derived from the radioresistant zone of regressing neuroepithelium. However, they stated that glia were very rarely labelled because of their continuing proliferation. This would explain the discrepancy between the findings of Das and those of other workers with regard to the time of origin of the Bergmann glia.

iii. Synaptogenesis

Although synaptogenesis has been studied primarily as a postnatal event (Larramendi, 1969; Woodard et al, 1971; Altman, 1972b), there is a growing volume of evidence that synapses form early in prenatal life throughout the CNS. Thus, synaptogenesis in the chick spinal cord is detectable from 45 hours after incubation (Oppenheim and Foelix, 1972). In the chick ciliary ganglion synapses begin to function from five days of incubation and show 100 per cent transmission by eight days (Landmesser and Pilar,

1972 and 1974a). In the human foetal brain cortical synapses have been detected as early as 8.5 weeks of gestation (Molliver et al, 1973). In the cerebellum prenatal synaptogenesis has also been reported. In the monkey, synaptic contacts were observed on the soma and axons of the deep cerebellar nuclei, and climbing fibres formed baskets around Purkinje cells by E75 (Komguth et al, 1968). In the chick very few axo-dendritic synapses were seen in the molecular layer at 8 days of incubation while the first axo-somatic synapses on a Purkinje cell were noted half a day later (Foelix and Oppenheim, 1974). Del Cerro and Snider (1972b) showed the presence of synapses in the molecular layer of the rat cerebellum from 15 min after birth, suggesting that their actual development took place prenatally. This was confirmed when synapses were found in the molecular layer throughout the entire rostro-caudal extent of the rat vermis (West and del Cerro, 1976). The failure of earlier workers to find prenatal synaptogenesis (Woodward et al, 1971) may have been due to the discovery that aldehyde-osmium stained synapses were invariably more frequent than those stained by ethanolic phosphotungstic acid. It was concluded that the latter pattern of staining represented a later stage in synaptic development.

In conclusion, while most of the growth and development of the cerebellum takes place in the rat after birth, key events occur prenatally; the proliferation, migration and differentiation of EGL cells, Purkinje and Golgi cell precursors are prenatal events, and both gliogenesis and synaptogenesis may also be initiated before birth. However, there remain many unanswered questions and conflicting findings which are beyond the scope

of this thesis to answer; for example, what is the source of the EGL cells and in what manner do they migrate to the pial surface? When does gliogenesis occur? What is the source of the Bergmann glial cells? Is cerebellar synaptogenesis a major prenatal process?

C. Postnatal development of the mammalian cerebellum

i. Morphology

During postnatal development the two most obvious changes in the gross morphology of the mammalian cerebellum are increase in size and the development of the ten major cortical lobules. Fissura preculminata divides the anterior lobe into dorsal and ventral segments. The vermal parts of these segments correspond respectively to the culmen and central lobules. Subsequently the ventral segment is divided by the precentral and intracentral fissures. The anterior lobe is in this way divided into lobules I, II and III. By 20 hours after birth, the prepyramidal fissure has formed, separating lobule VIII from the rostral part of the vermis, and at the same time lobule VII becomes delimited from lobule VI as the posterior superior fissure is formed. By the second postnatal day (Fig. 3) the intraculminate fissure divides the dorsal segment. Together with the already-formed preculminate and primary fissures, this forms lobules IV and V. The posterior lobe is first divided by the fissura secunda, the third fissure to form after preculminata and prima. Fissura secunda separates lobules IX and VIII. The posterolateral fissure separates lobules IX and X (the flocculonodular lobe) (Zeman and Innes, 1963; Larsell, 1970).

The two day old rat cerebellum possesses all of its subdivisions. From this time on, the major changes are an increase in the definition of the sulci and gyri, and an increase in the overall size of the cerebellum, illustrated in figure 4. It should be noted that here the cerebellar lobules

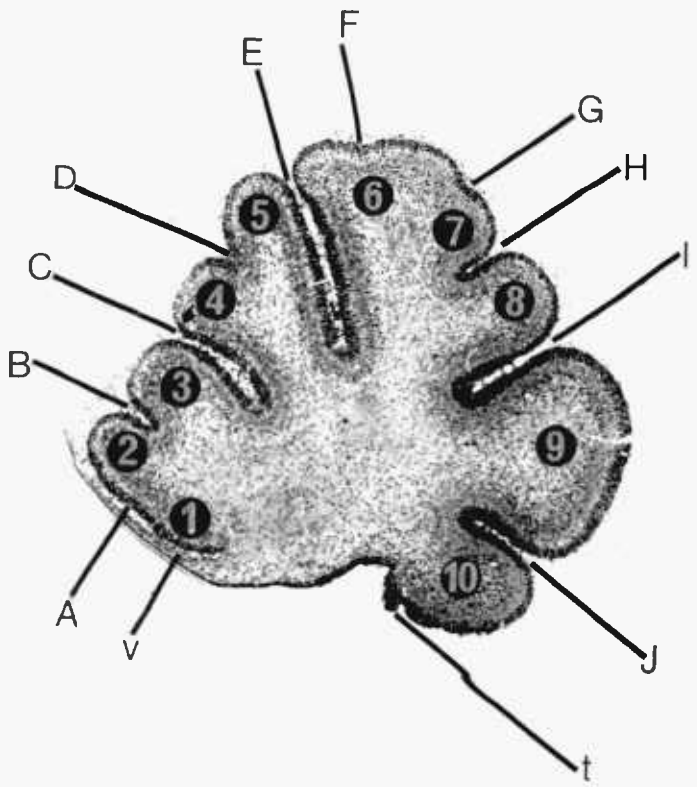

Fig. 3 Cerebellum of 2 day old rat
Mid-sagittal section vermian lobules.
Toluidine blue stained 1 μm resin section (x 37)
(for key see Fig. 4)

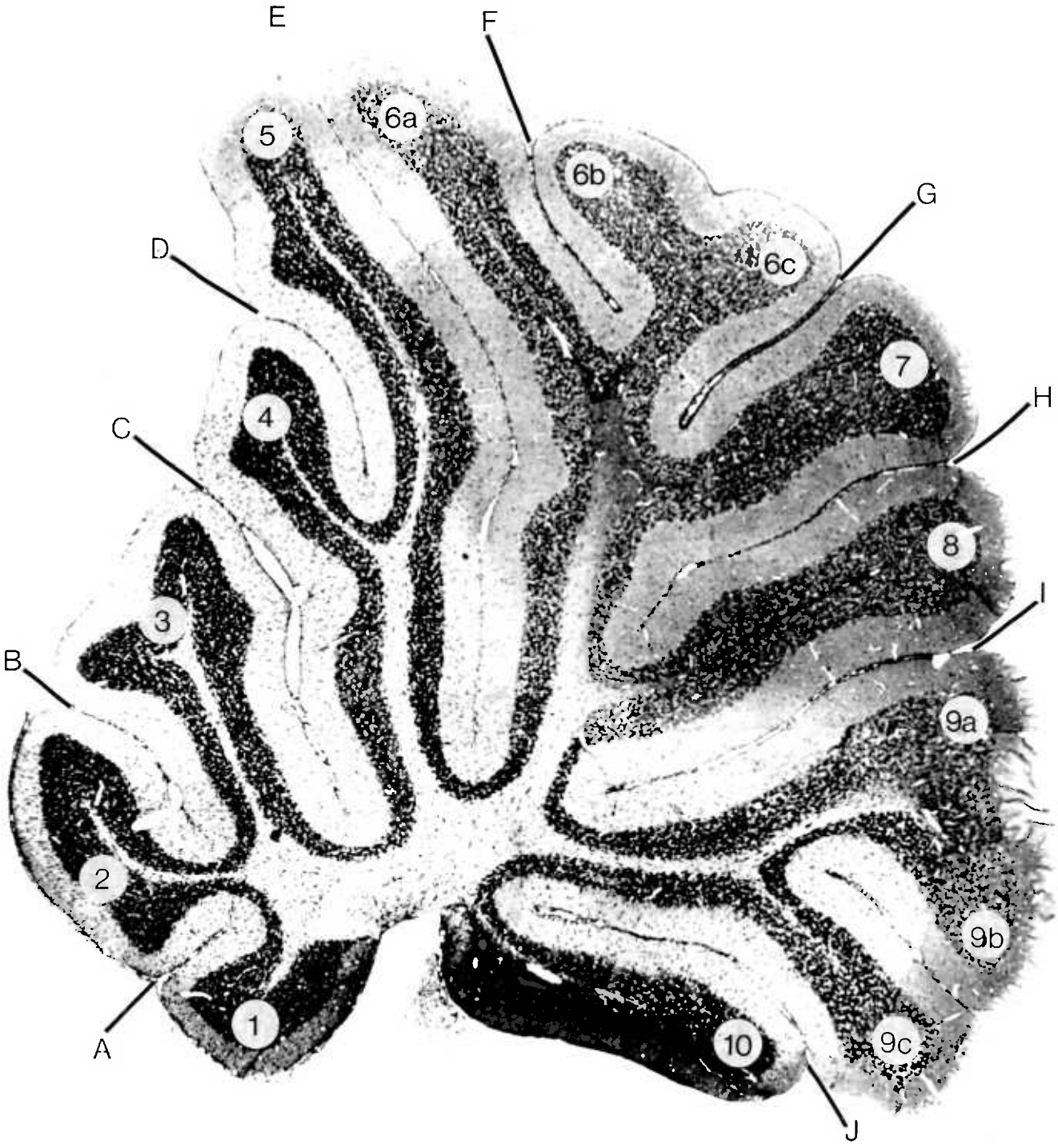

Fig. 4 Cerebellum of 21 day old rat

(for details please turn over)

Fig. 4 (details contd.)

Mid-sagittal section vermian lobules. Toluidine blue stained 1 μm resin section (x 37)

Key

1. Lobule I, lingula
2. Lobule II, ventral lobule of lobulus centralis
3. Lobule III, dorsal lobule of lobulus centralis
4. Lobule IV, ventral lobule of culmen
5. Lobule V, dorsal lobule of culmen
6. Lobule VI, declive
7. Lobule VII, tuber
8. Lobule VIII, pyramus
9. Lobule IX, uvula
10. Lobule X, nodulus

A. precentral fissure a
B. precentral fissure
C. preculminate fissure
D. intraculminate fissure
E. fissura prima
F. declival sulci 2
G. posterior superior fissure
H. prepyramidal fissure
I. fissure secunda
J. posterolateral fissure
t. telachoroidea
v. anterior medullary velum

Table II Nomenclature of the vermian lobules of the rat cerebellum

(from Larsell 1970)

Lobule number	Lobule name
I	Lingula
II	ventral lobule of lobululus centralis
IIa, IIb	sub lobules a and b
III	dorsal lobule of lobulus centralis
IIIa, IIIb	sub lobules a and b
IV	ventral lobule of culmen
V	dorsal lobule of culmen
VI	declive
VIa, VIb, VIc, VId	sub lobules a, b, c, and d
VII	tuber
VIIa, VIIb	sub lobules a and b
VIII	pyramus
IX	uvula
IXa, IXb, IXc, IXd	sub lobules a, b, c, and d
X	nodulus

have been numbered according to Larsell (1970). For their corresponding names see Table II.

ii. Development of EGL

The EGL is a layer of proliferating cells covering the pial surface of the cerebellum. Its presence is temporary (undisputed since Hess, 1858) but the time taken for it to disappear varies in different species. In the chick it has almost disappeared by hatching (Mugnaini and Forströnen, 1967), while in man it persists for up to 18 months after birth (Raaf and Kernohan, 1944; Gadsdon and Emery, 1976). In the rat it remains for 21 days after birth (e.g. Altman, 1972a) (see Table III).

In coronal sections of the cerebellum, two morphologically distinct zones are often recognisable in the EGL (Cajal, 1911; Mareš et al, 1970; Altman, 1972a). From Fig. 5 it can be seen that in the newborn rabbit cerebellum there is a layer, three or four cells deep with irregular rounded nuclei, amidst which can be seen several mitotic figures (A). Below this there is another layer, also three or four cells deep, in which the nuclei are elongated in the horizontal plane. Mitoses are less frequent among these cells. Below this layer, in the molecular layer, there are cells with nuclei which are elongated in the certical plane. From his studies of Golgi impregnated material from animals of several postnatal ages, Cajal (1911) proposed a sequence of events (shown compositely in Fig. 6). The cells in the upper zone of the EGL are only infrequently impregnated, but when visualised are seen to possess a short process reaching vertically to

Table III The duration of the external granular layer in various species of animal

Species	Duration of EGL	Source
trout	several months, into adulthood	Pouwels (1978 a-e)
frog		Gona (1976)
chick	day 18-20 of incubation*	Hanaway (1967)
	post hatching*	Mugnaini and Forströnen (1967)
	2nd day post hatching*	Ishida (1973)
mouse	20 days after birth	Fujita et al (1967)
rat	22+ days after birth	Addison (1911)
	21 days after birth	Altman (1972a)
rabbit	90 days after birth	Smith (1962)
cat	end of 2nd month after birth	Shofer et al (1964)
	9+ weeks after birth*	Smith and Downs (1978)
dog	10+ weeks after birth	Phemister and Young (1968)
monkey	3rd month after birth	Rakic (1971)
man	10-12 months after birth	Ellis (1920)
	18-20 months after birth	Raaf and Kernohan (1944)
	12-18 months after birth	Gadsdon and Emery (1976)

*EGL persisting in oldest animals examined

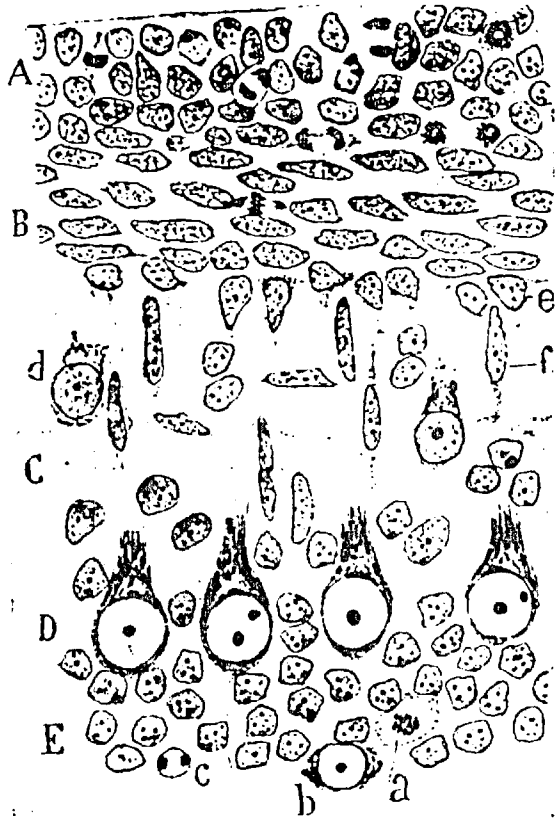

Fig. 135. Section of a cerebellar convolution of a newborn rabbit. Nissl method. A) Zone of indifferent cells, some cells of which are undergoing mitosis; B) zone of fusiform horizontal cells; C) plexiform zone, D) zone of Purkinje cells; E) granular zone; a) dividing cell in the midst of the granules; b) Golgi cell; f) fusiform granule cell migrating towards the granular zone (E).

Fig. 5 The cerebellar cortex of a newborn rabbit.

Note the relatively deep zone of elongated cells of the EGL (B) amongst which there can be seen a mitotic figure. Nissl stained coronal section. (From Cajal, 1960).

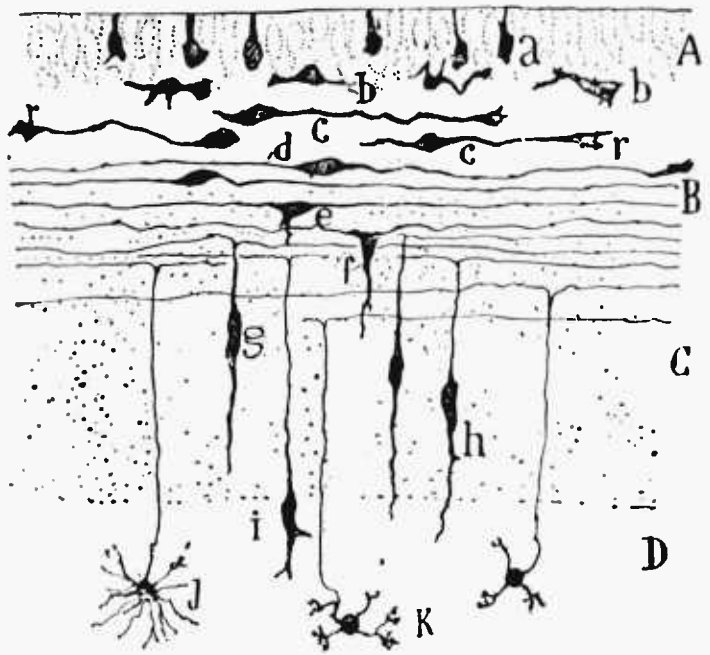

Fig. 134. Schematic figure to show all the forms and locations adopted by the granule cells during their development. A) Indifferent cell layer; B) layer of granule cells in the horizontal bipolar stage; C) plexiform layer; D) granular layer; g, h) stage of vertical bipolarity; i, j) embryonic granule cells; k) almost fully-developed granule cell.

Fig. 6 The development of the granule cell.

Composite picture taken from Golgi stained preparations of cerebella of rats at various postnatal ages to show the development of the granule cells. (from Cajal, 1960).

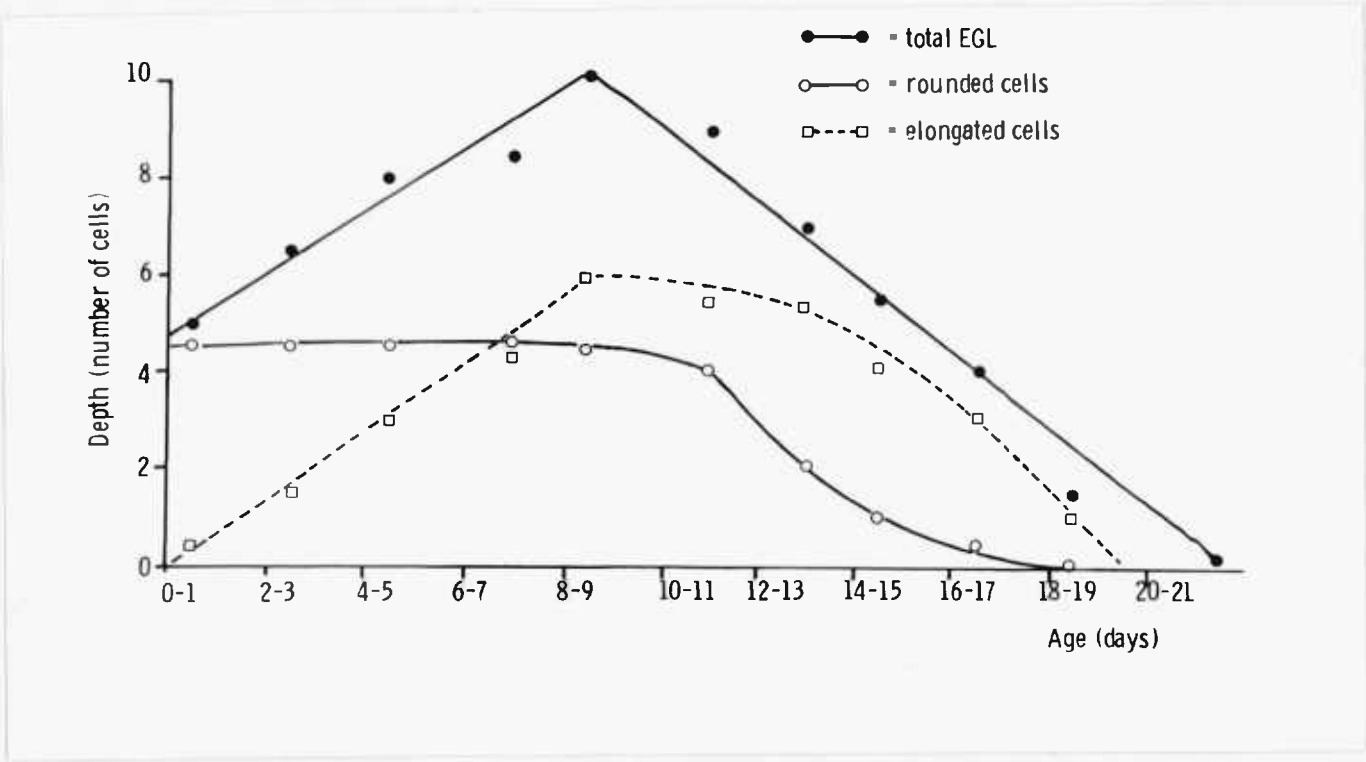

Fig. 7 Changes in the depth of the rat external granular layer and its zones as a function of age.

(redrawn from Altman, 1972a)

the pial surface. Below these is a zone of frequently impregnated cells with a horizontal orientation. As these cells mature they extend horizontally first one process, becoming unipolar, and then a second process at the opposite pole to become bipolar cells. Then, below the nucleus, a dendrite is extended vertically downwards and this process eventually traverses the molecular layer to reach the internal granular layer. The nucleus travels down the dendrite into the IGL and the cell matures into a granule cell.

Altman's (1972a) description of the developing rat cerebellum provided the foundation of the current schema of the EGL (see for example Eccles, 1977). He added to, and modified Cajal's (1911) observations in many ways. At the light microscope level, from a study of cresyl violet and haematoxylin stained 6 μm sections he described the EGL of newborn rats as consisting of rounded, darkly staining cells showing mitotic activity throughout the layer. This layer, which he termed the proliferative zone, remained constant in thickness until about the tenth postnatal day, when it began to decrease, disappearing by about postnatal day 19 (Fig. 7). From birth the appearance of a second, deeper layer was noted. The cells of this layer, termed by Altman the premigratory zone, were described in sagittal sections as being smaller than the cells of the proliferative zone, while in coronal section they had an elongated, spindle-shaped appearance. This zone increased in thickness at a linear rate until about the eighth postnatal day after which it decreased in thickness, at a slower rate than the proliferative zone until it too has disappeared by about postnatal day 20. Unlike Cajal

(1911), Altman (1972a) was unable to detect any mitotic figures amongst the fusiform cells of the premigratory zone.

The published ultrastructural studies on the EGL cells do not appear to make more than the most subtle distinctions between the cells of the two zones. Altman (1972a) omitted to describe the ultrastructure of the pre-migratory cells separately from proliferative cells, which he observed to show similar characteristics from birth and throughout the later stages of development. These characteristics were : a polymorphous nucleus from 4 to 5 µm in diameter; a thin rim of perykaryon sometimes with irregular protrusions, rich in free ribosomes, containing mitochondria and a Golgi apparatus but without rough endoplasmic reticulum and microtubules. Basal bodies and cilia were often seen, but coated vesicles and multivesicular bodies were rarely found. The cells were either contiguous with each other or separated by intercellular spaces of various sizes. Infrequently desmosomal junctions between the cells were seen but never synapses. Del Cerro and Snider (1972a) are largely in agreement with Altman (1972a) in their ultrastructural observations though they are at odds in some other respects. They reported the EGL as passing through three phases. The first phase, lasting to postnatal day 5, was characterised by a homogeneous population of germinal cells in the EGL. The second phase lasted until postnatal day 17; neuroblasts started to accumulate below the germinal cells; this corresponded to the formation of the "premigratory zone". The third phase, lasting from postnatal day 17 until the disappearance of the EGL cells at postnatal day 20, was the period in which proliferation of germinal cells and formation of

neuroblasts ended. However, the ultrastructural observations of del Cerro and Snider confirmed those of Altman (1972a). They reported a thin rim of cytoplasm around the nucleus, the presence of cilia, specialised membrane contacts, and the absence of synaptic junctions. At variance with Altman (1972a), they suggested a gradient of organelle differentiation between the outermost and the innermost cells of the EGL starting at about postnatal days 7 - 8, increasing numbers of mitochondria, increasing volume of cytoplasm and the appearance of neurotubules being noted. However, the most striking feature was the change of shape - the flattening of cells. These flattened cells were identified as neuroblasts by del Cerro and Snider. They also observed the existence of a true neuropil separating clusters of cells. This is not detectable at the light microscope level, although it had been previously described (Mareš et al, 1970).

Ultrastructural studies of the EGL in species other than the rat may not be entirely relevant since in addition to the artifactual and interpretative differences which may account for conflicting observations, there may also be species-specific differences. Most prominent of these are of course the differences in the postnatal survival of the EGL for different species (see Table III).

As well as generating cortical microneurons the EGL may well be at least partly responsible for the dramatic folding that the cerebellum undergoes. Studies in mice have shown that the cerebellar cortex grows about four times faster than the white matter as a result of the increase in the production of cells by the EGL; this in turn increases the volume of the

molecular layer and IGL (Haddara and Noereddin, 1966). The mechanism by which this might be achieved was suggested by Mareš and Lodin (1970), who found that in the mouse the average rate of proliferation in the fissures was higher (by 38 per cent in two-day old animals and 13 per cent in seven-day old animals) than at the more superficial parts of the gyri. This increase in proliferation rate was partly caused by a reduction in cell cycle times (a 10 per cent reduction in the two-day old animals, 9 per cent in the seven-day old animals). Foliation might also be caused in part by differential rates of cell death. The relationship between the EGL and cerebellar foliation is well demonstrated in experimentally induced hypo- and hyperthyroidism in postnatal rats. Hyperthyroidism leads, among other things, to an early termination of cell proliferation in, and disappearance of the EGL. This in turn appears to cause fewer fissures in the cerebellum, although the fissues are of normal depth. Hypothyroidism prolongs cell proliferation in the EGL and retards its disappearance (amongst its several effects), with a concomitant increase of the number of fissures which are of decreased depth (Nicholson and Altman, 1972; Lauder et al, 1974).

iii. Development of basket and stellate cells

The production of these cerebellar interneurons by the EGL and their subsequent migration was first described by Cajal (1890, 1911, 1960) and Athias (1897). These light microscopic observations were later confirmed by electron microscopy (Larramendi, 1969; Mugnaini, 1969; Rakic, 1972b and 1973) and by autoradiography (Miale and Sidman, 1961; Fujita et al,

1966; Altman, 1969). In the monkey the production of stellate and basket cells is mainly a prenatal event (Rakic, 1973) whereas in rodents it is exclusively a postnatal one (Miale and Sidman, 1961; Fujita, 1967; Altman, 1969).

Developing basket cells are distinguished by their fusiform shape and two polar projections, one short and thick, the other thinner and often possessing a growth cone. The basket cells sink gradually until they reach their final destination, with the earliest generated cells being distributed in the deeper reaches of the molecular layer, and the progressively older cells tending to be more superficially situated. Having reached their destination the basket cells extend their dendrites which ascend toward the pial surface. The axon elongates, and forms collaterals around the Purkinje cell soma. In the rat, basket cell production takes place mainly during the first postnatal week.

Stellate cell production is a similar process but it takes place after most of the basket cells have been generated, in the rat reaching a peak toward the end of the second postnatal week. The stellate cells are also bipolar and also appear to sink into the molecular layer at which point their dendrites grow both upward and downward to make contact with the parallel fibres.

iv. Organization and maturation of Purkinje cells

Purkinje cells are generated prenatally in the rat (Altman and Bayer, 1978) and, like many other nerve cells are presumed never to divide after birth, except allegedly under pathological conditions (see Blackwood, 1976). However, spatial reorganisations take place postnatally and the cells mature.

The Purkinje cells of the neonate rat are arranged in a layer two or three cells deep. The basal pole projects the axon while the apical pole projects short fine processes into the molecular layer (Addison, 1911). By the fifth postnatal day the Purkinje cells have been organised into their adult configuration of a layer one cell deep. The dendritic tree arborises over the first three weeks of postnatal life as the apical projections thicken and ramify (Cajal, 1911; Altman, 1972b). The axon produces its collaterals which extend into the lower molecular layer and the upper internal granular layer (Cajal, 1911).

v. Development of Golgi II cells

Golgi cell differentiation in the rat commences on E19 and continues slowly until after birth; the Golgi cells which are formed as late as two days postnatally were often found to be located superficially, near the Purkinje cell layer (Altman and Bayer, 1978). At birth the cells are bipolar and are located in the internal granular layer. The varicose axon, of variable length, projects into the internal granular layer, often projecting collaterals. The apical layer extends into the molecular layer. With increasing age, the cell body increases in volume, the axon elongates and ramifies and the dendrite arborises, acquiring spines, until it eventually reaches the pial surface (Cajal, 1911).

vi. Development of granule cells

As previously described the granule cells are a product of the external

granular layer and migrate to their destination through the molecular and Purkinje cell layers (Cajal, 1911; Miale and Sidman, 1961; Mugnaini and Forströnen, 1967; Mugnaini, 1969; Altman, 1969, 1972a, 1972b; Smith and Downs, 1978). The bulk of the granule cells are formed during the second and third postnatal weeks in the rat (Altman, 1969). The migration of the developing granule cells has raised the question of how the cells are able to thread their way through the developing neuropil that separates their source from their destination. During the period that the cells are making their journey basket cells and stellate cells are developing, and parallel fibres are accumulating, while the dendritic trees of Purkinje cells contribute to the obstacles in the migratory path. Studying the monkey cerebellum, which, with its relatively slow development, permitted easier recognition of cells and their interrelationships, Rakic (1971) observed that migrating granule cells were directly opposed to Bergmann fibres (which were present, from E80, in all ages studied). He, therefore, proposed that the Bergmann fibres act as guides for the migrating granulecells, avoiding potential obstacles. Since such a large number of granule cells migrate (Rakic, 1971 cites a figure of 1.7 billion in Macacus) each Bergmann fibre would have to serve for several generations of granule cells, and this observation was also made. A theory of glial guidance mechanism was later proposed for the neocortex (Rakic, 1972a; Sidman and Rakic, 1973; Meller and Tzetzlaff, 1975).

However, the concept of such a mechanism has not found universal acceptance. Das et al (1974), using autoradiographic techniques, were unable to demonstrate Bergmann fibres in the rat cerebellum before postnatal day nine,

despite Cajal's (1911) observation of them in Golgi-stained sections of the cerebellum of newborn mice. As a result an alternative guidance mechanism had to be proposed to account for the migration of the granule cells occurring before postnatal day nine. They thus proposed that until the Bergmann fibres developed, the migrating cells could travel down the radially oriented processes of almost any other cell, for example Purkinje cell dendrites and other migrating granule cells, as well as Purkinje cell somata. However, the absence of Bergmann fibres noted by Das and his co-workers in the first postnatal week is probably due to the difficulty of labelling glia as their rapid proliferation quickly dilutes out the label (Altman and Bayer, 1978). In contrast to the observations of Das et al, 1974, radial glial fibres have been detected in the human foetal cerebellum as early as 7 - 10 weeks of gestation (Choi and Lapham, 1978) and in the E13 - 15 mouse cerebellum (Swarz and Oster Granite, 1978), though Bignami and Dahl (1973), using an indirect immunofluorescence technique, were unable to detect astrocyte-specific protein in the rat cerebellar cortex for the first three postnatal days. The other criticism of the hypothesis of neuronal processes acting as guides (Das et al, 1974) is that the data they presented showed material cut only in the sagittal plane. This means that the true relationship between the migrating cells and the processes to which they are apposed cannot be determined. That is, a migrating cell may be in close contact with a neuronal process (dendrite), but from sections cut in only one plane it cannot be stated unequivocally that the cell is wrapped around the process and is using it as a guide.

Hattori and Fujita (1974) used scanning electron microscopy to study the developing cerebral cortex of foetal mice. They challenged the idea that the fibres along which the migrating neurons were travelling were glial in origin, claiming that the fibrous elements were the thinned processes of matrix cells. Altman (1975) suggested that in addition to using the Bergmann fibres as guides, the migrating cells might use their own processes, extended ahead of the descending nuclei, in order to reach their destinations. Certainly it is not disputed that descending granule cells have a cytoplasmic process which extends beyond the nucleus. However, Altman's (1975) light microscopic data are not sufficient to confirm that vertically projected processes are separate from the Bergmann fibres, as distinct from being in apposition to them, which would confirm the observations of Rakic (1971).

vii. Development of glia

The main emphasis in the studies of postnatal histogenesis of the cerebellar cortex has fallen on the production of neurons; gliogenesis has received relatively little attention.

The question of whether the EGL cells are capable of producing glial cells as well as neurons has already been mentioned, as has the controversy over postnatal production of Bergmann glial fibres. The origin of the Bergmann glial cells themselves remains unsolved; Ishida (1973) and Das (1976) are of the opinion that they are produced by the EGL. Basco et al (1977) suggested that the Bergmann glial cell bodies are, in the first postnatal week

of the rat, in the internal granular layer and furthermore that the cell bodies are capable of proliferation even though their fibre network is well established. Only after the first week do the cell bodies begin to appear in the region of the Purkinje cells. This would explain the failure of Das et al (1974) to detect Bergmann glia during the first post-natal week. Lewis et al (1977) support the view that the cerebellar astrocytes are in the main formed before the granule cells. The site of origin of these cells is not known, but the subependymal layer is most probably the source of at least some of the astrocytes of the cerebellar cortex as well as the oligodendrocytes, though probably not the microglia (Privat, 1975).

8. MATERIALS AND METHODS

A. Animals

Albino Wistar rats were housed in breeding cages each containing one male and five females. The female rats were examined daily for the presence of both a vaginal plug and sperm in the vaginal tract. The latter were taken as evidence of successful mating, and successfully mated females were placed in isolation in littering cages. The day of mating was termed E0 (embryonic day 0). The littering cages were also examined daily for the offspring. The day of litter production was termed P0 (postnatal day 0).

Peat and wood wool were used as bedding material in the littering cages. All rats were provided with pelleted rat meal and tap water ad libitum and kept on a 12 hours light/dark cycle.

B. Labelling Procedures

Young rats of various ages were injected either intraperitoneally or subcutaneously with [^{3}H] thymidine (specific activity 21 Ci/mmole, Radiochemical Centre, Amersham), 2.5 μCi/g body weight.

The rats were killed at sixty minutes after injection by cardiac perfusion. The rats were anaesthetised by exposure to diethyl ether vapour in a desiccator. The thoracic cavity was then opened and the inferior vena cava severed. Formol-acetic fixative (1% glacial acetic acid in 10% neutral buffered formalin; Cavanagh and Lewis, 1969) was injected, via a 26 gauge needle attached to a 20 ml syringe, into the aorta via the left

ventricle. The head was detached, the cerebellum exposed, and the head was immersed in neutral buffered formalin for 24 hr postfixation at room temperature. The cerebellum was then removed by severing the peduncles, and bisected sagittally. One sagittal portion was further bisected to give two coronal pieces. All sections were dehydrated and embedded in brain wax in a Shandon tissue processor. After embedding, 7 µm sections were cut on a Sorvall rotary microtome. The sections were mounted on chrome-gelatin slides and stored under distilled water in a covered staining jar until all sections had been cut.

C. Autoradiography

The technique employed was a modification of that of Kopriwa and Leblond (1962) and Butler and Caley (1972). Under the illumination of a Kodak Wratten No. 1 safelight, Ilford L4 emulsion was melted in a Coplin jar in a water bath at 45°C and diluted 1 : 2 with distilled water. The solution was gently stirred with a glass rod and left for 30 mins to allow air bubbles to disappear. At the end of this time a clean glass slide was dipped into the emulsion and pulled out vertically. The emulsion coating was examined. If air bubbles were present the emulsion was allowed to stand for a further 15 min. When the emulsion was free of air bubbles, the slides bearing sections were dipped. This entailed dipping each slide into the emulsion for 2 secs and pulling it out at, as far as possible, at a constant speed. The slides were hung up vertically in a light-tight box for 1 hr before packing into light-tight slide boxes containing silica gel desiccant. The slide boxes were stored at 4°C for six weeks. Following exposure, slides

were returned to the darkroom to reach room temperature before development in Kodak D19 developer, fixation and staining.

The development schedule was as follows :

1. Kodak D19 at 19°C : 5 mins
2. Distilled water at 19°C : 10 sec
3. Johnson Fixsol (1 : 3 dilution) : 8 min
4. Distilled water at 19°C : 10 min x 4 changes

All done under safelight

The sections were stained with Mayer's haemalum for 10 min, followed by dehydration in graded alcohols, clearance in xylene and mounting in Styrolite resin (R.A. Lamb, London).

The autoradiographs were examined under a x100 oil immersion objective (N.A. 1.30). Labelling indices were derived from counts of the entire EGL in sagittal section. Positions of labelled nuclei were evaluated relative to the depth of the entire EGL at that point. For example, a labelled nucleus one cell down from the pial surface in an EGL 10 cells thick would be considered to be 20% into the EGL. Thus, the EGL at the pial surface was counted as 0-20%, and the EGL/molecular layer boundary was counted as 80-100%. Nuclei were scored as labelled if associated with four or more silver grains.

D. Fixation and processing for light- and transmission electron microscopy

Young rats were anaesthetised and fixed by perfusion-fixation as described above. However, the following modifications were made . To facilitate outflow of the perfusate an incision was made in the right auricle. A small

slit was made in the left ventricle and a plastic cannula was inserted into the aorta via the cut in the ventricle and secured with a suture. The cannula was connected to the perfusion apparatus by means of a two-way tap. The perfusion apparatus consisted of two bottles suspended at a height of 160 cm. One bottle contained normal saline, the other fixative. A brief wash (10 sec) was followed by a 15 min perfusion with half strength Karnovsky fixative (2% paraformalde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2) (Karnovsky, 1965). With younger animals too small to cannulate perfusion was achieved by injection of fixative into the left ventricle. After perfusion the skull was opened, the cerebellum removed and cut into 1 mm coronal or parasagittal slices which were then immersion fixed for a period of not less than 24 hours in half-strength Karnovsky fixative at room temperature. It has been found that in rats the perfusion fixation is not critical for animals below 12 days of age, and the temperature of the fixative does not appear important (del Cerro and Snider, 1972a).

The slices were then postfixed in 1% osmium tetroxide in 0.1M cacodylate buffer or distilled water for two hours followed by several washes in distilled water. The slices of cerebellum were dehydrated and embedded in resin in one of two ways :

A : 1. 50% ethanol 10 mins x 2
2. 70% ethanol 10 mins x 2
3. 90% ethanol 10 mins x 2
4. 95% ethanol 10 mins x 2
5. 100% ethanol 10 mins x 3
6. propylene oxide : resin 50:50 1 hr on rotator

7. propylene oxide : resin 25 : 75 1 hr on rotator
8. resin 100% overnight at room temperature on rotator
9. fresh resin in polypropylene capsules overnight at room temperature followed by polymerisation for 48 hr at 60°C.

B : 1. acidified 2,2 dimethoxypropane (DMP) 10 mins x 2 (Muller & Jacks, 1975; Maser & Trimble, 1977)
2. as step 8 above
3. as step 9 above

It can be seen that using acidified DMP (1 drop conc.HCL/100 ml DMP) as per schedule B was much less time-consuming than the conventional technique of using graded alcohols.

The resin used was Taab epoxy resin (Taab, Reading) made up to give a resin of medium hardness.

Thick (2.0 μm) sections were cut on an ultramicrotome (LKB UM2) using glass knives, mounted on glass slides, stained with toluidine blue (1% in 1% borax, pH 11), washed, dried, mounted in Taab resin and viewed in the light microscope.

Thin (50 - 100 nm) sections were cut on an ultramicrotome (LKB UM2) using glass knives, collected on 400 (hexagonal) mesh copper/palladium grids (Gilder, London), stained with uranyl acetate and lead citrate, or lead citrate (Reynolds, 1963) alone and viewed with an AEI 801 electron microscope.

E. Fixation and processing for scanning electron microscopy

After perfusion-fixation as described above, the cerebellum was dissected out and left in fixative for 72 hr. Having been washed in several changes of buffer over 12 hours, tissue was then either postfixed in a modified Parducz's fixative consisting of 12 volumes of 1% osmium tetroxide and one volume of saturated mercuric chloride buffered with 0.1M sodium cacodylate (Seymour and Berry, 1975), or treated with osmium-thiocarbohydrazide-osmium (OTO) technique (Kelly et al, 1973; Malick and Wilson, 1975; Murphy, 1978) to render it conductive without the need to subject it to metallic coating. The tissue was then rinsed in distilled water, the meninges removed and the cerebellum was fractured either along the folia (coronally) or across them (sagittally) using a pair of sharpened dissection probes (Flood, 1975). Specimens were dehydrated in graded ethanols or acidified DMP as described above and dried in a critical-point drying apparatus (Polaron Equipment Ltd., Watford) using liquid carbon dioxide. The dried specimens were mounted on aluminium stubs with silver dag. Those specimens not treated with the OTO technique were sputter-coated with gold, and all material was scanned at 10KV with a Cambridge Stereoscan MK II.

F. Stereological methods

The techniques of stereological analysis used were derived from Weibel (1969; 1973), De Hoff (1968), Underwood (1970). Electron micrographs of external and internal granule cells enlarged to give a constant magnification (x 22,000), were placed under a transparent acetate sheet on which was marked a 0.5 cm grid. For a given cell, the number of intersects

falling over the cytoplasm, nucleus, rough endoplasmic reticulum, mitochondria and Golgi apparatus were counted. The number of intersects overlying each organelle was proportional to the cross-sectional area of the organelle. The total number of intersects falling over the organelles in a given cell were proportional to the total area of the cell occupied by the organelle. The area of organelles measured in this way was proportional to their volume, and thus the number of intersects overlying each type of organelle was proportional to the volume of the organelle. Sufficiently large numbers of cells were analysed to minimise errors caused by tangential sectioning of the cells and their components.

For each age analysed 50 micrographs of EGL cells and 50 micrographs of IGL cells were so treated. The same procedure was used to analyse the differences between the round cells and the fusiform cells of the EGL in coronal section. Means, standard deviations and standard errors of the mean for each parameter were calculated. Lines of best fit were plotted by linear regression analysis, and the significant differences between points were calculated by the application of Student's t test for independent t. Differences were considered to be statistically significant where the probability was less than 0.05.

All material was analysed blind, by allocating each micrograph a code number. All micrographs were then analysed at random, the data being sorted into their relevant groups only after all analyses were complete. This precaution was taken to minimise subjective error.

It should be noted that in the internal granule layer, care was taken only to analyse those cells which were morphologically compatible with granule

cells; cells which had the ultrastructural appearance of astrocytes were ignored.

G. Techniques for the study of cerebellar vasculature

To visualise the vasculature of the developing postnatal cerebellum a modification of the technique described by Pape and Wigglesworth (1979) was used.

Under deep diethyl ether anaesthesia, rats of various ages were given intracardiac perfusions of a gelatin/indian ink solution. This was made by adding one part of 3% gelatin stock melted at 45°C to six parts of indian ink heated to 45°C. The perfusate was injected into the left ventricle under very low pressure, and the right auricle was cut to allow outflow of perfusate. The minimum amount of perfusate was injected, just sufficient for the outflow from the cut right auricle to appear black. This precaution, and the low injection pressure, was to minimised rupture of capillaries. The skull was then opened and the whole cerebellum removed, care being taken to preserve the meningeal membranes. The cerebellum was fixed in buffered neutral formalin for several weeks, bisected in the sagittal plane, and then taken through the following solutions :

1. 50% ethanol
2. 70% ethanol
3. 70% ethanol : Cellosolve (2 ethoxylethanol) 2:1
4. 70% ethanol : Cellosolve 1:2 (2 changes)
5. Cellosolve (2 changes)
6. methyl salicylate (2 changes)

At the end of this process, which took about 2 weeks, the tissue was cleared.

Thin slices (about 1 mm) were cut freehand with a fresh scalpel blade, and kept under methyl salicylate for examination under a stereoscopic microscope with both incident and substage illumination. One half of each cerebellum was taken through toluene to remove the methyl salicylate before embedding in wax, sectioning ot 7 μm and staining with haematoxylin and eosin for conventional histological examination.

H. Immunocytochemical techniques

To investigate the distribution of various neuronal and glial marker proteins in the developing cerebellum a range of techniques were used.

i. Glial fibrillary acidic protein (GFAP) (antiserum provided by Dr. A. Bignami)

Rats were perfuse-fixed with 4% formaldehyde, 0.25% glutaraldehyde in phosphate-buffered saline (PBS) at pH 7.4 or with periodate-lysine-paraformaldehyde fixative (McLean and Nakane, 1974). The cerebellum was removed and 100-150 μm sagittal sections were cut using a Vibrotome (Oxford, USA). The sections were cut and collected in ice cold PBS. The slices were then subjected to the following treatments:

1.	incubation in 0.1M $NaIO_4$	15 min
2.	wash in PBS x 3	5 min
3.	incubation in $NaBH_4$ 10 mg/ml in PBS	10 min
4.	wash in PBS x 3	5 min
5.	incubation in DMSO 5%	10 min
6.	wash in PBS x 3	5 min
7.	incubation in normal 1:200 or anti-GFA specific antiserum 1:250 and 1:500	30 min

8. wash in PBS x 3 — 5 min
9. incubation in rabbit anti IgG 1:50 — 30 min
10. wash in PBS x 3 — 5 min
11. incubation in horseradish peroxidase/rabbit anti-horseradish peroxidase (rabbit PAP) 1 : 50 — 30 min
12. wash in PBS x 3 — 5 min
13. pre-incubation in inactivated diaminobenzidine (DAB) (0.2 mg/ml TRIS buffer pH 7.6) — 30 min in dark at 4°C
14. incubation in DAB activated with H_2O_2 (0.5 µl/ml) — 15 min in dark at 4°C
15. DAB replaced with TRIS buffer
16. wash in PBS x 3 — 5 min
17. post-fix in 1% OsO_4 in PO_4 buffer — 1 hr
18. dehydrate in graded alcohols
19. embed in Spurr resin

From blocks of sagittal cerebellar slices, 1.0 µm coronal sections were cut with glass knives on an ultramicrotome and stained with toluidine blue for light microscopy. 50 - 100 nm coronal sections were cut, collected on grids and examined unstained, or stained with lead citrate, by electron microscopy.

ii. Glutamine synthetase (antiserum provided by Dr. P. L. Woodhams)

The technique used was similar to that described for GFAP above with the following modifications :

1. 4% formaldehyde, 0.25% glutaraldehyde in PBS was used as the perfusion fixative,

2. anti-glutamine synthetase was diluted 1:400 and 1:200 with PBS.

iii. Galactocerebroside (antiserum provided by Dr. M.C. Raff)

The technique used was that described for glutamine synthetase but the antiserum was diluted 1:100, 1:500 and 1:1000 in PBS.

iv. Enolases ("neuron-specific" and "neuron-non-specific") (NSE and NNE) (antisera provided by Dr. P. Marangos)

Rats of various ages were perfuse fixed with 4% paraformaldehyde 0.5% glutaraldehyde in 0.1M PO_4 buffer at pH 7.4 or 0.2% picric acid/4% formalin on 0.1M PO_4 buffer at pH 7.4. After removal and postfixation in the same buffer the cerebella were incubated overnight in 10% sucrose in PBS. The following day the tissue was incubated for 15 min in 12% glycerol, 4% sucrose in PBS. After parasagittal bisection, halves of the cerebella were mounted on cork stubs in Tissue-Tec II OCT compound (R.A. Lamb, London) and frozen in isopentane held at the temperature of liquid nitrogen. Sections (10 μm) were then cut in a cryostat and collected on glass slides. The slides were placed on perspex pedestals in a perspex box in the base of which was added some PBS. During incubations a close-fitting lid was placed on the box. The purpose of this procedure was to prevent the drops of reagents applied to the sections from drying out during incubation. The incubations were as follows :

1. Incubate with 0.3% H_2O_2 in PBS — 15 min
2. Wash in 0.2% Triton-X in PBS — 15 min
3. Incubate with normal swine serum : PBS + 0.2% Triton-X 1 : 20 — 30 min
4. Blot dry and incubate with specific antisera overnight.

5.	wash in PBS	5 min
6.	incubate with anti-rabbit IgG 1: 50	30 min
7.	wash in PBS	5 min
8.	incubate with anti-rabbit PAP 1:50	30 min
9.	wash in PBS	5 min
10.	incubate with DAB (0.5 mg/ml TRIS buffer + 5 µl H_2O_2/20 ml buffer)	15 min in dark at 4°C
11.	stop reaction by transferring slides to large volume of distilled water	
12.	tone reaction product by immersing sections in 0.1% osmium tetroxide for 20 sec	
13.	wash in distilled water	
14.	dehydrate in graded alcohols	

Sections for light microscopy were then cleared in xylene and mounted with DPX mountant (BDH Chemicals, Poole).

v. D2 protein (antiserum provided by Dr. P.L. Woodhams)

The technique used was identical to that described for the enolases above.

vi. Myelin basic protein (antisera provided by Dr. S. Cohn and Dr. P. Glynn)

The technique used was identical to that described for the enolases above. However, for the anti-myelin basic protein serum raised in goats, anti-goat IgG and PAP were employed.

I. Experimentally induced cell death

Nine day old rats were injected with 2.0 mg/g or 0.5 mg/g body weight of hydroxyurea (BDH, Poole). At various times after injection rats were killed by intra-cardiac perfusion of formol acetic fixative (for paraffin wax preparations) or half-strength Karnovsky fixative (for resin embedding). The details of the subsequent stages in the processing were as described in the section 'fixation and processing for light-and electron microscopy.'

J. Measurement of pyknotic indices

The pyknotic indices in the EGL of rats of different ages were obtained by counting 500 - 1000 cells in sagittal sections prepared as described above in the section on fixation and processing for light-and electron microscopy. To determine whether there were regional differences in cell death, lobules I and II, lobule VI and lobules IX and X were counted separately. The pyknotic index for each group of lobules for each age was expressed as the percentage of pyknoses per 100 normal (non-pyknotic) cells. In toludine blue-stained 2 µm resin sections the distinction between pyknotic and mitotic cells (a source of possible error) is apparent.

9. RESULTS

A. Morphology of the cerebellar external granular layer

i. Introduction

Although brief descriptions of the ultrastructure of the EGL have formed part of several studies (notably those of Smith, 1962; Shofer et al, 1964; Meller and Glees, 1969; Altman, 1972a), the morphology of this layer has been subjected to few extensive investigations. In particular, changes in the ultrastructure of the constituent cells with age have not been examined in detail. One of the ultrastructural studies concentrating on the EGL was made on cerebellar histogenesis in the chick (Mugnaini and Forströnen, 1967). As has been pointed out elsewhere in this work, interspecific differences may be sufficiently significant to invalidate comparisons between different species (see Table III). Mugnaini and Forströnen concluded that there was a continuum of transitional forms between the cells of the EGL and the granule cells of the IGL. They were also of the opinion that the outer cells of the EGL were undifferentiated. As evidence for this they described large number of free ribosomes in the cytoplasm, poorly developed membrane systems and chromatin-rich nuclei often containing several nucleoli. They also cited the presence of DNA-filaments and dense granules in the mitochondrial matrix as evidence of lack of differentiation (Mugnaini and Forströnen, 1967).

From their ultrastructural study of the EGL in the rat del Cerro and Snider (1972a) drew somewhat different conclusions. They described the EGL as

passing through three distinct phases. The first period lasted until postnatal day 5, the second until postnatal day 17 and the third until the EGL had disappeared at about postnatal day 20. In the first period the EGL cells formed a homogeneous population of germinal cells. The second period was characterised by the appearance of neuroblasts (sic) which accumulated in rows beneath the germinal cells. The last period was signalled by the cessation of germinal cell proliferation and neuroblast formation (del Cerro and Snider, 1972a).

Both studies follow the consensus of opinion that holds the EGL cells to be primitive and undifferentiated. As proliferation may not be incompatible with neuronal differentiation (see below), this study set out to examine the ultrastructure of the constituent cells of the EGL while keeping in view the possibility that they may be differentiating neurons.

ii. Results *

On the day of birth (day 0), the EGL is morphologically homogenous, i.e. there is no zone of elongated cells (see Fig. 7). Scanning electron microscopy revealed the EGL to contain a complex mesh of processes (Fig. 8). The orientation of the processes was either parallel with or vertical to the pial surface. Transmission electron microscopy confirmed the presence of the processes (Fig. 9) but did not allow the identification of the grid-like pattern. The loose packing of cells and processes gave rise to large and frequent

*Note : all sections described were taken from lobule VI. The term superficial EGL describes the zone of rounded cells belonging to the proliferative zone as described by Altman (1972a). The term deep EGL describes the zone of elongated cells belonging to the proliferative zone as described by Altman (1972a).

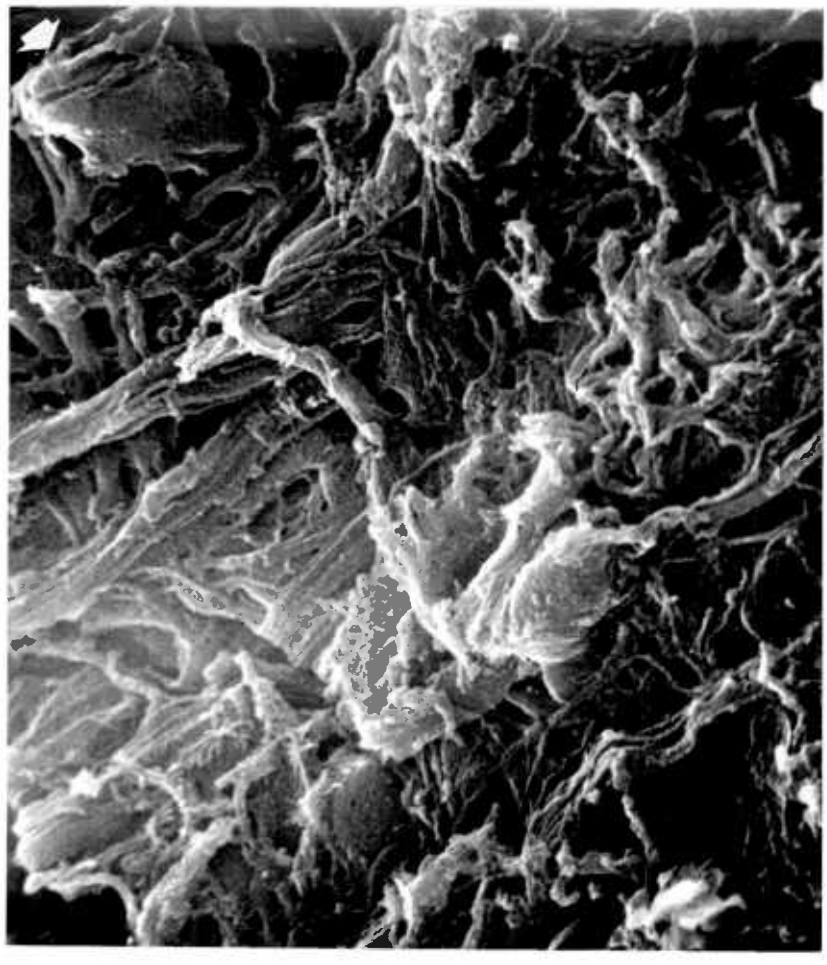

Fig. 8 Ultrastructure of the 0 day old rat external granular layer.

At birth the EGL has not achieved the organisation apparent at later ages. However, orientation of processes both radially and horizontally is clearly seen in this scanning electron micrograph. Arrow = pial surface

Au/Pd sputter coated coronal fracture (x 3700)

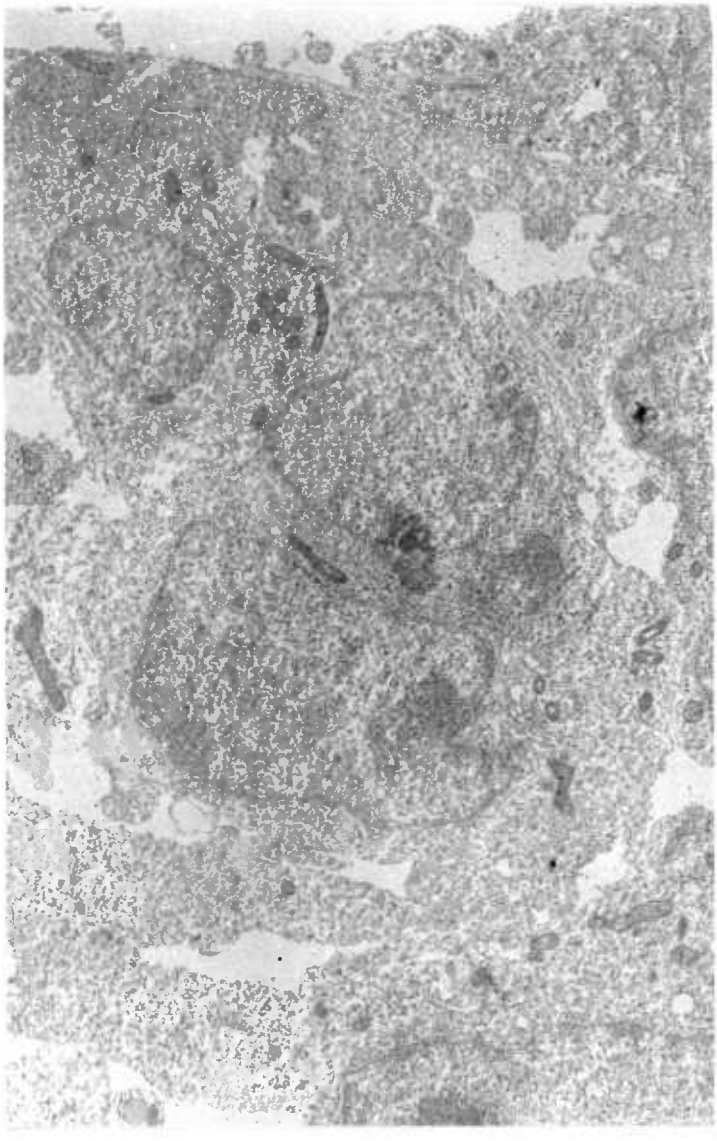

Fig. 9 Ultrastructure of the 0 day old rat external granular layer.

EGL in the proximity of the pial surface. Uranyl acetate/lead citrate stained sagittal section (x 8,750)

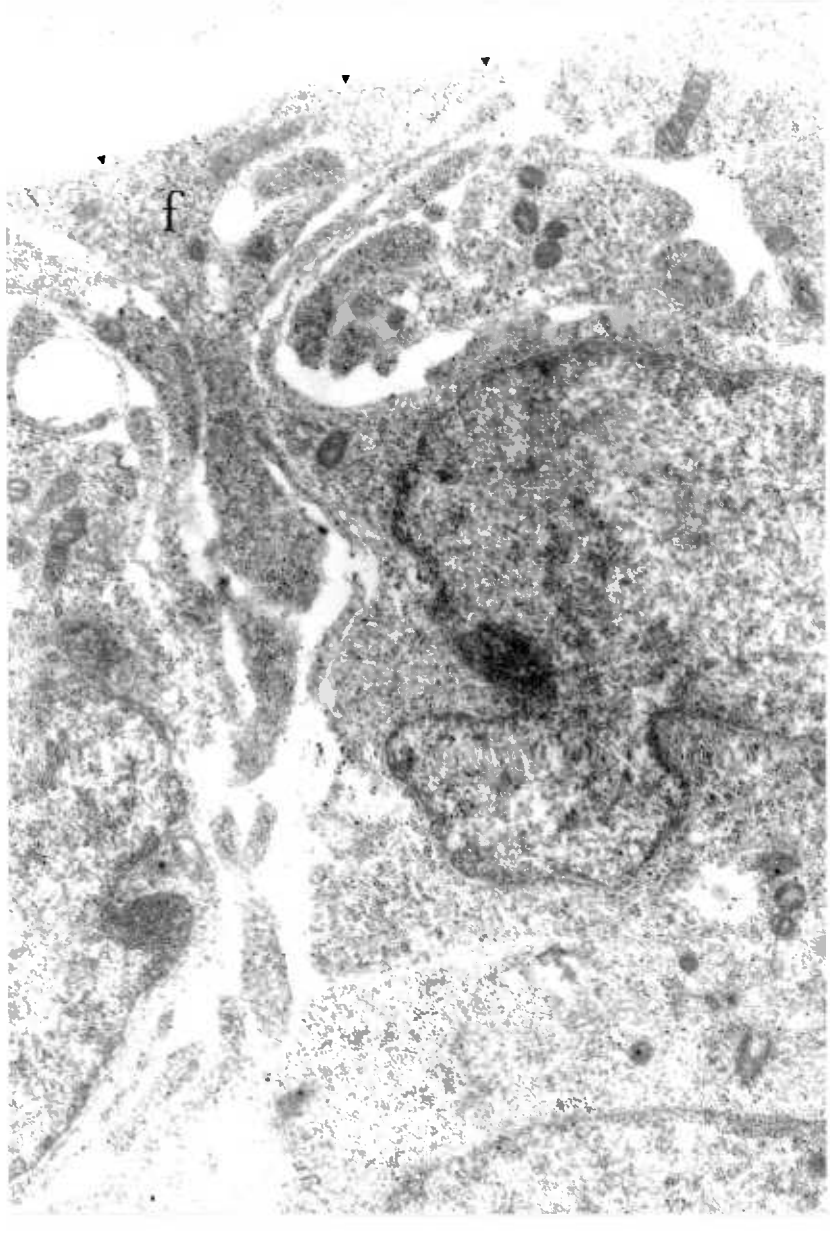

Fig. 10 Ultrastructure of the 0 day old rat external granular layer.

Bergmann glial end-foot (f) at the pial surface. The basement membrane is distinguishable (arrowheads) Uranyl acetate/lead citrate stained coronal section (x 17,000)

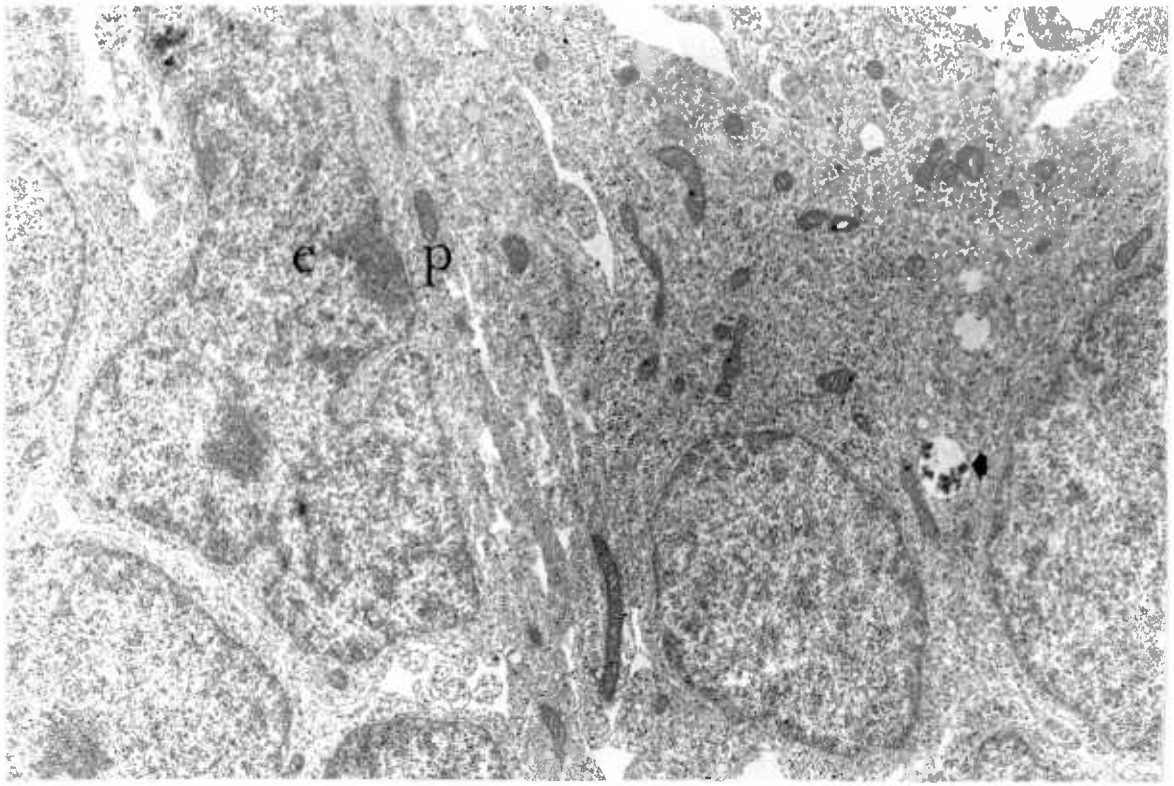

Fig. 11 Ultrastructure of the 0 day old rat external granular layer. EGL cell (e) aligned along glial process (p). A second EGL cell contains a phagosome (arrow).
Uranyl acetate/lead citrate stained sagittal section (x 13,600)

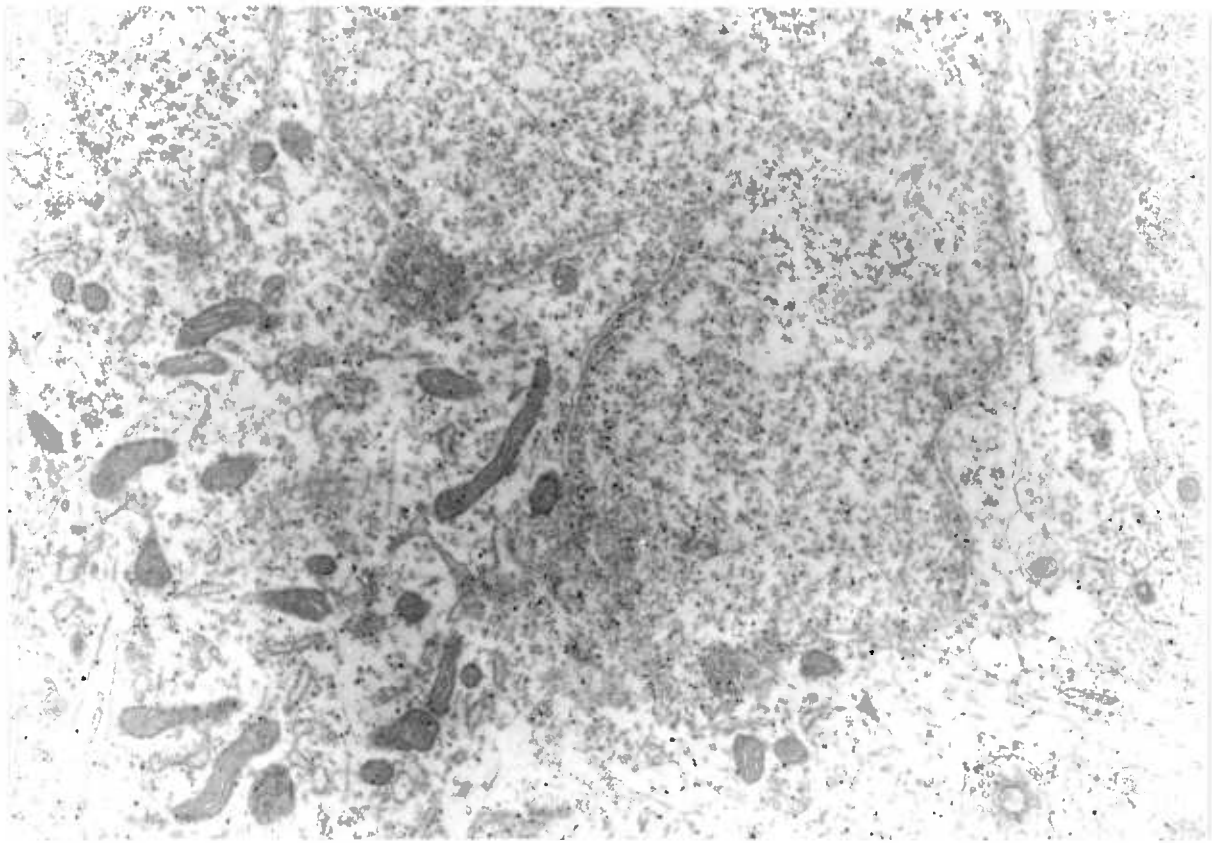

Fig. 12 Ultrastructure of the 0 day old rat external granular layer.

Portion of EGL cell showing high concentration of mitochondria.

Uranyl acetate/lead citrote stained sagittal section (x 17,000)

intercellular spaces (Figs. 8 and 9). The cells themselves were of variable shape; the nuclei were pleomorphic with unevenly distributed chromatin, the greatest concentrations being around the rim of the nucleus (Figs. 10 and 11). The amount of cytoplasm was variable but in many cells it was abundant (Fig. 11). The mitochondria had round or rod-shaped profiles, and in several cells they were found in high concentrations (Fig. 12). Rough endoplasmic reticulum was present (Figs 11 and 12). The EGL cells were interspersed with Bergmann glial fibres (Fig. 11) which terminated in end-feet at the pial surface (Fig. 10).

By 3 days, the rat EGL has acquired a zone of elongated cells (see Fig. 7). The cells of the superficial EGL appear similar to those of the 0 day old rat. Where cells were sectioned at the point where the cytoplasm was thickest, the organelles were seen to be in their highest concentrations. Ribosomes were now seen throughout the cytoplasm usually arranged in rosettes, and extensive Golgi bodies were present (Fig. 13). Between the superficial cells there were spaces, often filled with processes (Fig. 13). The EGL cells appeared to be making frequent double walled coated vesicles by contact with processes adjacent to them (Figs. 16 and 17). Other intercellular specialised contacts often seen were zonulae adhaerentes (Fig. 13). A less frequent cell-to -cell contact was a fused-membrane-like junction (Fig. 13); whether this actually represented a true fusion, or a variation of the tight junction contact was not known. Amongst the deeper cells, apart from the elongation (seen only in coronal sections) very little ultrastructural differentiation was apparent. Some cells possessed thin rims of cytoplasm, poor in organelles (Fig. 14) while others had

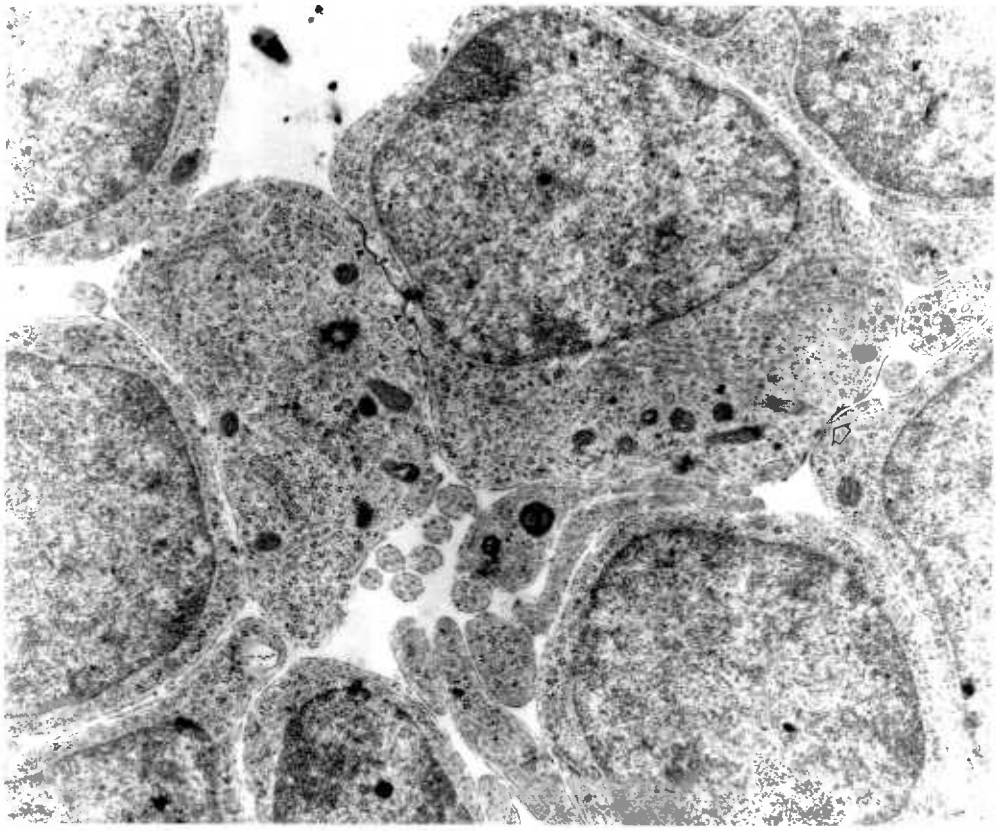

Fig. 13 Ultrastructure of the 3 day old rat external granular layer

Rounded cells in the superficial EGL showing specialised contacts : fused-membrane-like (arrowheads) and asymmetric zonula adhaerens (open arrow)
Lead citrate stained sagittal section (x 14,500)

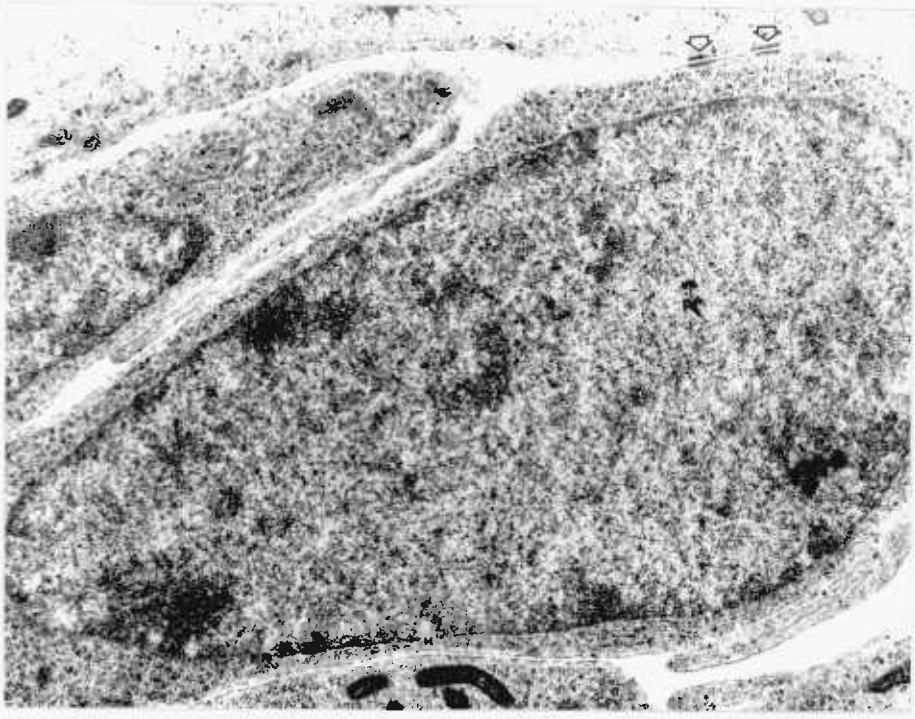

Fig. 14 Ultrastructure of the 3 day old rat external granular layer

Elongated cell in the deep EGL in contact (puncta adhaerentia - open arrows) with parallel fibre in molecular layer.

Lead citrate stained coronal section (x 19,500)

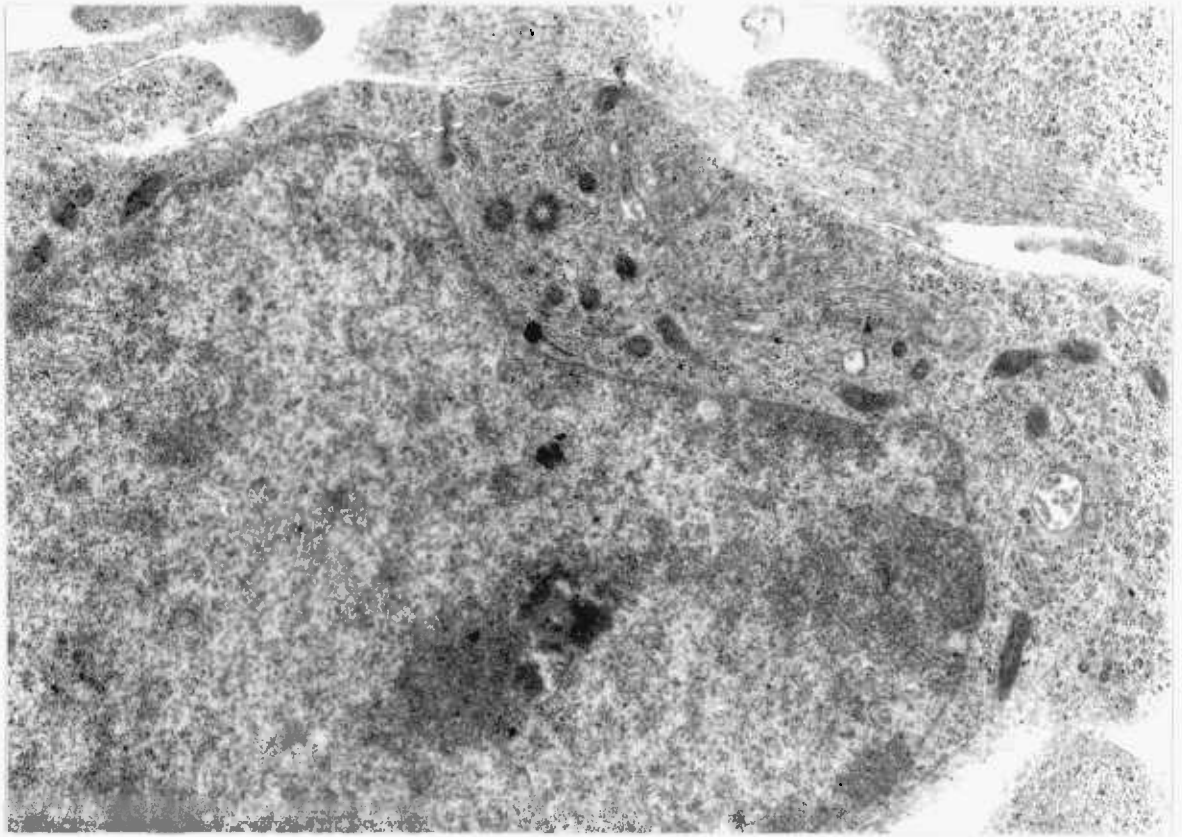

Fig. 15 Ultrastructure of the 3 day old rat external granular layer

Elongated cell in deep EGL. Lead citrate stained sagittal section (x 19,000)

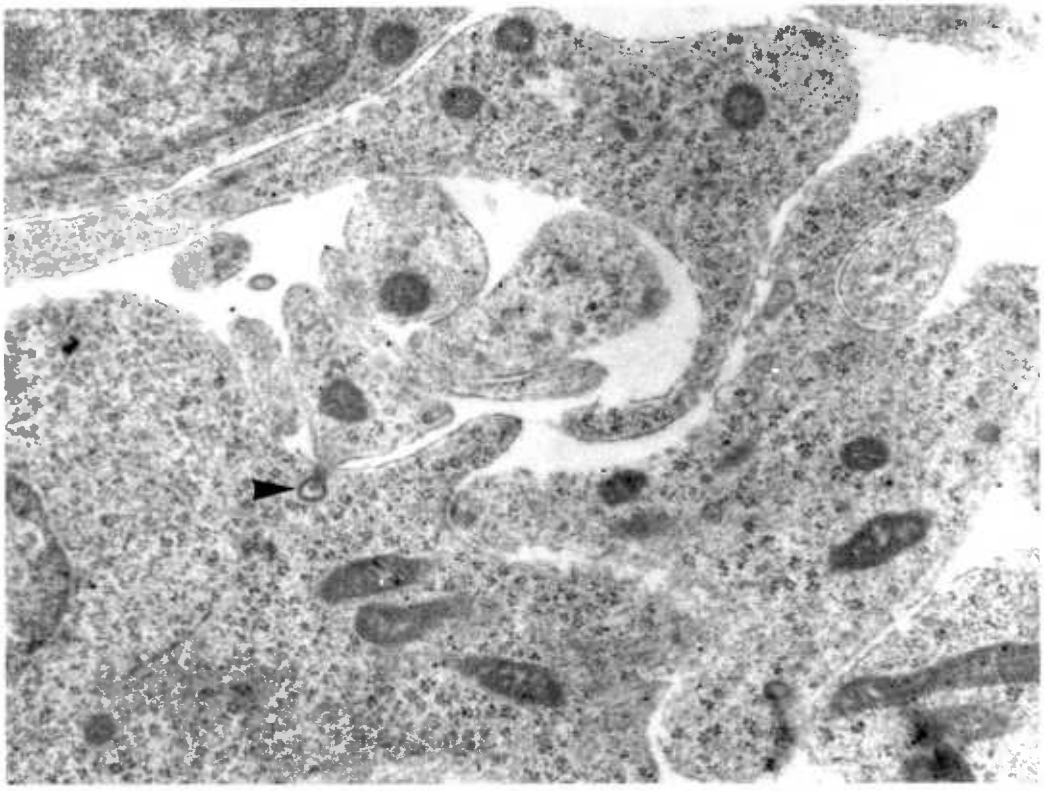

Fig. 16 Ultrastructure of the 3 day old rat external granular layer

Superficial EGL. Double walled coated vesicle formation (arrowhead).

Lead citrate stained sagittal section (x 25,200)

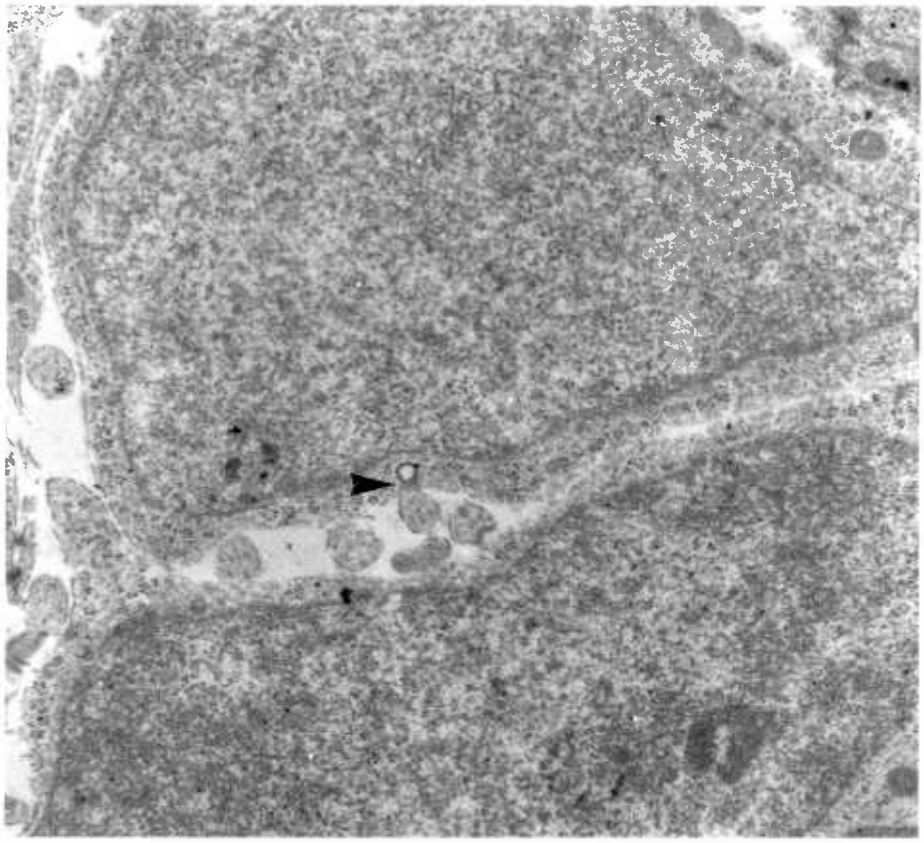

Fig. 17 Ultrastructure of the 3 day old rat external granular layer.

Superficial EGL. Double walled coated vesicle formation (arrowhead)

Lead citrate stained sagittal section (x 20,800)

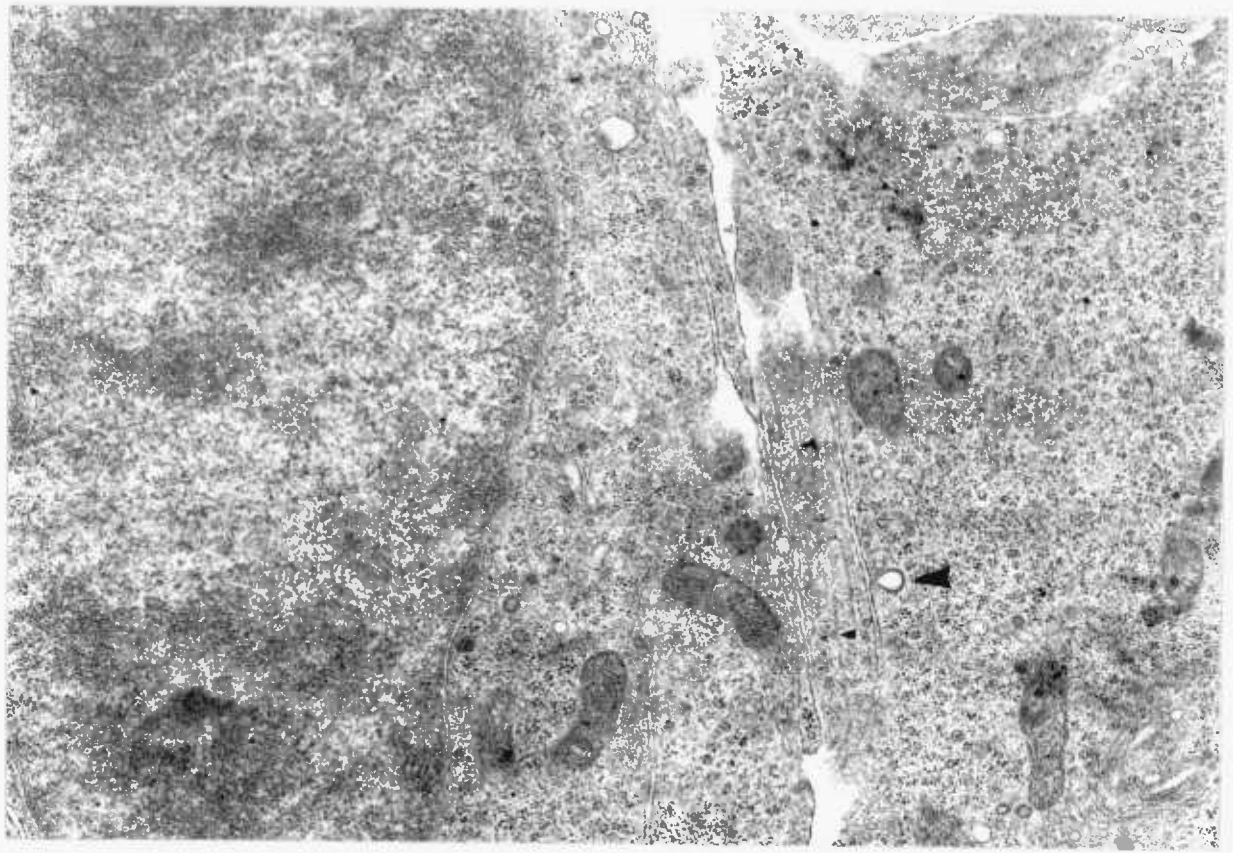

Fig. 18 Ultrastructure of the 5 day old rat external granular layer.

Superficial EGL. Double walled coated vesicle (large arrowhead). The process which possibly contributed the vesicle contains neurotubules (small arrowheads). Lead citrate stained coronal section (x 21,400)

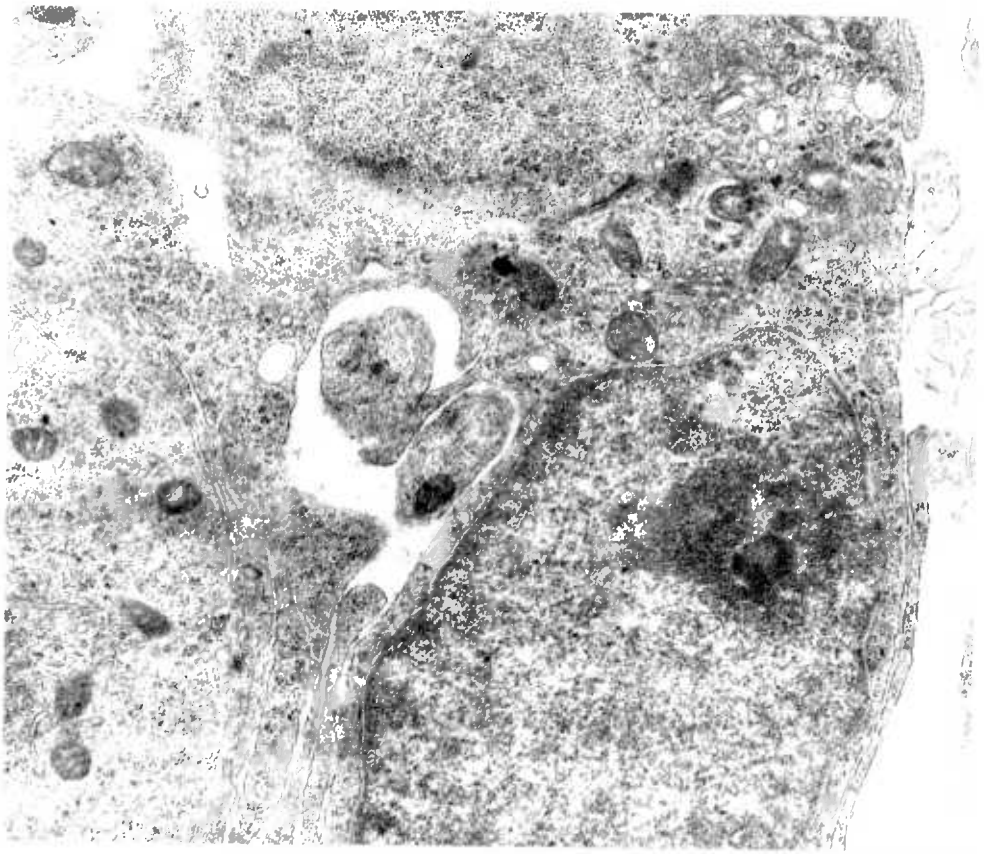

Fig. 19 Ultrastructure of the 5 day old rat external granular layer.

Superficial EGL. Rounded cell. Polar concentration of mitochondria and membrane system. Zonula adhaerens contact with adjacent rounded cell. Lead citrate stained coronal section (x 18,900)

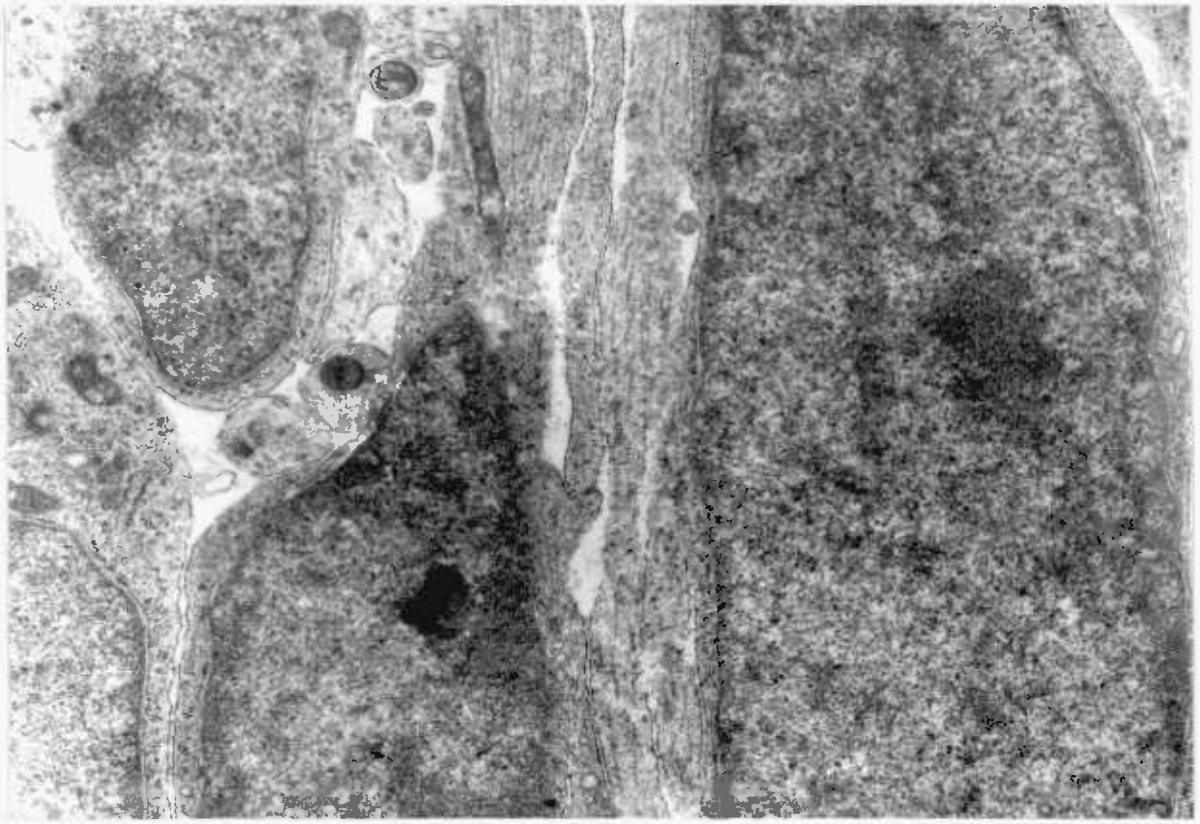

Fig. 20 Ultrastructure of the 5 day old rat external granular layer. Deep EGL. Elongated cells. Lead citrate stained coronal section (x 18,900).

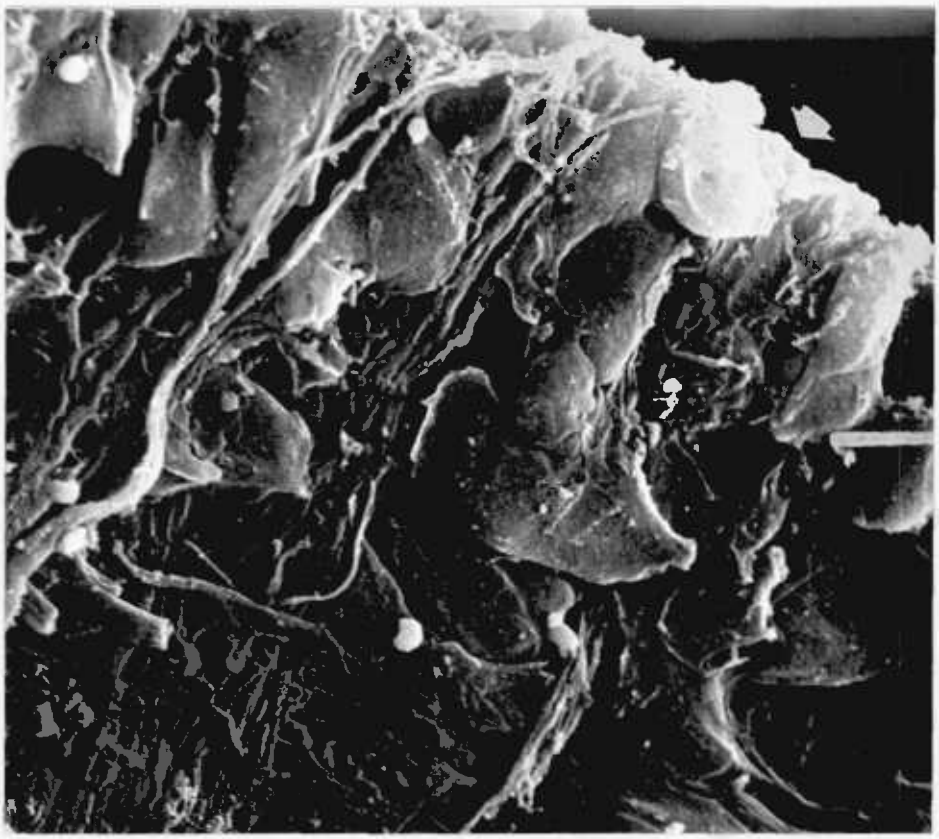

Fig. 21 Ultrastructure of the 7 day old rat external granular layer

Cells near the pial surface show pleomorphic morphology and appear quite bosely packed. Deeper into the EGL the packing density is greater and the horizontal orientation is more apparent. Bundles or palisades of Bergmann glial fibres run vertically up through the EGL to the pial surface.

Au/pd sputter coated coronal fracture (x 3,100)

abundant cytoplasm with a full range of organelles, including centrioles and multivesicular bodies (Fig. 15).. The elongated cells often made contact with parallel fibres by means of puncta adhaerentia (Fig. 14).

Within the next two days many of the processes of the superficial EGL had acquired the characteristics of parallel fibres; in particular, the development of neurotubules (Fig. 18). In some cases it appeared that the EGL cells were in communication with the fibres through the formation of double walled coated vesicles (Fig. 18). In other respects the appearance of the superficial EGL and its constituent cells showed no significant change from that described for day 3 (Fig. 19). In the deep EGL, production of parallel fibres from the apical poles of the elongated cells, conforming to conventional description, was noted (Fig. 20).

Scanning electron microscopy of the 7 day old rat (Fig. 21) EGL confirmed the electron microscopic observations of thin sections; in particular, the loose packing of the superficial EGL cells, accounting for the intercellular spaces (Fig. 19). In the deep EGL the cells appeared closer together, and the orientation of cells parallel to the pial surface was more pronounced (Fig. 21). Also clearly visible were the Bergmann glial fibres, traversing the EGL at a right angle to the pial surface (Fig. 21).

At 8 days the superficial cells appeared to show a greater degrée of polarisation than hitherto, with the majority of organelles concentrated in the polar accumulation of cytoplasm (Fig. 22). Rarely, collections of clear vesicles were found at the apex of the cytoplasmic bulb, possibly representing growth cones (Fig. 23). The arrangement of intercellular

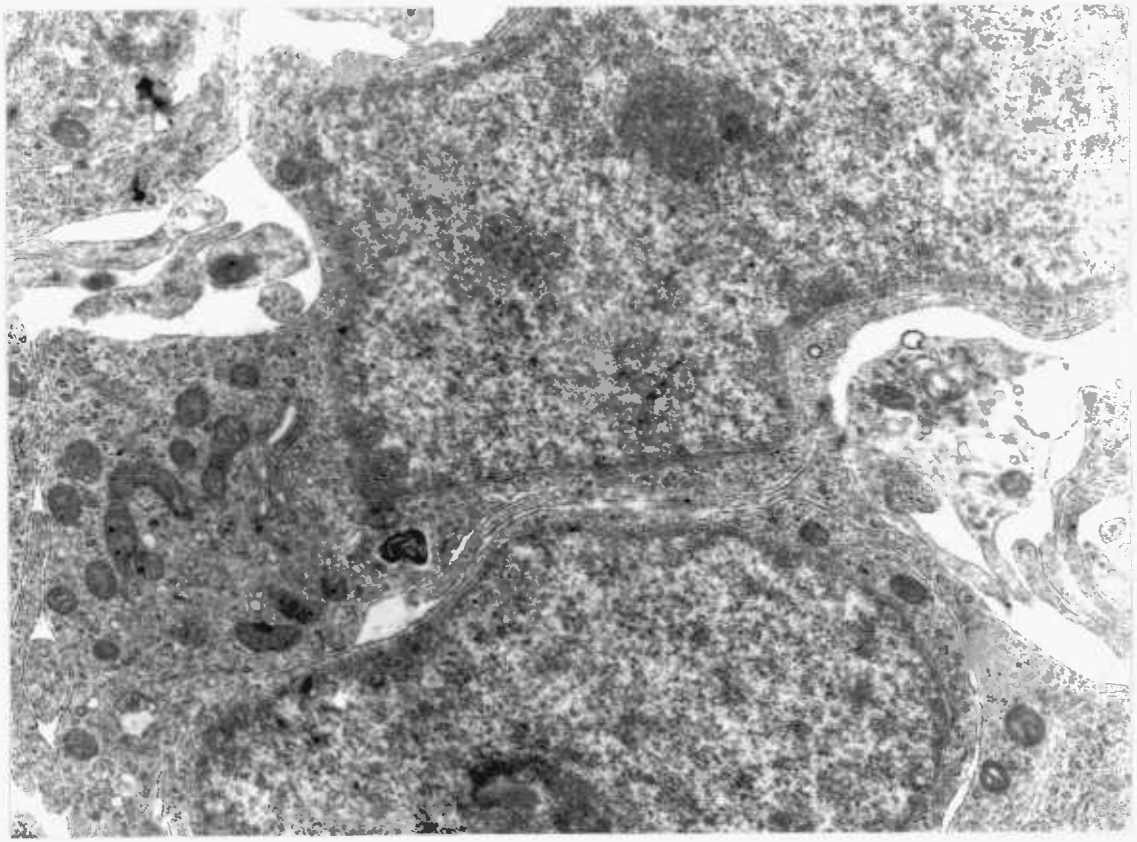

Fig. 22 Ultrastructure of the 8 day old rat external granular layer.

Rounded cell in superficial EGL. The organelles are concentrated in the polar cytoplasm.

Lead citrate stained coronal section (x 18,900)

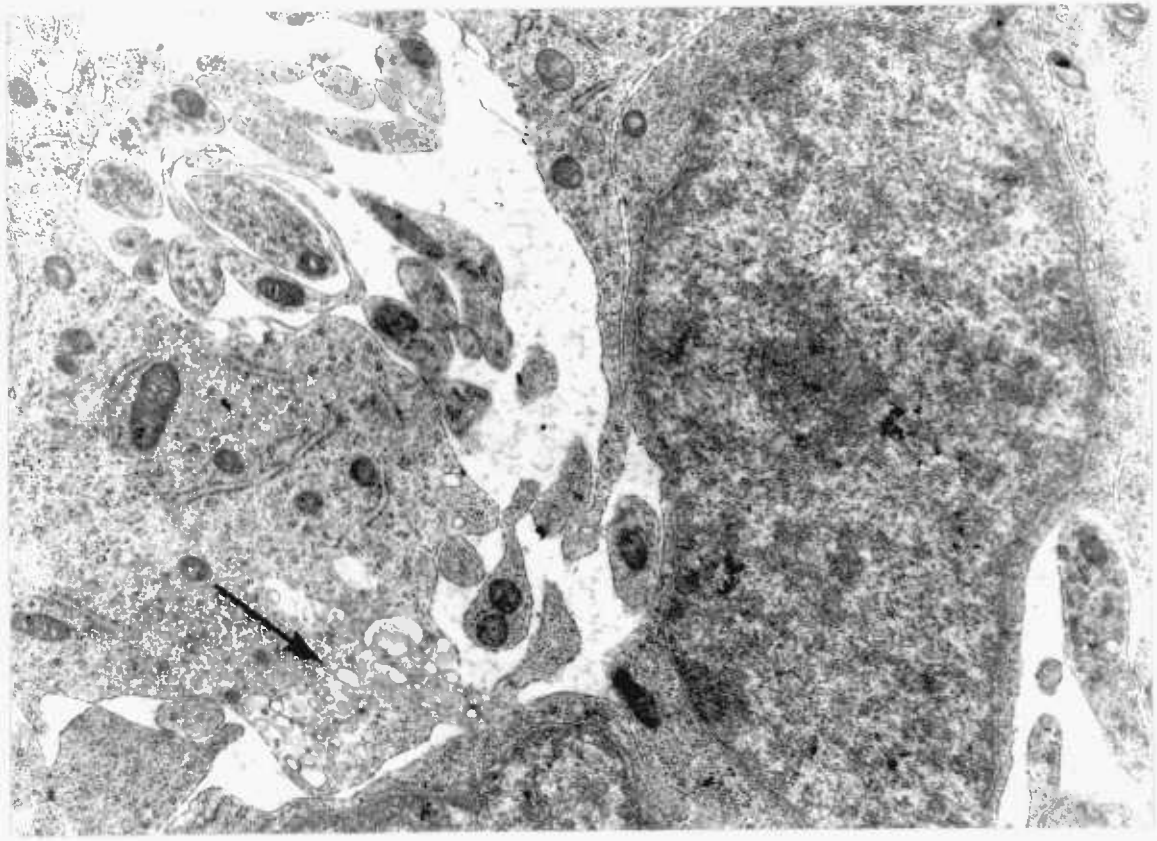

Fig. 23 Ultrastructure of the 8 day old rat external granular layer. Superficial EGL. Concentration of large vesicles (arrow) may be a growth cone. Lead citrate stained coronal section (× 18,900)

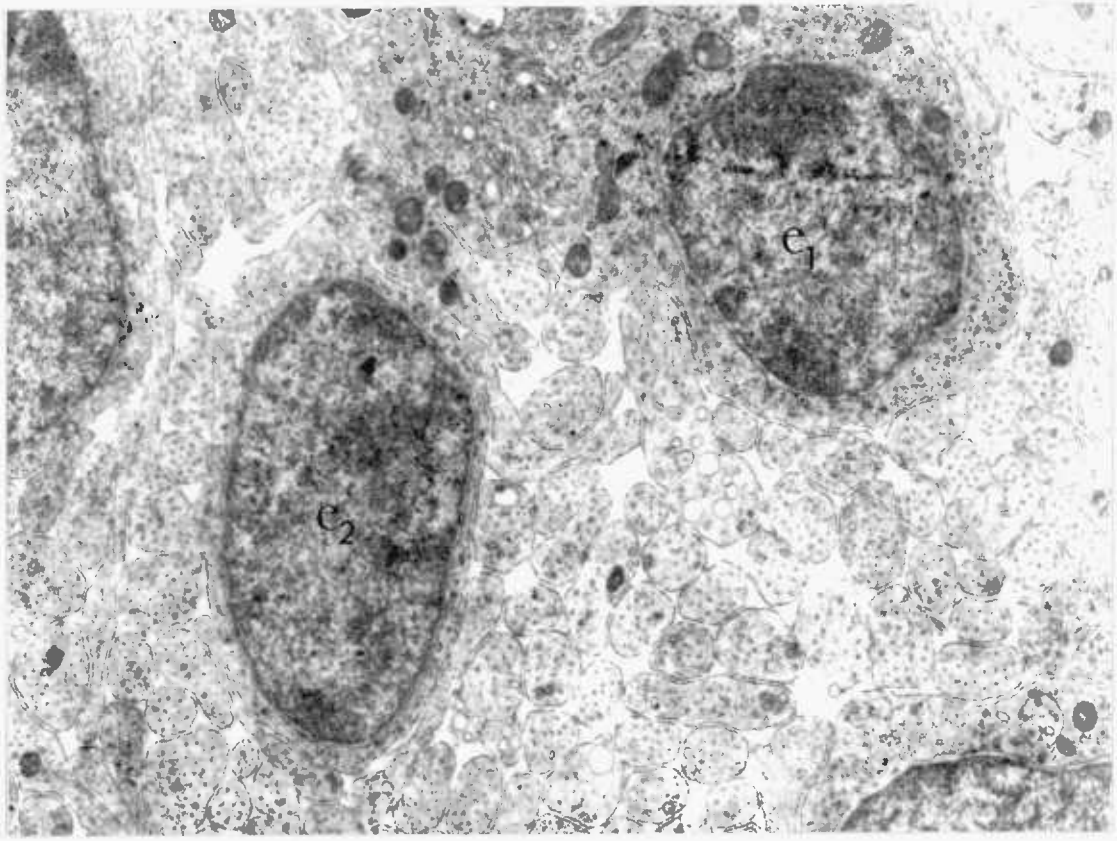

Fig. 24 Ultrastructure of the 8 day old rat external granular layer.

Deep EGL. Elongated cells may appear to have a large volume of cytoplasm with many organelles (e_1) or only a thin rim of cytoplasm with few organelles (e_2). Lead citrate stained sagittal section (x 18,900)

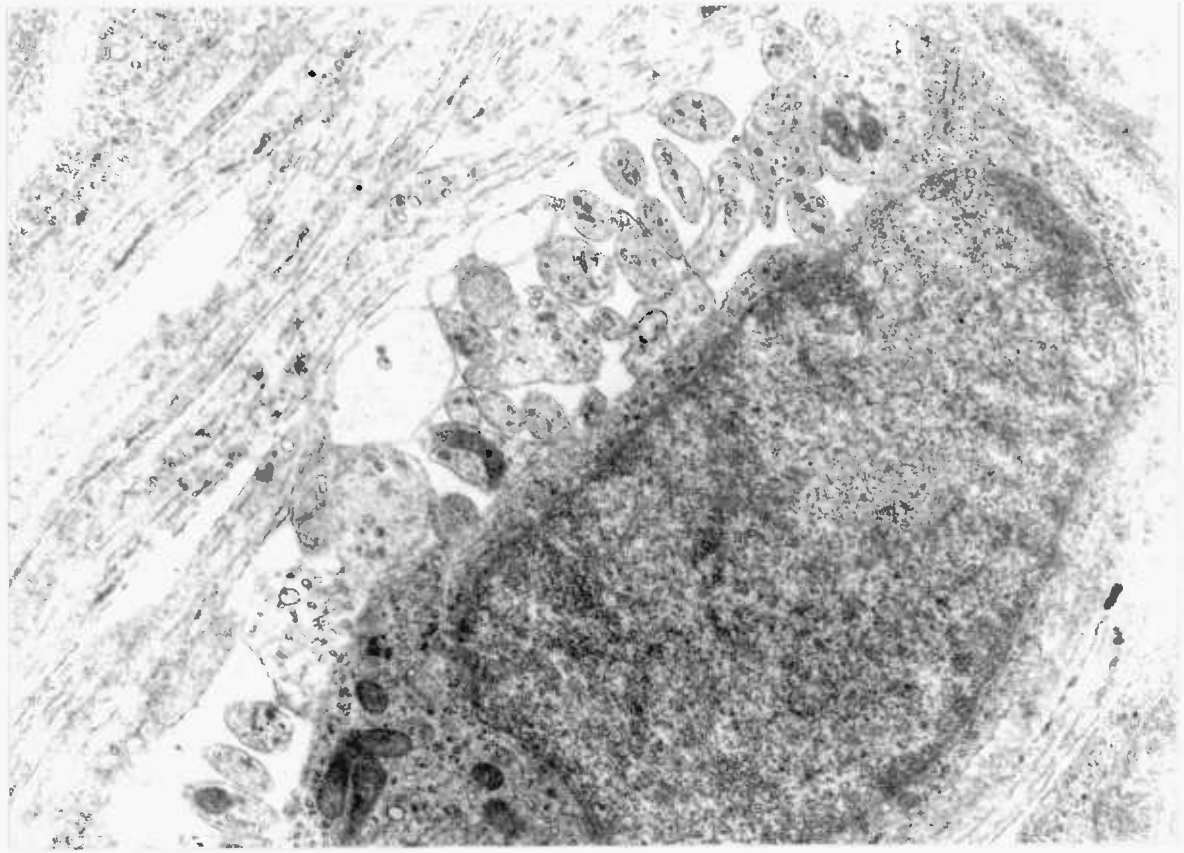

Fig. 25 Ultrastructure of the 8 day old rat external granular layer. Deep EGL. Elongated EGL cell in close proximity to parallel fibres. Between the elongated cell and the parallel fibres are other processes in this section cut transversly. They may originate from the EGL cell. Lead citrate stained coronal section (x 18,900)

spaces occupied by processes, some containing tubules, was unchanged from that described for earlier ages. The deep EGL cells also remained similar to those in younger animals. In sagittal section cells rich in cytoplasm and organelles were found in close proximity to cells whose nuclei were surrounded by a thin rim of cytoplasm containing only ribosomes (Fig. 24). The network of processes first described in the EGL of the newborn rat appeared to persist amongst the elongated cells in the deep EGL; in coronal section, elongated cells were seen whose closest neighbours were not parallel fibres as expected, but processes running at right angles to them (Fig. 25). This may have been a result of tangential sectioning, but the cytoplasm of these fibres was more dense than that of the parallel fibres and it is possible that the elongated cells were extending short processes other than the two major polar ones.

The appearance of the EGL at 9 days in the scanning electron microscope was not markedly different from that of 2 days earlier. The cells appeared pleomorphic and loosely packed near the surface, horizontally oriented, elongated and with little intercellular space in the depths of the layer (Fig. 26). Extension of processes running parallel to the pial surface, and parallel to the folia at all levels of the EGL were seen (Fig. 26). The alignment of the cells made sagittal fracturing difficult, but the EGL broken open in this plane revealed the lack of any apparent production of processes across the folia in a rostro-caudal plane (Fig. 28). The other striking feature of the scanning electron micrographs was the clarity with which they demonstrated the distribution and morphology of the Bergmann glial processes and end-feet. Such processes were seen to travel vertically

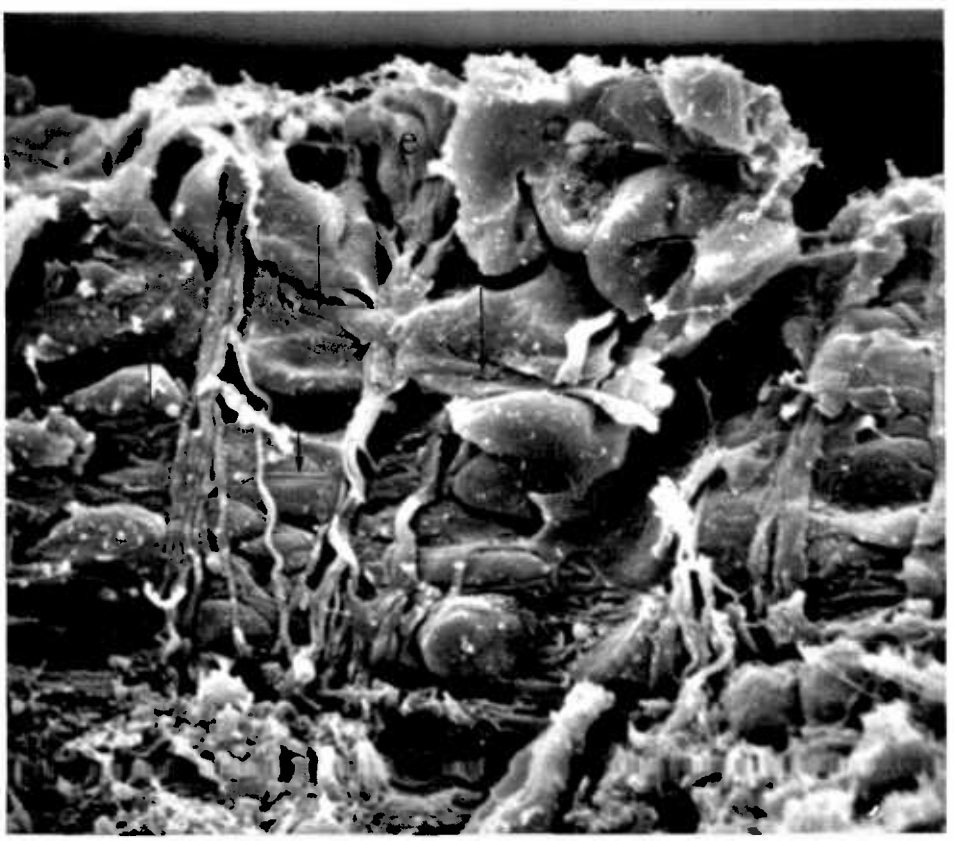

Fig. 26 Ultrastructure of the 9 day old rat external granular layer.

Horizontal processes (arrows) are extended at all levels of the EGL. Bergmann glial fibres traverse the layer to terminate at the pial surface in club-shaped end-feet (e). The diagonal furrow probably represents the space occupied by a radial capillary.

Au/Pd sputter-coated coronal fracture (x 3,000)

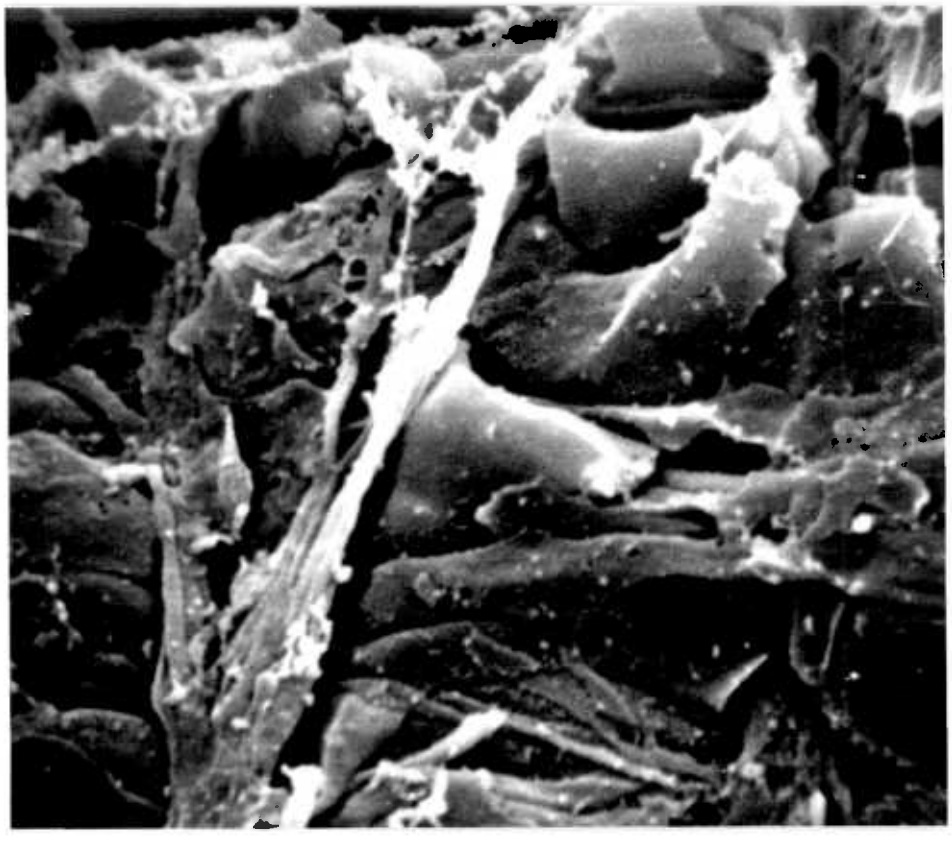

Fig. 27 Ultrastructure of the 9 day old rat external granular layer.

Bergmann glial process diverging near the pial surface. Au/Pd sputter-coated coronal section (x 7,800)

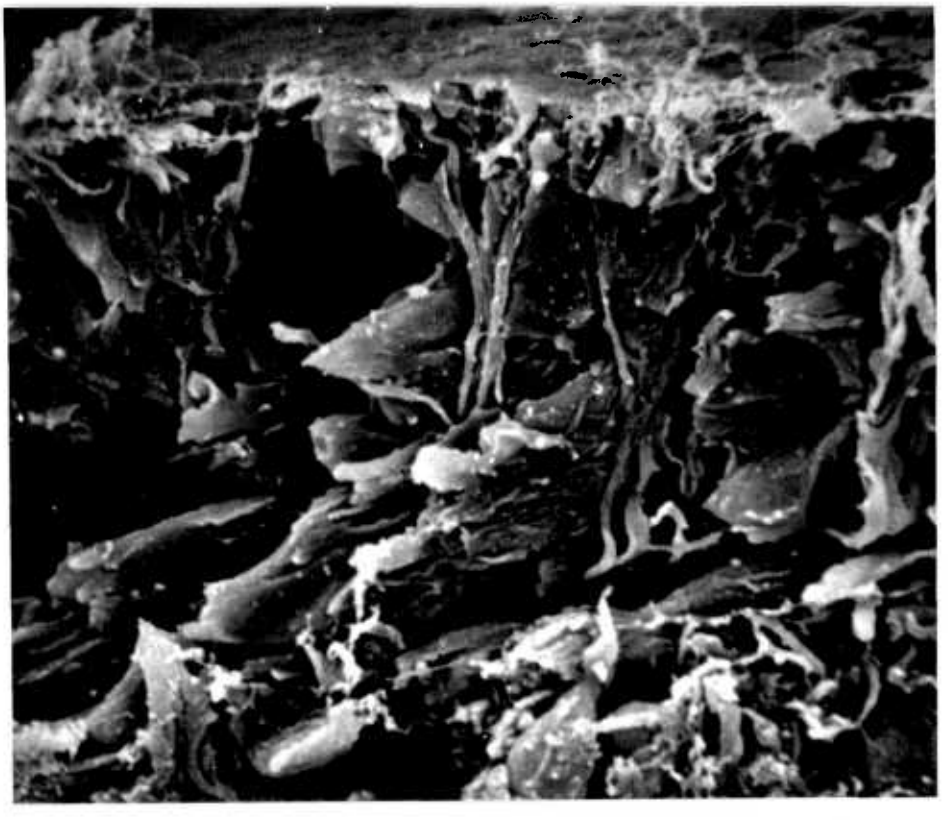

Fig. 28 Ultrastructure of the 9 day old rat external granular layer.

The coronal orientation of the entire EGL is apparent, as are the regularly spaced bundles of Bergmann glial fibres.

Au/Pd sputter-coated sagittal fracture (x 3,000)

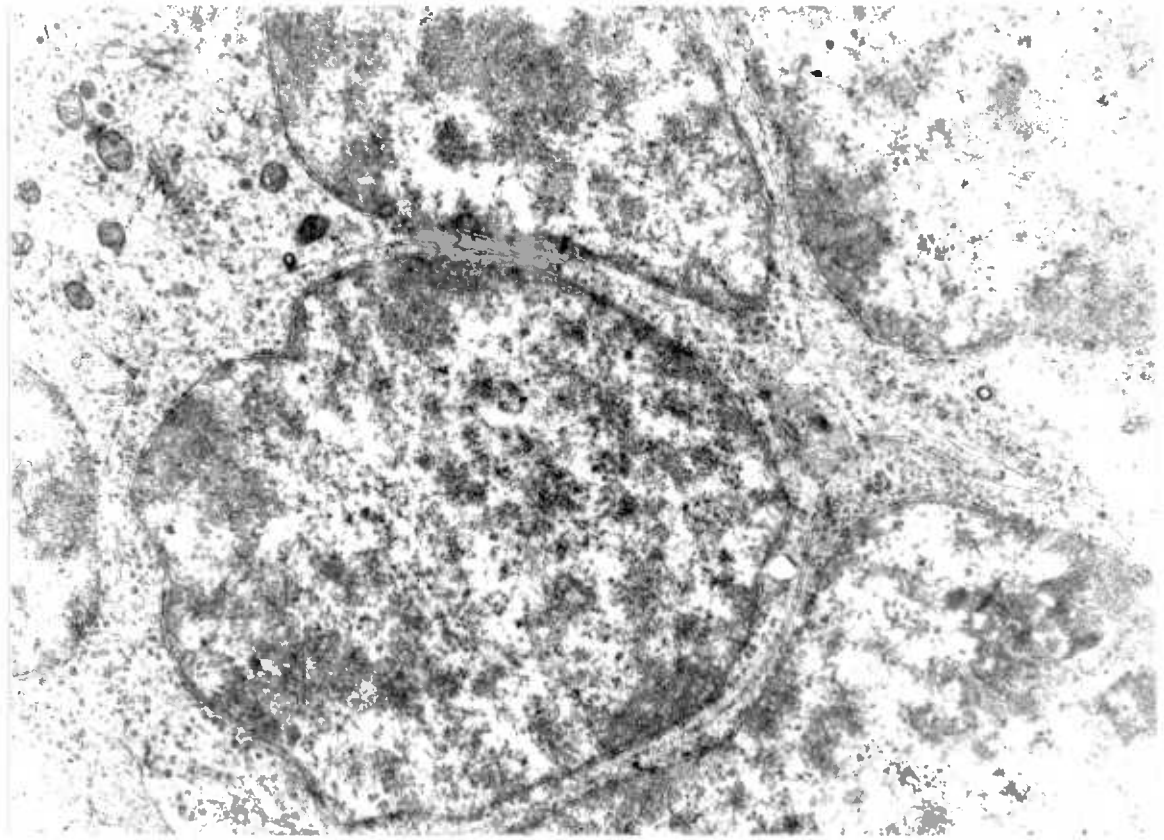

Fig. 29 Ultrastructure of the 13 day old rat external layer.

Round cells in the superficial EGL. Lead citrate stained sagittal section (x 15,800)

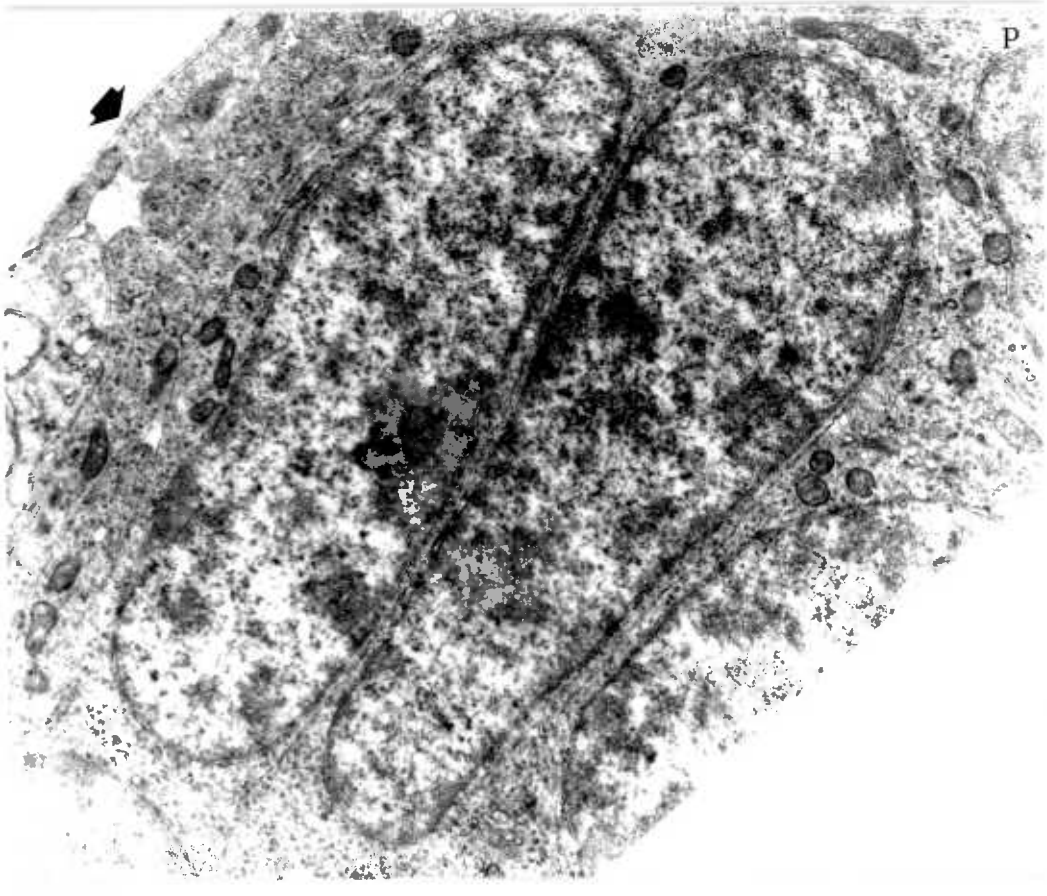

Fig. 30 Ultrastructure of the 13 day old rat external granular layer.

Superficial EGL. Note the elongated cells, one producing a polar process (p), at the pial surface (arrow).
Lead citrate stained coronal section (x 14,500).

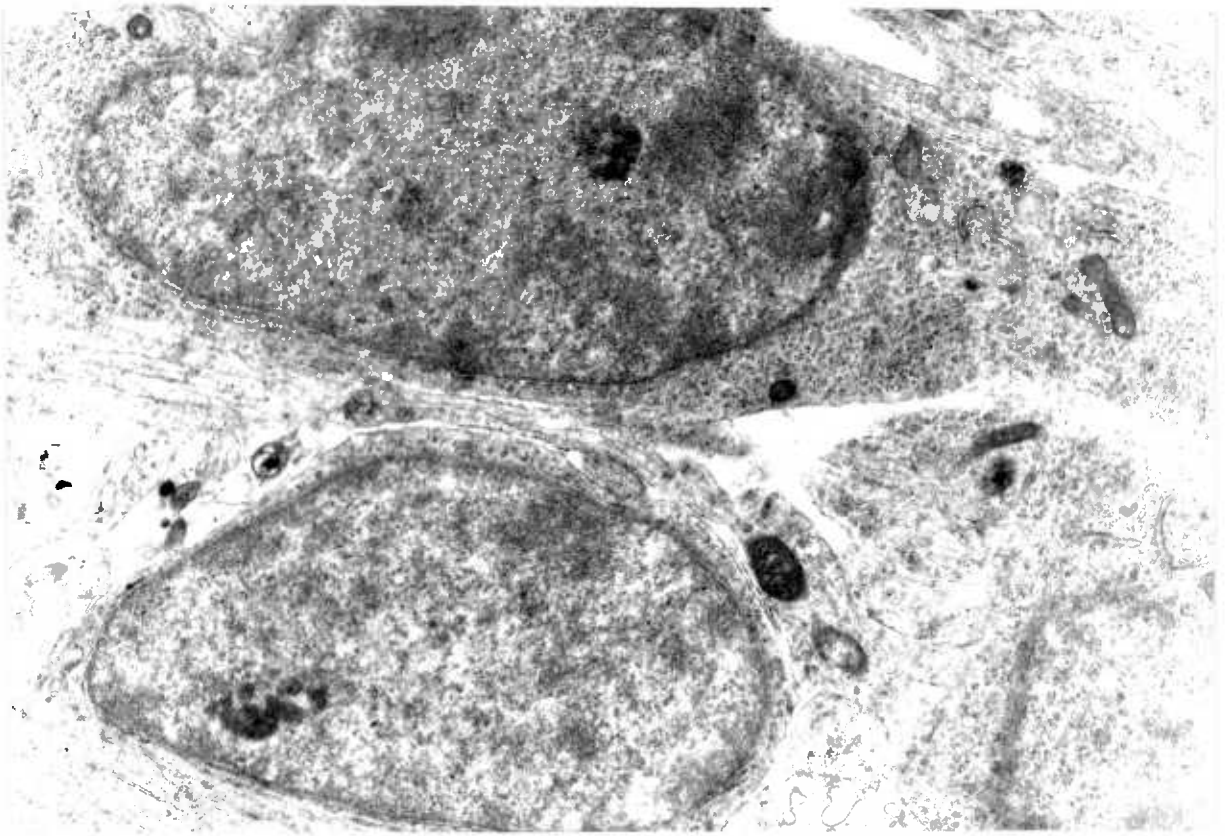

Fig. 31 Ultrastructure of the 13 day old external granular layer.

Deep EGL. Lead citrate stained coronal section (x 15,800)

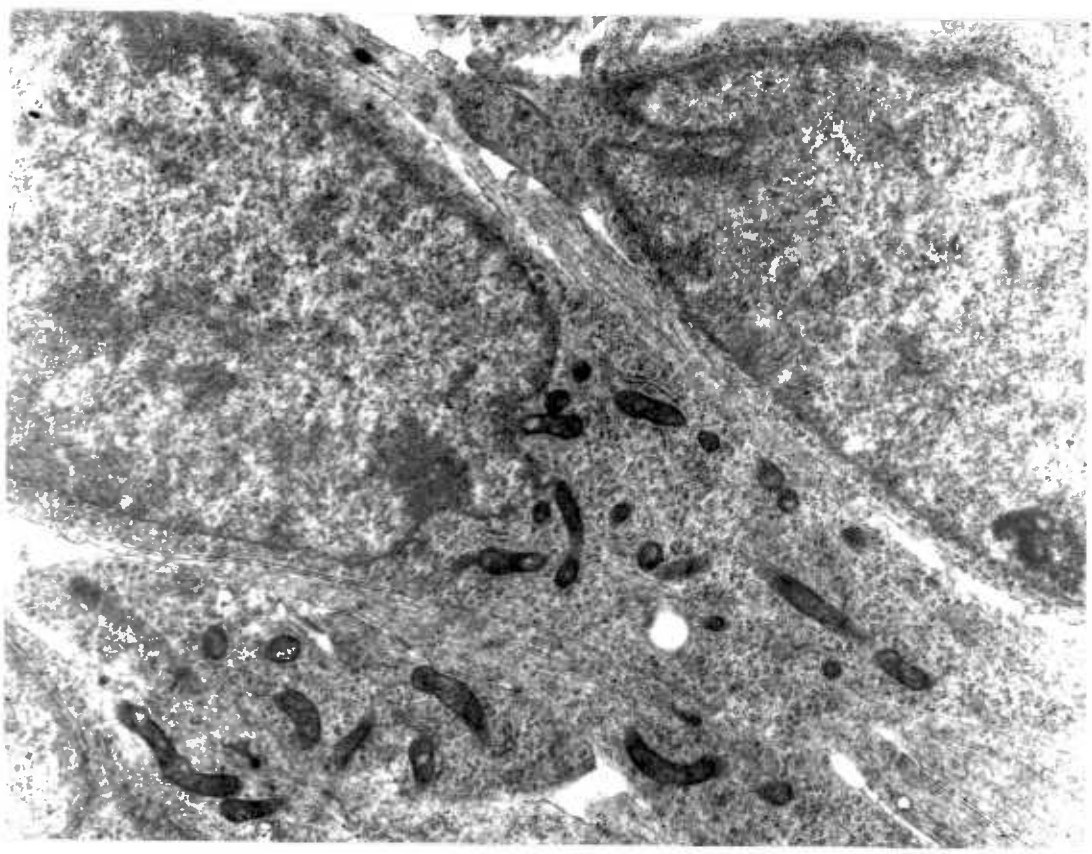

Fig. 32 Ultrastructure of the 13 day old rat external granular layer.

Deep EGL. Lead citrate stained coronal section (x15,800)

through the EGL in bundles bending around the EGL cells in such a way as to achieve a gentle corkscrew-like appearance (Figs. 26 and 28). At some distance from the EGL, each fibre divided into several thinner processes (Fig. 27) which reached to the pial surface, terminating there in bulbous end-feet (Figs. 26 and 27). The Bergmann glial fibres appeared to be spaced at regular intervals, but the terminal divisions and the swelling of the end-feet ensured that the entire pial surface was completely occupied by glial processes (Fig. 28).

By 13 days the superficial cells in the EGL were more closely packed than before (Fig. 29), with a morphology closely resembling that of the granule cell of the internal granular layer (Fig. 30). On a qualitative basis it was not possible to say whether there was an increase in number, size or density of the constituents of the cells; most of the features described in EGL cells of younger animals were still detectable , for example double walled coated vesicles (Fig. 29). A new development at this age was the detection of elongated cells producing polar processes at, or just below most superficial layer of EGL cells (Fig. 30). These had every appearance of the elongated cells in the deep EGL (Figs. 3I and 32).

Though the superficial EGL had become more densely packed at 13 days, even at 16 days there were abundant intercellular spaces filled with processes within one or two cells of the pial surface (Fig. 34). Beyond this level, however, no spaces remained (Fig. 33). The cells of the deep EGL retained all the features described at earlier ages (Fig. 35) in particular the production of processes at 90° to the parallel fibres (Fig. 36), first observed in the 8 day old animal (Fig. 25).

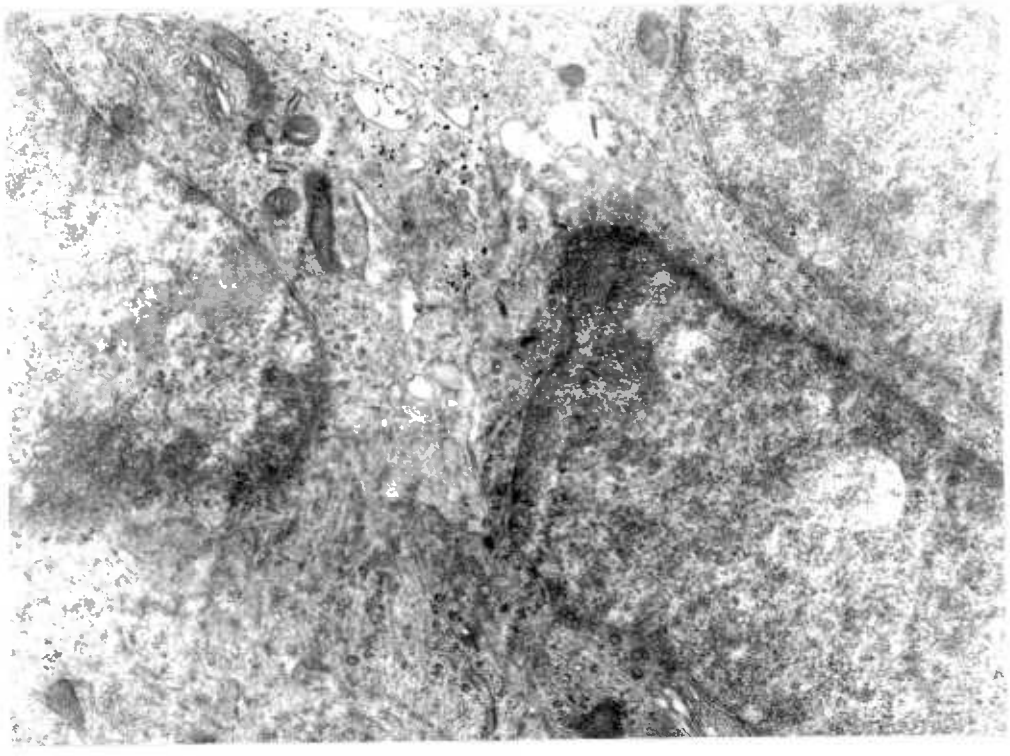

Fig. 33 Ultrastructure of the 16 day old rat external granular layer.

Superficial EGL. Bergmann glial fibres contain glycogen granules. Lead citrate stained coronal section (x 17,600)

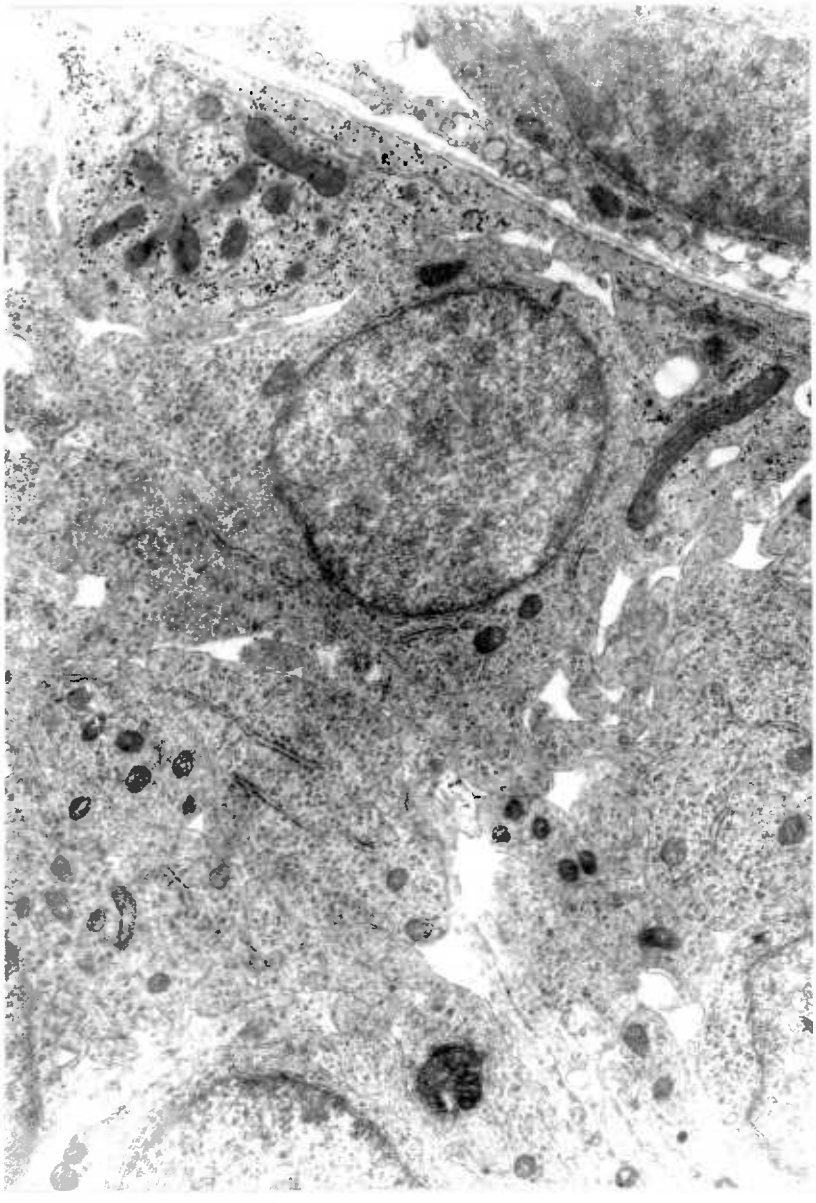

Fig. 34 Ultrastructure of the 16 day old rat external granular layer.

Superficial EGL. Bergmann glial end-feet contain glycogen granules. Lead citrate stained sagittal section (x 15,000).

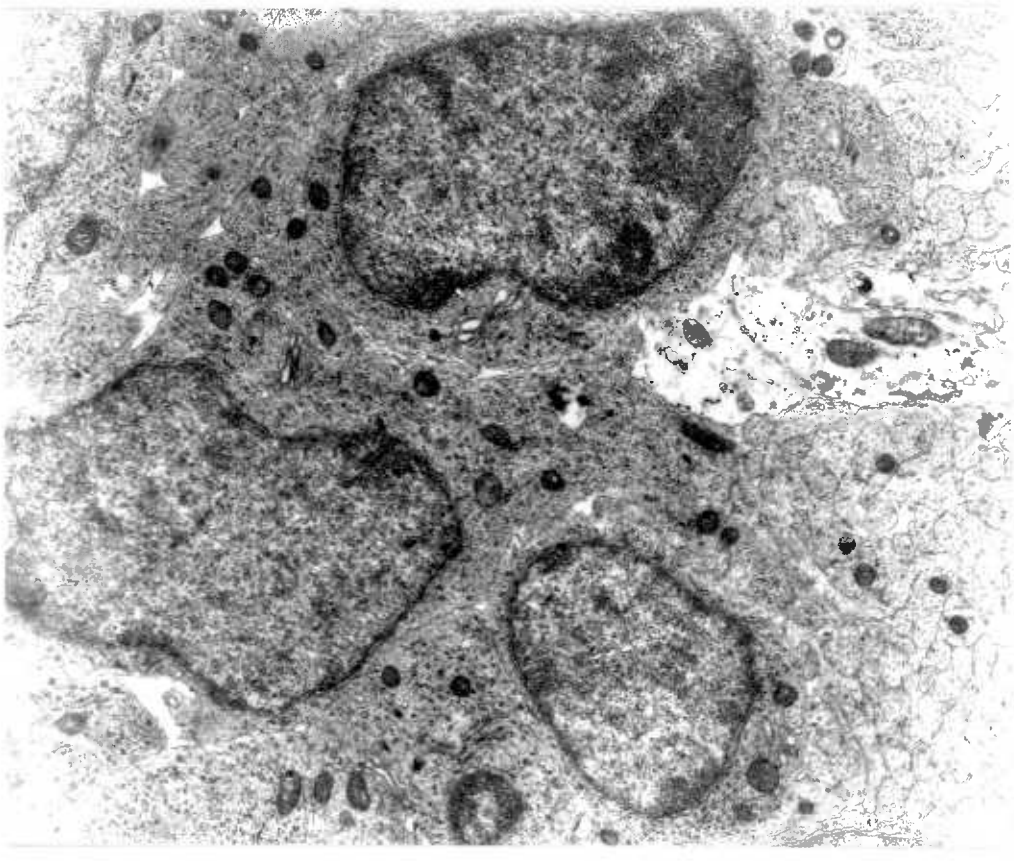

Fig. 35 Ultrastructure of the 16 day old rat external granular layer.

Deep EGL. Lead citrate stained sagittal section (x 13,200)

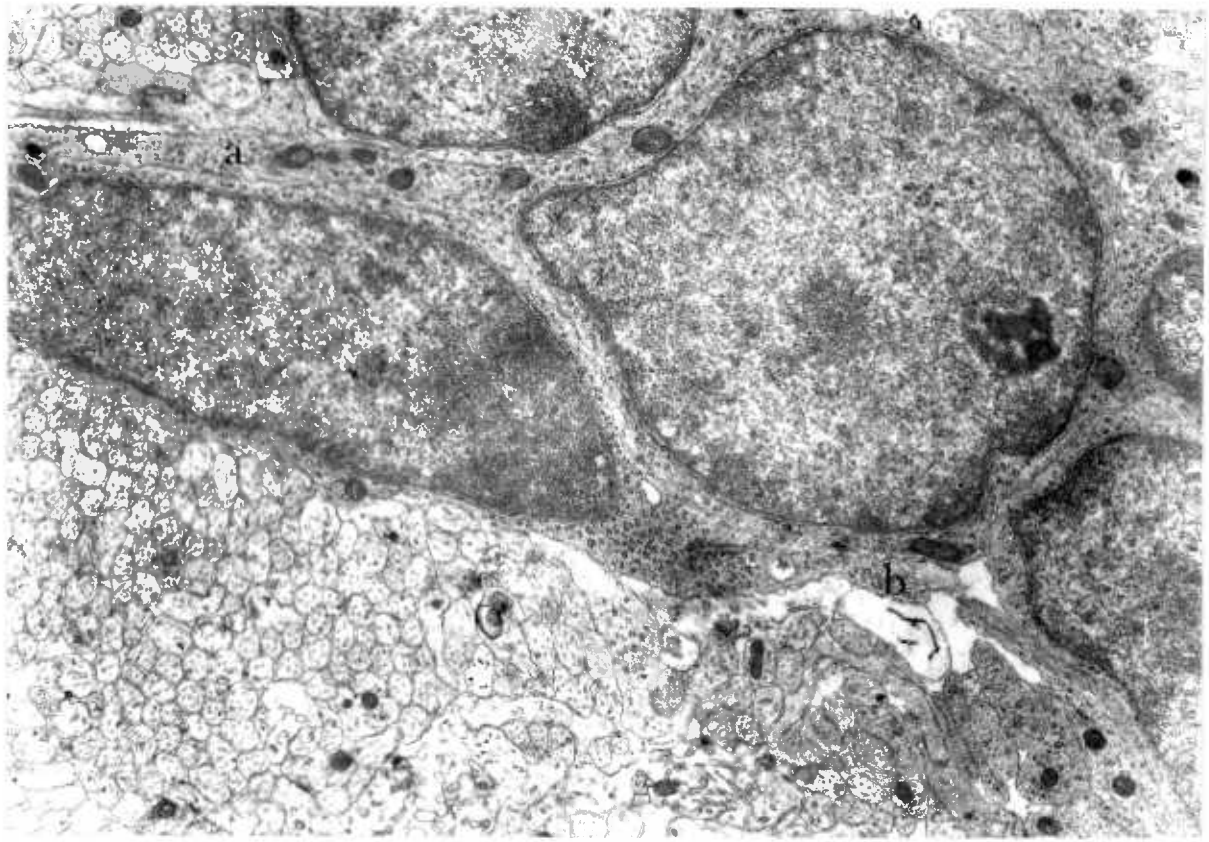

Fig. 36 Ultrastructure of the 16 day old rat external granular layer. Deep EGL. The elongated cells produce processes (a, b) which run at 90° to the parallel fibres seen at the bottom of the micrograph. Lead citrate stained sagittal section (x 14,500).

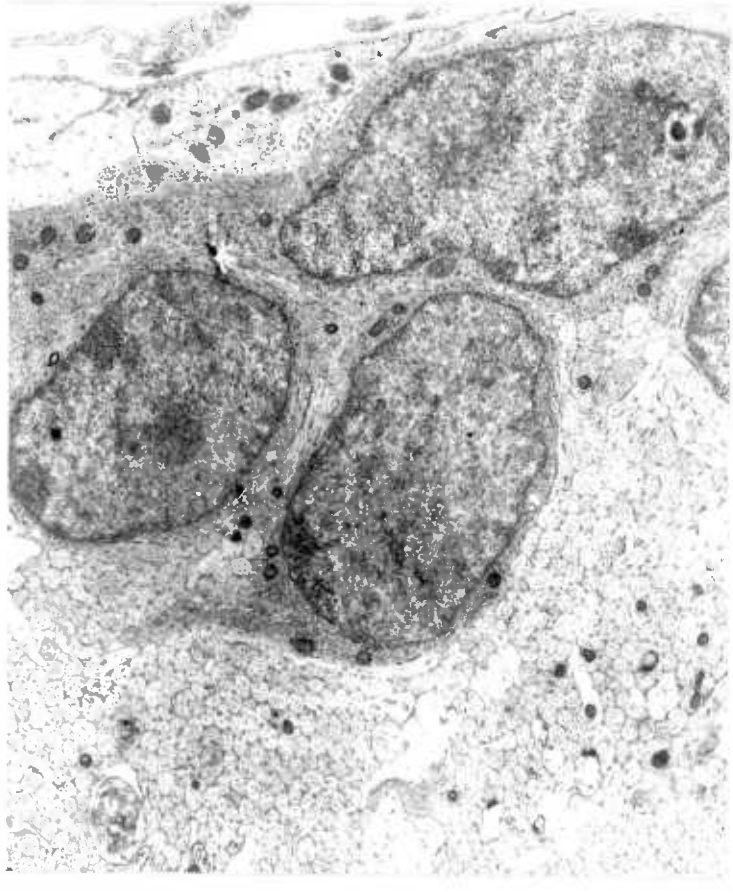

Fig. 37 Ultrastructure of the 20 day old rat external granular layer.

Only a few EGL cells now remain.

Lead citrate stained sagittal section (x 12,000).

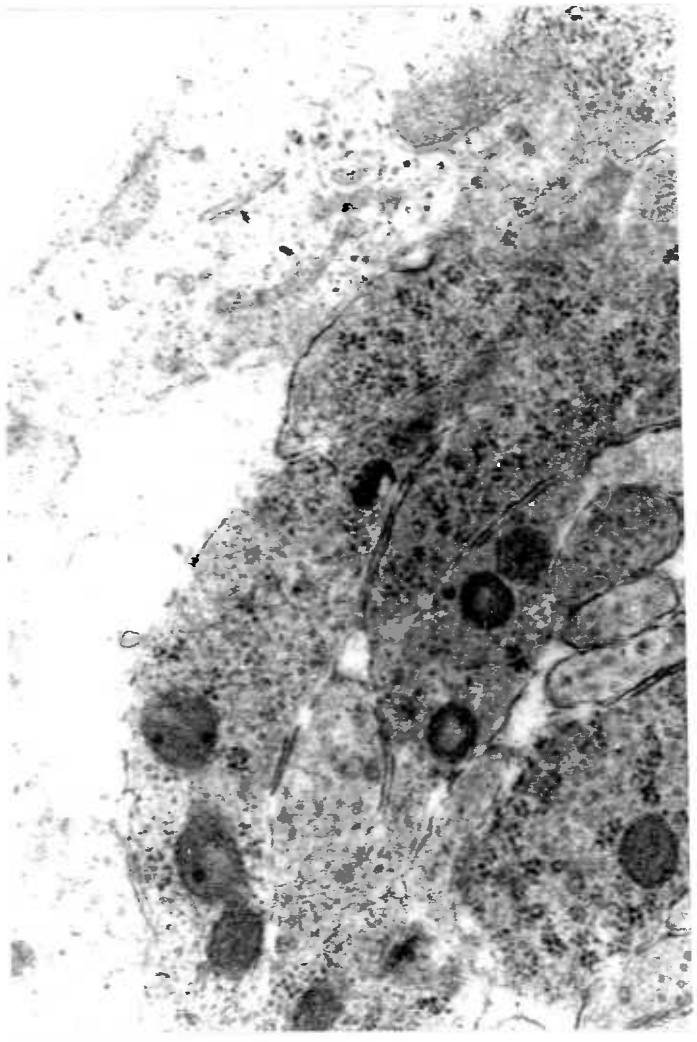

Fig. 38 Ultrastructure of the 20 day old rat external granular layer.

In some places no EGL cells remain and molecular layer begins directly beneath glial end-feet (distinguishable by their pale cytoplasm).

Lead citrate stained sagittal section (x 38,400).

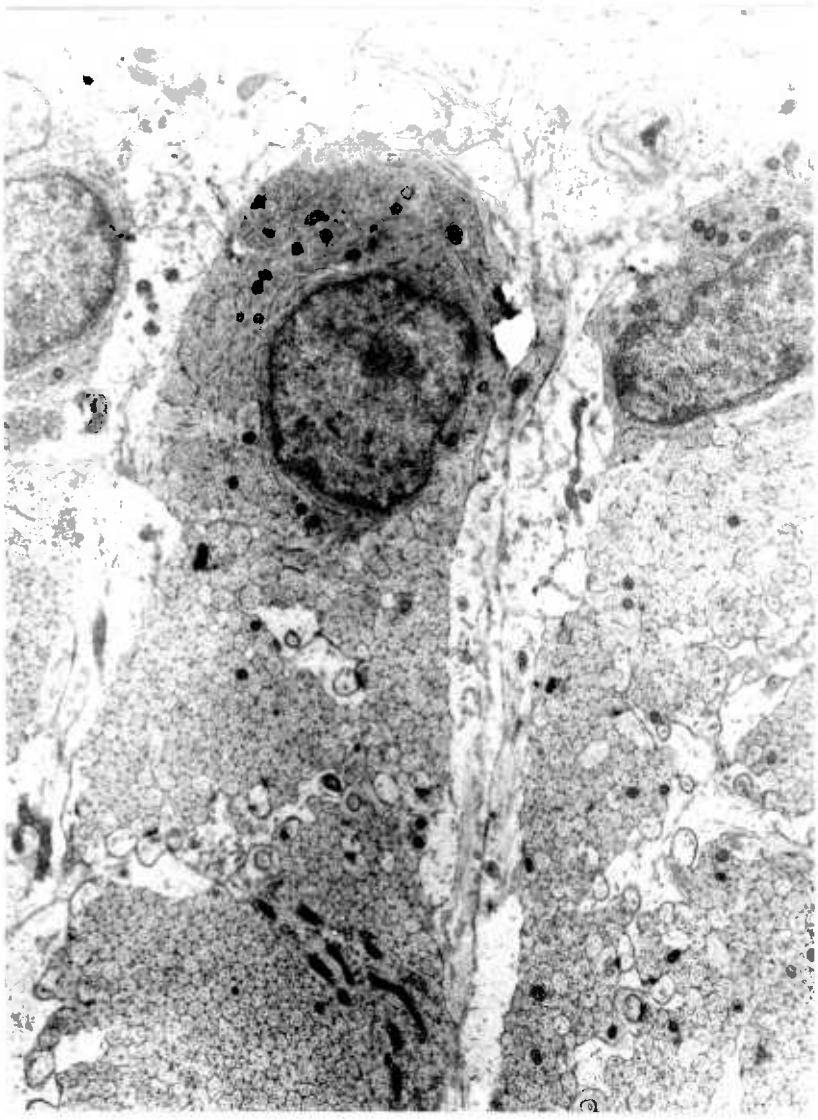

Fig. 39 Ultrastructure of the 20 day old rat external granular layer.

The molecular layer is interspersed with Bergmann glial processes.

Lead citrate stained sagittal section (x 9,600).

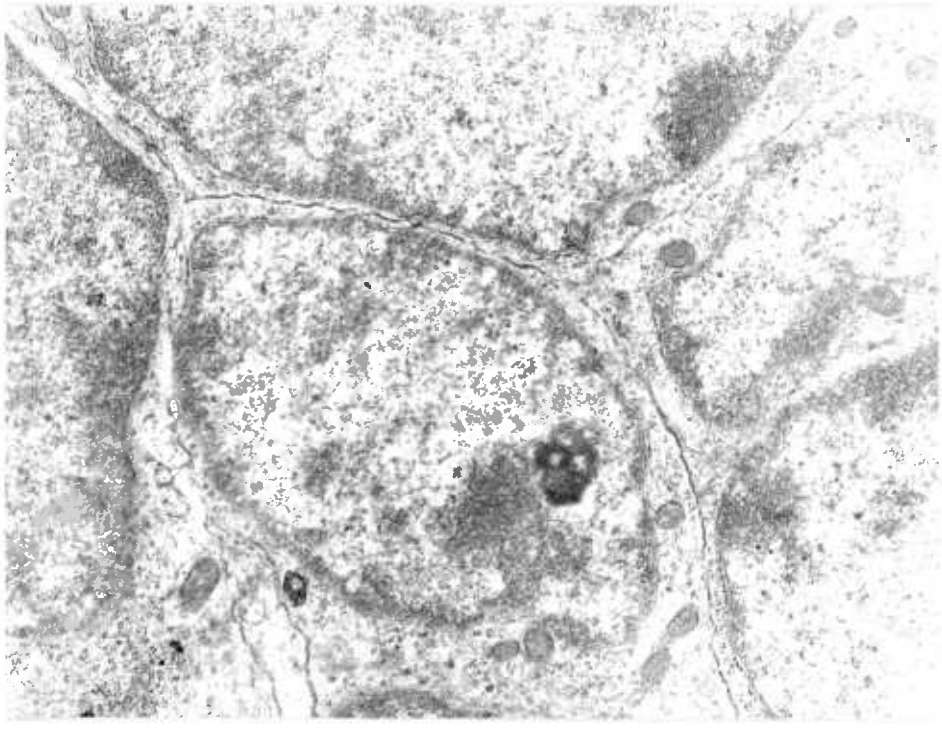

Fig. 40 Ultrastructure of the 20 day old rat internal granular layer.

Granule cell neurons in the IGL. Note the morphological similarity with the EGL cells.
Lead citrate stained sagittal section (x 15,100).

At 20 days only a few cells remained between the molecular layer and the pial surface (Fig. 37) and in some places the EGL had completely disappeared (Fig. 38). With only a few EGL cells remaining, the regularly spaced columns of Bergmann glial fibres were apparent (Fig. 39).

iii. Comments

To date the morphological studies of the EGL using electron micrascopy have faithfully followed the conventional descriptions laid down at the tum of the last century by light microscopists. Indeed, this is acknowledged by del Cerro and Snider (1972a). The fact that the superficial EGL cells are proliferating has inclined electron microscopists to favour the theory that the EGL cells are primitive, and they have sought ultrastructural evidence to support this. Mugnaini and Forströnen (1967) observed a high number of free ribosomes in the cytoplasm of EGL cells, a poorly developed membrane system, nuclei rich in chromatin and often with several nucleoli and DNA filaments and dense granules in the matrix of mitochondria. In their opinion, these ultrastructural features were in keeping with the undifferentiated nature of the EGL cells. However, they made no reference to the ultrastructural features of these cells at their final destination, i.e. as the granule cells of the IGL. Species specific differences (Table III) make direct comparisons difficult, but in the rat certain features cited by Mugnaini and Forströnen (1967) as evidence of the immaturity of EGL cells are also to be observed in mature granule cells. The latter also contain numerous free ribosomes (Fig. 40 this study; Palay and Chan-Palay, 1974). Mature granule cells have a poorly developed membrane system and few mitochondria in their perikaryon (Fig. 40 this study;

Palay and Chan-Palay, 1974). In the rat both EGL cells and granule cells have only one nucleolus, and in no instance were DNA filaments or dense granules found in mitochondria.

Since the observation that the EGL consisted of two zones (Obersteiner, 1869) evidence for a functional differentiation within the layer has been sought; emphasis has been placed on the proliferative nature of the upper zone and the postmitotic, premigratory nature of the lower zone (Mareš et al, 1970; Altman, 1972a). Apart from confirming that, in coronal section, the superficial cells are usually rounded and the deep cells elongated, no morphological differences between the cells of the two zones were found in the present study. The stereological analyses of the constituent cells of the superficial and deep EGL presented below entirely support this. Furthermore, the observation that process production occurs in the superficial layers of the EGL (Figs. 26 and 30) casts further doubt on the clear-cut distinction between the two zones.

Double-walled coated vesicle (DWCV) formation has not been previously reported in the EGL, although the vesicles themselves are said to be commonly found in EGL cytoplasm (Mugnaini and Forströnen, 1967). The formation of DWCV has been shown to occur in the molecular and internal granular layers of the rat cerebellum, in which a peak formation was observed at 20 days after birth (Eckenhoff and Pysh, 1979). It is known that there are cell surface differences between neonatal and adult cerebellar neurons (Zanetta et al, 1978). DWCV formation may represent a means for the internalisation of surface moieties, important in neuromorpho-

genesis, which are present in young animals but have disappeared by adulthood. It has therefore been suggested that DWCV formation represents a mechanism for the remodelling of neural membranes.

At no age were there found any cells in the EGL which resembled astrocytes or oligodendrocytes. This is contrary to the view of Fujita et al (1966) who reported that the morphology of the cells in the mouse EGL just before its disappearance was similar to that of glioblasts found elsewhere in the central nervous system. This may be a species-specific difference, but more probably is due to the difficulty of accurate identification of cells at the light microscope level since ultrastructural evidence for gliogenesis by the EGL was not found in the mouse (Swarz and del Cerro, 1977).

The only glial presence detected in the EGL was the fibres and end-feet of the Bergmann astrocytes. These were observed from the day of birth, and were presumably present in the foetal EGL (Swarz and Oster-Granite, 1978; Choi and Lapham, 1978). The view that Bergmann glial process production is a postnatal phenomenon in the rat cerebellum (Das et al, 1974; Das, 1976) must be seriously doubted. Since the radial fibres are present from birth, the proposal by Rakic (1971, 1972a) that they have an important role in acting as guides for the migrating EGL cells is given added support. However, the arrangement of the Bergmann glial fibres, especially as seen in scanning electron micrographs confirms the observations of Altman (1975). In this study, Altman noted fibres present from birth in the rat EGL, but did not detect alignment of them until several days later. He suggested that they became aligned into longitudinally orientated palisades,

at regular intervals. The sizeable distance between the palisades raises the possibility of alternatives to the glial processes as migration guides, and it was suggested that the migrating granule cells might use their own extending processes as a guidance mechanism (Altman, 1975). Since specialised contacts were not seen between glial processes and EGL cells, but were seen between EGL cells and their neighbours, this study lends tentative support to Altman's (1975) theory. Finally, the observations presented here do not lend support to the description of the Bergmann glial processes becoming tortuous and branched with increasing age (del Cerro and Snider, 1972a). There was certainly no evidence of the columnar arrangement of the cells within the EGL being lost (del Cerro and Snider, 1972a). Rather, the processes become straighter and more regularly spaced, supporting the observations of Bignami and Dahl (1973). From the scanning electron micrographs it can be seen that any tortuousity in the glial processes occurs only in order to avoid EGL cells, while branching only takes place near the pial surface.

In summary, the EGL is considerably more homogeneous than previously described. There is a change in the shape of its constituent cells across its width, concomitant with a decrease in extracellular space. This suggests that the major difference between the two zones is an increase in the packing density, with deeper cells being pushed closer together, thereby accentuating their bipolar shape. The cells show an ultrastructural similarity to mature granule cells and since there is no evidence of gliogenesis in the EGL, it seems probably that its components are a clone of neurons, still

capable of mitotic activity - though, since no synaptogenesis was found, not as yet functional. Their presence at the cerebellar surface has several possible explanations : to be near the high concentration of blood vessels in the pial capillaries which supply the factors necessary for their proliferation, growth and maturation; to permit the high level of mitotic activity required to generate the large number of granule cells present in the adult IGL, local generation of cells requiring only short migration paths and avoiding clogging of radial migratory guides from the periventricular region; to allow the intercellular communication (notably DWCV formation) which may be an important feature of neuromorphogenesis; and to enable the elaborate contacts between the parallel fibres and the dendritic trees of the Purkinje cells to be formed.

B. Stereology of the external and internal granular layer

i. Introduction

Though there is not complete agreement as to their source or role, it is recognised that the cells of the EGL are unique among the proliferating cells of the developing central nervous system in that they undergo a two-stage migration; proliferating at their source (generally assumed to be the rhombic lip, or germinal trigone), migrating to the pial surface of the cerebellum, proliferating at this site and then undergoing a second migration to their final destination (the lower molecular layer and the internal granule layer (IGL). Though they have already completed one stage of migration, the cells of the EGL are considered to still be immature. They have been variously described as undifferentiated or primitive cells (Altman, 1972a), germinal cells and neuroblasts (del Cerro and Snider, 1972a). This is surprising since in other parts of the central nervous system, a single migration precedes the maturation process (see Sidman and Rakic, 1973). However, all the studies of the morphology of the EGL cells, in particular the ultrastructural observations of Mugnaini and Forströnen (1967), del Cerro and Snider (1972a) and Altman (1972a), present only subjective qualitative observations; to date, no quantitative data has been present which would confirm the view that the EGL cells are "primitive". In contrast to the EGL cells, the microneurons of the IGL have never been described as being undifferentiated. The maturational changes that occur, distinguishing EGL cells from the cortical microneurons must be assumed to take place immediately preceeding, or during the migration of the cells to the IGL. Two types of

changes would be expected to occur : functional changes and morphological changes. The functional changes might include the development of synapses and the synthesis of neurotransmitter substances, while the morphological changes would include an increase in the number of mitochondria, an increase in amount of rough endoplasmic reticulum and an increase in size of the Golgi apparatus (Jacobson, 1978). Since no synaptic contacts were observed in the EGL it was decided to investigate the morphological changes occurring in the cells of the EGL through the postnatal lifespan of the layer, and to compare these with the changes, if any, in the cells which they are destined to become - the microneurons of the IGL. Since comparisons between the EGL and IGL cells for any given age were made by measurements made on the same section, it was not found necessary to convert the results to absolute figures, hence the results are all given as relative volumes in arbitrary units.

Since Obersteiner's (1883) description of two morphologically distinct zones within the EGL, attempts have been made to ascribe functional correlates to the two zones (Mareš et al, 1970, Altman, 1972a). It was considered to be of interest therefore to compare the stereological differences, if any, between the two zones.

ii. Results

General observations : As a result of the wide variations of all parameters measured in all populations of cells the standard deviations were very large. However, since a large number of cells were analysed, standard errors of

Table IV Relative volumes of components of external and internal granular layer cells (for number of cells per group (n) see table VII).

Component / Age	Cytoplasm (x9)	Nucleus (x9)	Mitochondria	RER	Golgi
Day 3 EGL	14.37 ± 11.5*	35.49 ± 25.2	4.77 ± 6.6	0.67 ± 1.6	1.89 ± 4.5
	$p = 1.0 \times 10^{-4}$	$p = 3.4 \times 10^{-4}$	$p = 0.61$	$p = 0.21$	$p = 0.87$
Day 3 IGL	21.49 ± 12.5	48.48 ± 18.2	5.29 ± 6.8	1.00 ± 1.8	1.77 ± 5.2
Day 5 EGL	14.20 ± 8.7	34.86 ± 18.0	5.43 ± 6.8	0.55 ± 1.2	2.15 ± 4.8
	$p = 1.3 \times 10^{-3}$	$p = 6.1 \times 10^{-7}$	$p = 0.40$	$p = 6.1 \times 10^{-4}$	$p = 2.6 \times 10^{-2}$
Day 5 IGL	20.85 ± 13.1	57.63 ± 29.1	6.71 ± 9.0	2.00 ± 3.1	5.54 ± 11.23
Day 8 EGL	10.54 ± 8.8	25.87 ± 16.1	5.15 ± 7.0	0.73 ± 1.7	3.29 ± 7.4
	$p = 1.6 \times 10^{-10}$	$p = 1.4 \times 10^{-10}$	$p = 1.4 \times 10^{-3}$	$p = 4.5 \times 10^{-7}$	$p = 1.4 \times 10^{-2}$
Day 8 IGL	22.59 ± 12.0	48.79 ± 18.2	9.44 ± 9.8	2.98 ± 3.7	7.00 ± 11.9
Day 13 EGL	13.86 ± 11.6	31.46 ± 18.2	6.24 ± 6.9	1.20 ± 1.8	2.67 ± 6.6
	$p = 0.34$	$p = 2.0 \times 10^{-3}$	$p = 6.1 \times 10^{-3}$	$p = 3.9 \times 10^{-3}$	$p = 1.4 \times 10^{-2}$
Day 13 IGL	15.28 ± 9.3	39.07 ± 15.8	8.30 ± 7.7	2.21 ± 2.8	5.63 ± 9.7
Day 16 EGL	7.49 ± 4.0	19.90 ± 10.6	4.05 ± 4.3	0.51 ± 0.9	2.85 ± 4.4
	$p = 9.0 \times 10^{-7}$	$p = 2.0 \times 10^{-10}$	$p = 2.9 \times 10^{-5}$	$p = 3.7 \times 10^{-6}$	$p = 4.0 \times 10^{-2}$
Day 16 IGL	12.02 ± 7.2	36.92 ± 18.4	7.80 ± 6.9	1.76 ± 2.2	5.20 ± 9.4

* ± standard deviations

Table V Volume of component per unit volume cytoplasm (density) of external and internal granular layer cells

Component: / Age	Nucleus (x 9)	Mitochondria (x 10^3)	RER (x 10^3)	Golgi (x 10^3)	number of cells per group (n)
Day 3 EGL	3.30 ± 2.3*	29.35 ± 30.4	7.27 ± 26.1	8.85 ± 23.7	110
	p = 0.25	p = 0.21	p = 0.41	p = 0.39	
Day 3 IGL	2.92 ± 1.8	23.42 ± 29.6	4.55 ± 7.1	5.92 ± 17.8	66
Day 5 EGL	3.42 ± 2.7	34.52 ± 32.2	4.55 ± 10.3	12.18 ± 29.1	71
	p = 0.96	p = 0.18	p = 4.9×10^{-2}	p = 0.27	
Day 5 IGL	3.44 ± 2.4	26.74 ± 27.3	8.56 ± 10.8	19.00 ± 38.4	48
Day 8 EGL	3.60 ± 2.64	42.64 ± 40.1	5.82 ± 13.8	20.62 ± 45.1	104
	p = 6.6×10^{-2}	p = 0.88	p = 1.5×10^{-2}	p = 0.84	
Day 8 IGL	2.85 ± 2.3	41.70 ± 31.7	11.05 ± 11.9	22.02 ± 37.1	61
Day 13 EGL	3.62 ± 3.5	47.42 ± 37.5	9.63 ± 16.0	12.99 ± 34.6	91
	p = 0.98	p = 0.20	p = 2.5×10^{-2}	p = 2.1×10^{-2}	
Day 13 IGL	3.60 ± 2.6	54.51 ± 40.8	14.73 ± 19.0	27.5 ± 46.5	86
Day 16 EGL	3.48 ± 2.6	57.31 ± 55.1	7.54 ± 16.5	40.69 ± 58.4	80
	p = 0.22	p = 0.11	p = 7.0×10^{-3}	p = 0.29	
Day 16 IGL	4.30 ± 5.5	69.54 ± 47.5	14.33 ± 16.4	31.93 ± 54.1	106

* ± standard deviations

Table VI Relative volumes of components of round and elongated external granular layer cells

(for numbers of cells per group (n) see Table IX)

Component	Cytoplasm (x 0.1)	Nucleus (x 0.1)	Mitochondria	RER	Golgi
Age					
Day 3 round cells	17.74 ± 14.6*	44.30 ± 29.6	7.43 ± 9.9	0.70 ± 1.3	0.43 ± 2.1
	p = 0.27	p = 0.15	p = 0.97	p = 0.53	p = 4.4×10^{-2}
Day 3 elong. cells	23.06 ± 14.7	58.44 ± 29.0	7.31 ± 8.7	1.00 ± 1.7	3.13 ± 5.7
Day 5 round cells	17.28 ± 9.3	39.78 ± 17.3	6.89 ± 7.5	0.78 ± 1.5	1.78 ± 4.4
	p = 0.64	p = 0.33	p = 0.97	p = 0.37	p = 0.70
Day 5 elong cells	18.73 ± 8.5	45.60 ± 16.0	7.00 ± 9.3	0.33 ± 1.3	2.53 ± 6.7
Day 8 round cells	18.02 ± 12.6	34.14 ± 15.8	10.76 ± 8.6	1.62 ± 2.9	8.05 ± 11.5
	p = 0.54	p=1.6×10^{-2}	p = 0.62	p = 0.83	p = 0.32
Day 8 elong cells	16.08 ± 8.6	48.17 ± 13.8	9.08 ± 10.6	1.42 ± 2.1	4.42 ± 6.2
Day 13 round cells	19.33 ± 15.6	34.00 ± 21.4	8.20 ± 8.4	1.63 ± 2.3	3.62 ± 8.3
	p = 0.71	p=1.4×10^{-2}	p = 0.46	p = 0.40	p = 0.85
Day 13 elong cells	21.00 ± 10.9	50.79 ± 17.4	10.29 ± 8.9	1.07 ± 1.5	4.14 ± 8.0

Probability values calculated from Student's test for independent t

* ± standard deviations

Table VII Volume of component per unit volume cytoplasm (density) of round and elongated external granular layer cells

Component	Nucleus (x 0.1)	Mitochondria (x 10^3)	RER (x 10^3)	Golgi (x 10^3)	number of cells per group (n)
Age					
Day 3 round cells	5.03 ± 5.1*	35.00 ± 38.6	2.74 ± 5.1	0.96 ± 4.6	123
	p = 0.19	p = 0.32	p = 0.20	p = 3.1×10^{-2}	
Day 3 elong cells	3.16 ± 1.8	24.00 ± 23.6	7.31 ± 15.9	9.44 ± 17.4	116
Day 5 round cells	3.19 ± 3.2	32.61 ± 29.1	4.11 ± 7.2	8.00 ± 20.9	118
	p = 0.71	p = 0.99	p = 0.21	p = 0.71	
Day 5 elong cells	3.61 ± 3.3	32.40 ± 32.9	1.27 ± 4.9	11.46 ± 31.7	115
Day 8 round cells	2.88 ± 2.9	64.57 ± 31.4	8.48 ± 15.8	35.43 ± 41.1	121
	p = 0.32	p = 0.32	p = 0.68	p = 0.89	
Day 8 elong cells	3.83 ± 2.0	51.17 ± 44.1	6.42 ± 8.8	37.92 ± 56.9	112
Day 13 round cells	2.96 ± 4.4	43.87 ± 29.3	9.06 ± 13.8	13.00 ± 39.0	130
	p = 0.86	p = 0.69	p = 0.32	p = 0.64	
Day 13 elong cells	3.18 ± 1.9	47.71 ± 31.5	5.14 ± 7.2	18.79 ± 34.5	114

probability values calculated from student's test for independent t

* ± standard deviations

the mean were small.

Since, after the end of the second postnatal week, the rat EGL has become very attenuated, analyses were restricted to the period up to postnatal day 16.

Comparison of volumes of cell constituents in EGL and IGL : The relative volume of cytoplasm per EGL cell became gradually smaller with age. Likewise, the relative volume of cytoplasm per IGL cell was gradually reduced over the same period (Fig. 10). However, the cytoplasm of the IGL cells was slightly but consistently larger than that of the EGL cells. Exactly the same pattern applied to the nuclear volumes (Fig. 41); the nuclei of both the EGL and IGL cells became slightly smaller with age, though the IGL cell nuclei were significantly larger than those of the EGL cells throughout. The relative volume of mitochondria per EGL cell remained constant for the first two postnatal weeks, after which it showed a decline, while in the IGL the relative volume of mitochondria per cell increased for the first week before commencing a gradual decrease (Fig. 42). In the first postnatal week there was no significant difference between the mitochondrial volumes per cell of the two populations but from postnatal day 8, the mitochondrial volume per cell in the IGL was significantly larger than that in the EGL (Fig. 43). This pattern was mirrored in the changes in relative volume of both rough endoplasmic reticulum and Golgi apparatus; the relative volumes per cell of both organelles remained constant in the EGL cells while in the IGL cells they rose to a peak at postnatal day 8, declining from then to postnatal day 16. The IGL cells possessed a

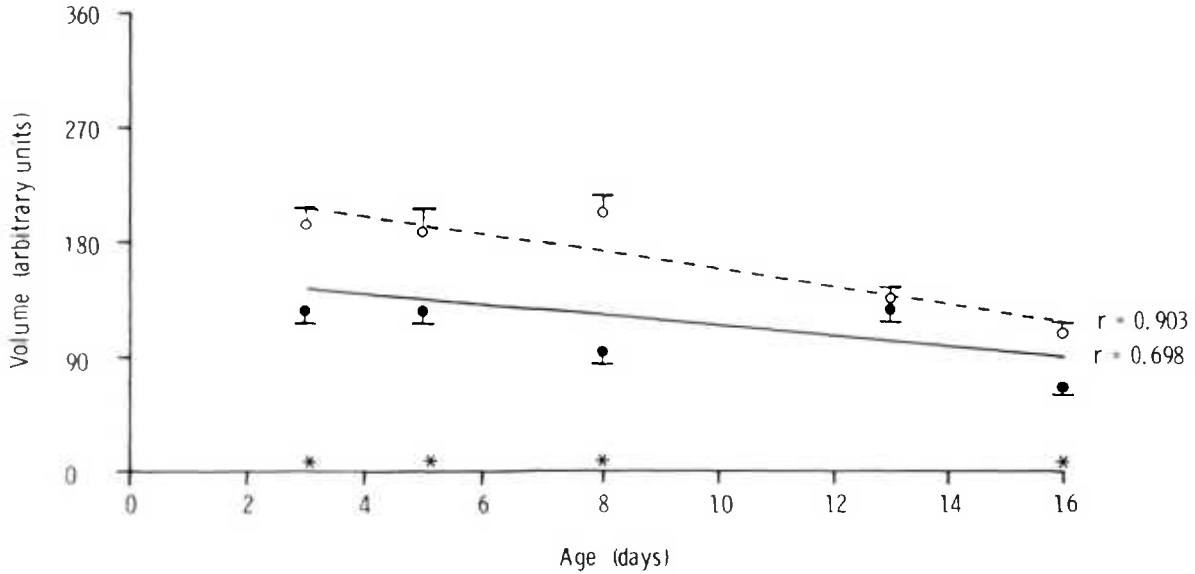

Fig. 41 Stereological comparison between the cells of the external and internal granular layers. (i) Cytoplasmic volumes.

The figure shows the mean and standard error for each age point. ●——● external granular layer; ○——○ internal granular layer; * $p < 0.05$; r = coefficient of linear regression.

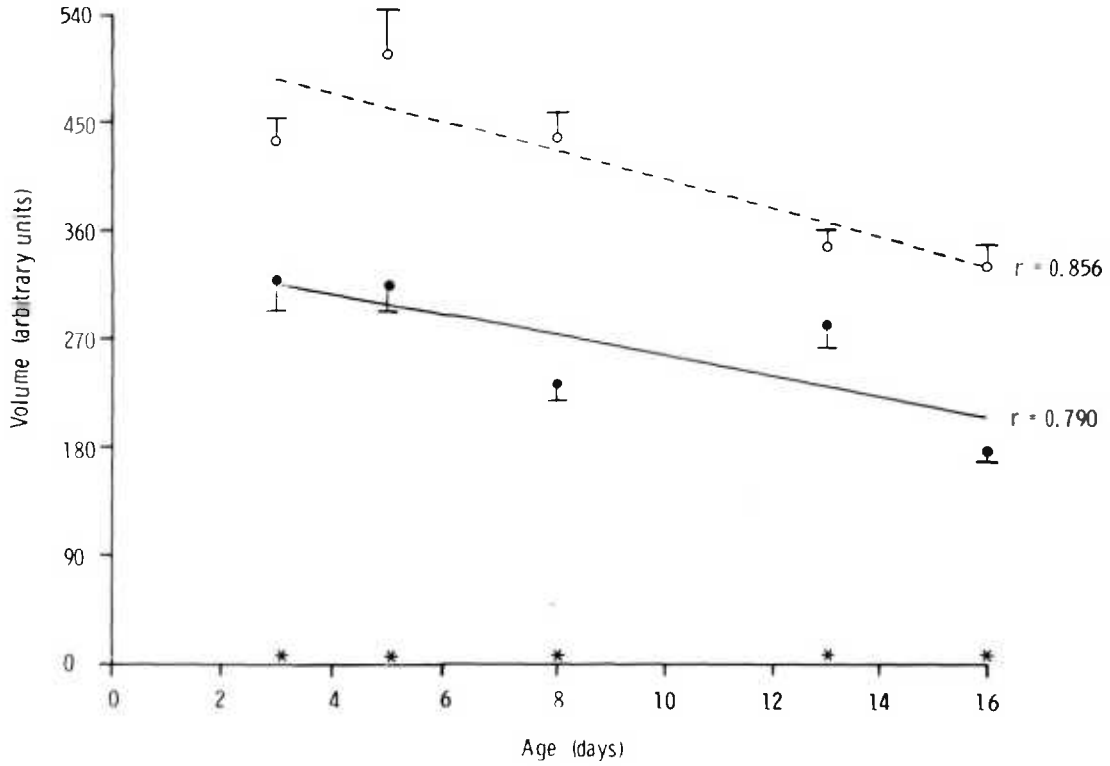

Fig. 42 Stereological comparison between cells of the external and internal granular layers. (ii) Nuclear volumes.

The figure shows the mean and standard error for each age point. ●——● external granular layer; ○——○ internal granular layer; * $p < 0.05$; r = coefficient of linear regression.

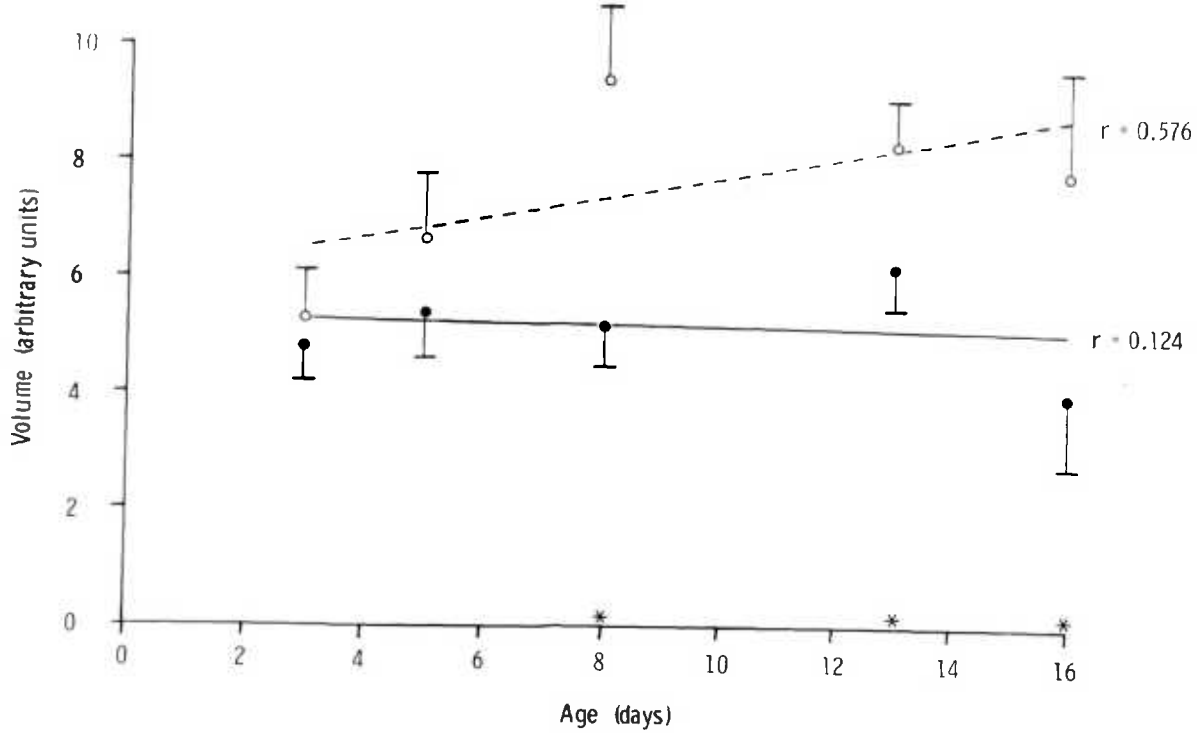

Fig. 43 Stereological comparison between cells of the external and internal granular layers. (iii) Mitochondrial volumes.

The figure shows the mean and standard error for each age point. ●——● external granular layer; ○——○ internal granular layer; * $p < 0.05$; r = coefficient of linear regression.

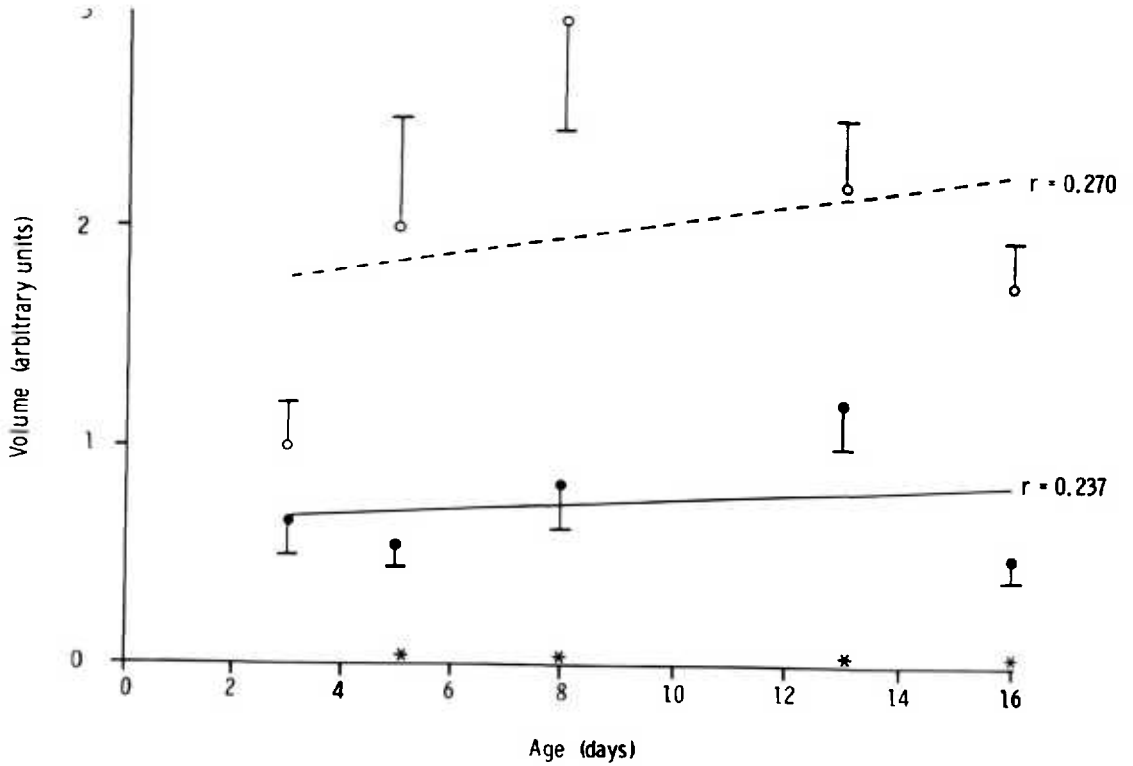

Fig. 44 Stereological comparison between cells of the external and internal granular layers. (iv) Rough endoplasmic reticular volumes.
The figure shows the mean and standard error for each age point. ●——● external granular layer; ○——○ internal granular layer; * $p < 0.05$; r = coefficient of linear regression.

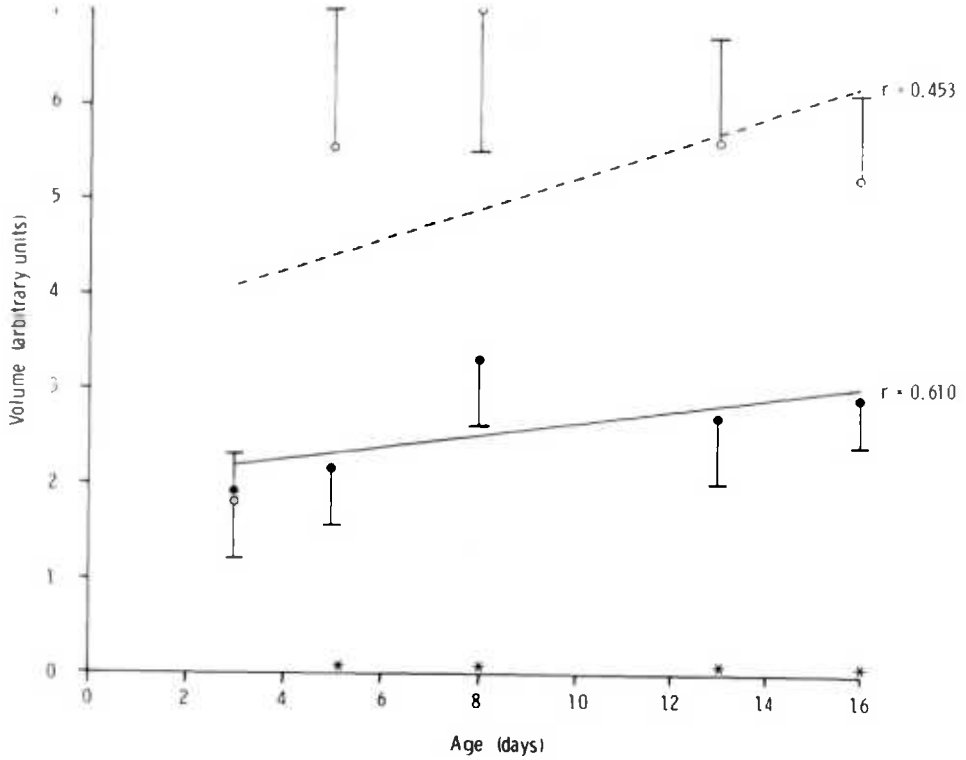

Fig. 45 Stereological comparison between cells of the external and internal granular layers.

(v) Golgi body volumes.

The figure shows the mean and standard error for each age point. ●——● external granular layer; o- - - -o internal granular layer; * $p < 0.05$; r = coefficient of linear regression.

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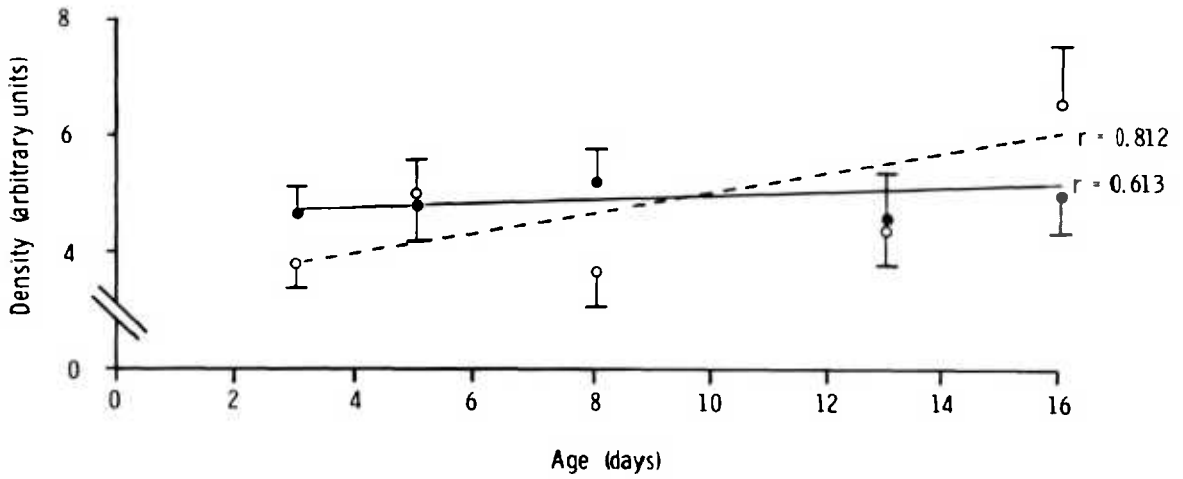


Fig. 46 Stereological comparison between cells of the external and internal granular layers. (vi) Nuclear densities.

The figure shows the mean and standard error for each age point. ●——● external granular layer; ○- - - -○ internal granular layer; r = coefficient of linear regression.

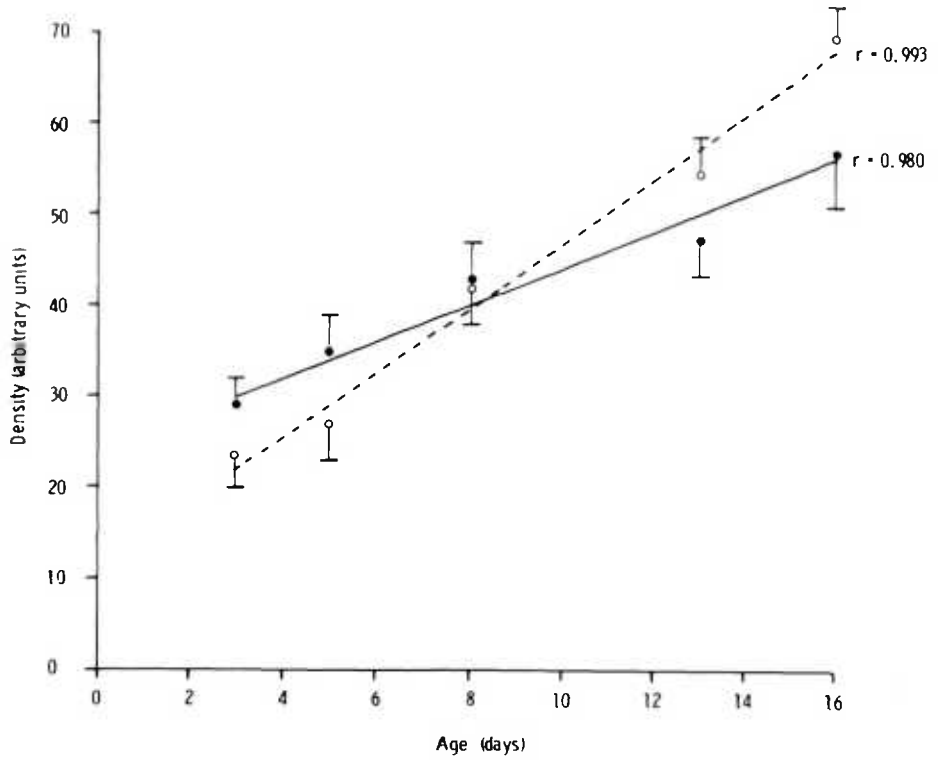


Fig. 47 Stereological comparison between cells of the external and internal granular layers.

(vii) Mitochondrial densities.

The figure shows the mean and standard error for each age point. ●——● external granular layer; ○——○ internal granular layer; r = coefficient of linear regression.

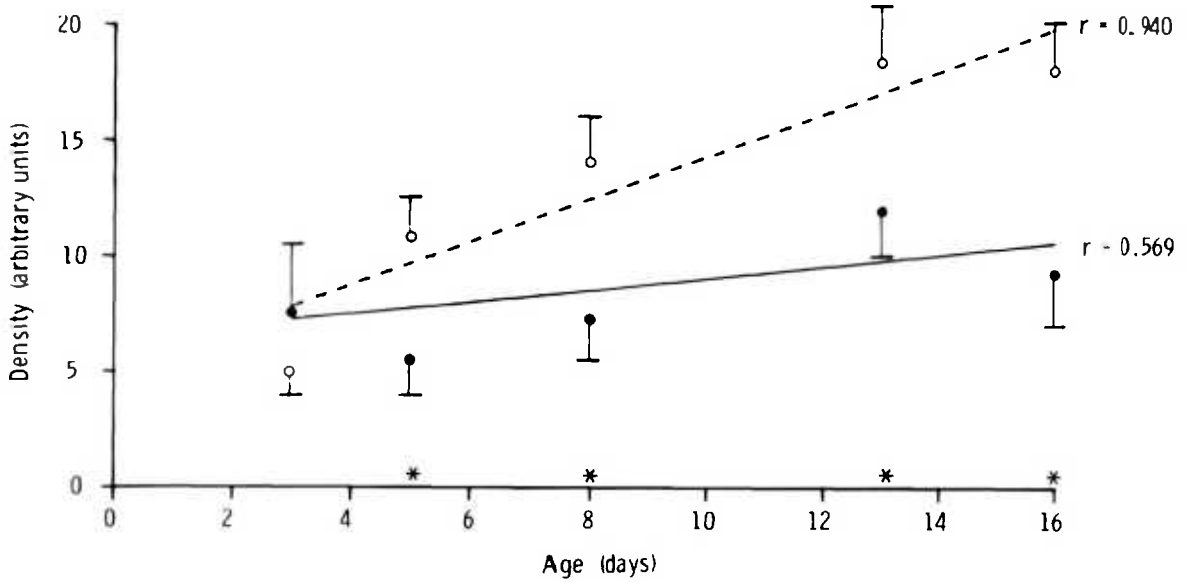


Fig. 48 Stereological comparison between cells of the external and internal granular layers. (viii) Rough endoplasmic reticular densities.
The figure shows the mean and standard error for each age point. ●——● external granular layer; ○——○ internal granular layer; * $p < 0.05$; r = coefficient of linear regression.

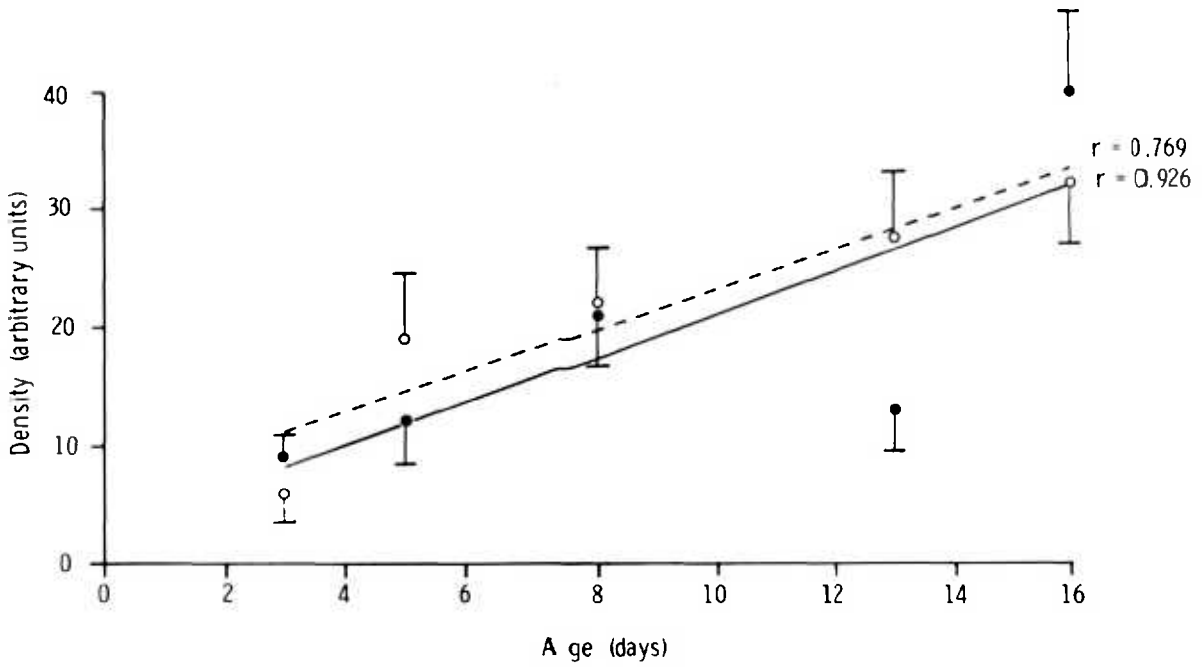


Fig. 49 Stereological comparison between cells of the external and internal granular layers.

(ix) Golgi body densities.

The figure shows the mean and standard error for each age point. ●——● external granular layer; ○——○ internal granular layer; r = coefficient of linear regression.

significantly larger relative volume per cell of RER and Golgi apparatus (Figs. 44 and 45) (Table IV).

Comparison of organelle density in EGL and IGL : Since, from the calculations of relative volumes, it was apparent that from the outset the IGL cells were larger than the EGL cells, it was not surprising that the relative volumes of organelles per IGL cell were found to be larger than those of the EGL cells. As an index of cell maturation it was considered more appropriate to examine organelle density, i.e. organelle per unit volume of cytoplasm. This was calculated by dividing the relative volume of each organelle by the relative volume of the cytoplasm of its cell.

In the cases of nuclei, mitochondria and Golgi apparatus there is no significant difference between the densities per IGL cell and the densities per EGL cell, and the densities per cell increase with age (Figs. 46, 47, 49). However, the RER is different; except for postnatal day three, there is a higher density of RER in the IGL cells and a trend for the rate of increase of RER density to be higher in the IGL cells than in the EGL cells (Fig. 48) (Table V).

Comparison of volumes of round and elongated EGL cells : The same parameters were used to compare the round cells of the EGL with the elongated cells of the EGL in coronal sections. The round cells corresponded to the cells of the proliferative zone and the elongated cells to those of the premigratory zone (Altman, 1972a).

The nuclei of the elongated cells were significantly larger in relative volume than the nuclei of the round cells at postnatal days 8 and 13 (Fig. 51).

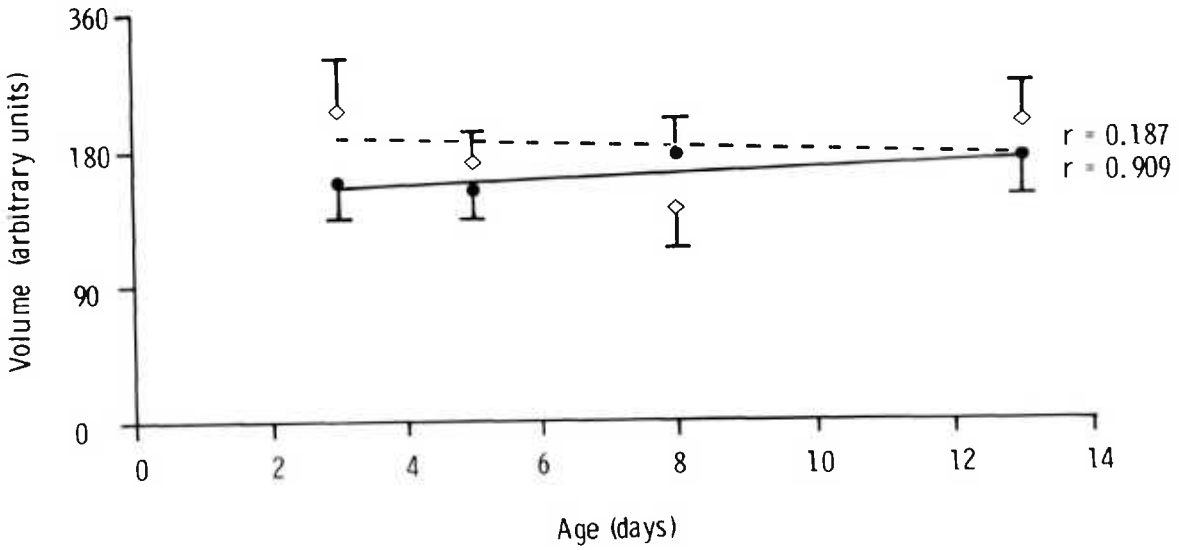


Fig. 50 Stereological comparison between the rounded and elongated cells of the external granular layer. (i) Cytoplasmic volumes.
The figure shows the mean and standard error for each age point. ●——● rounded cells; ◇——◇ elongated cells; r = coefficient of linear regression.

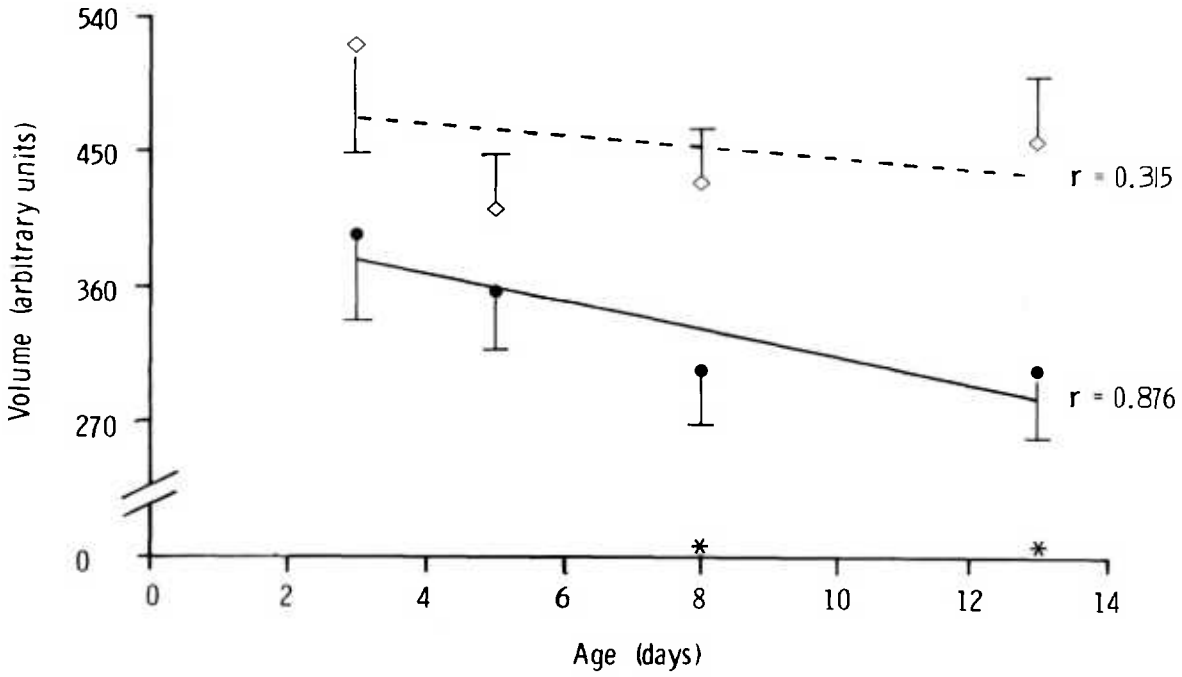


Fig. 51 Stereological comparison between the rounded and elongated cells of the external granular layer. (ii) Nuclear volumes.

The figure shows the mean and standard error for each age point. ●——● rounded cells; ◇——◇ elongated cells; * $p < 0.05$; r = coefficent of linear regression.

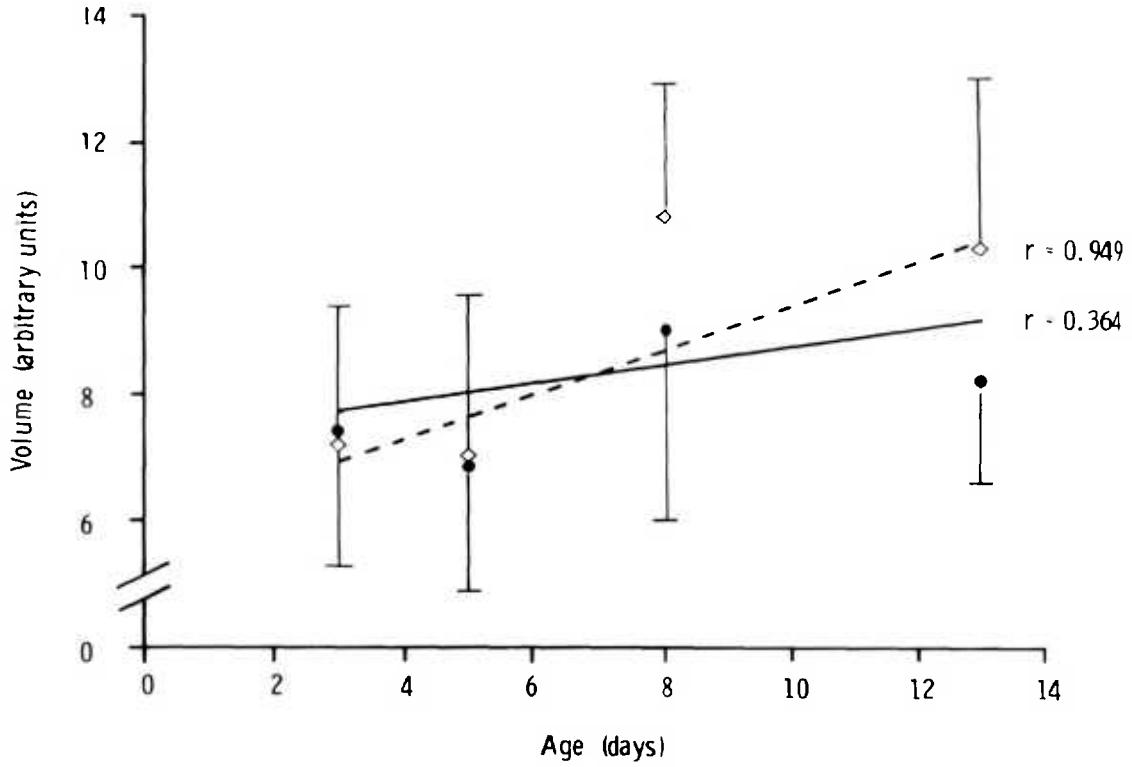


Fig. 52 Stereological comparison between the rounded and elongated cells of the external granular layer. (iii) Mitochondrial volumes.
The figure shows the mean and standard error for each age point. ●——● rounded cells; ◇——◇ elongated cells; r = coefficient of linear regression.

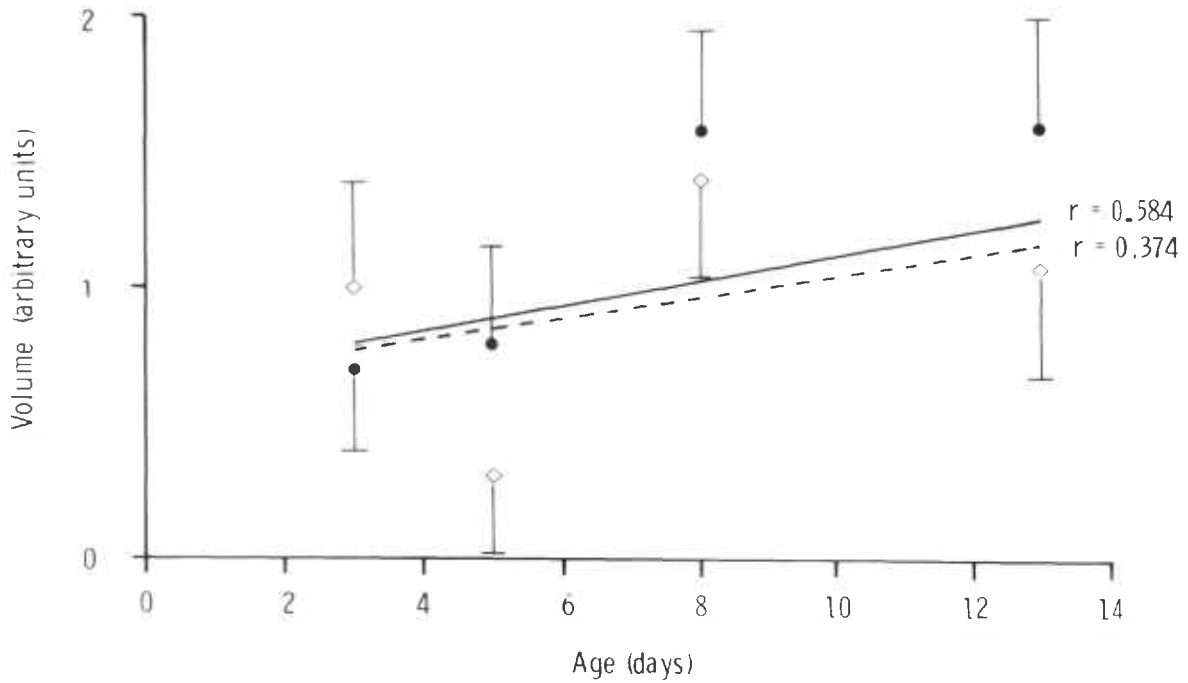


Fig 53 Stereological comparison between the rounded and elongated cells of the external granular layer. (iv) Rough endoplasmic reticular volumes.
The figure shows the mean and standard error for each age point. ●——● rounded cells; ◇——◇ elongated cells; r = coefficient of linear regression.

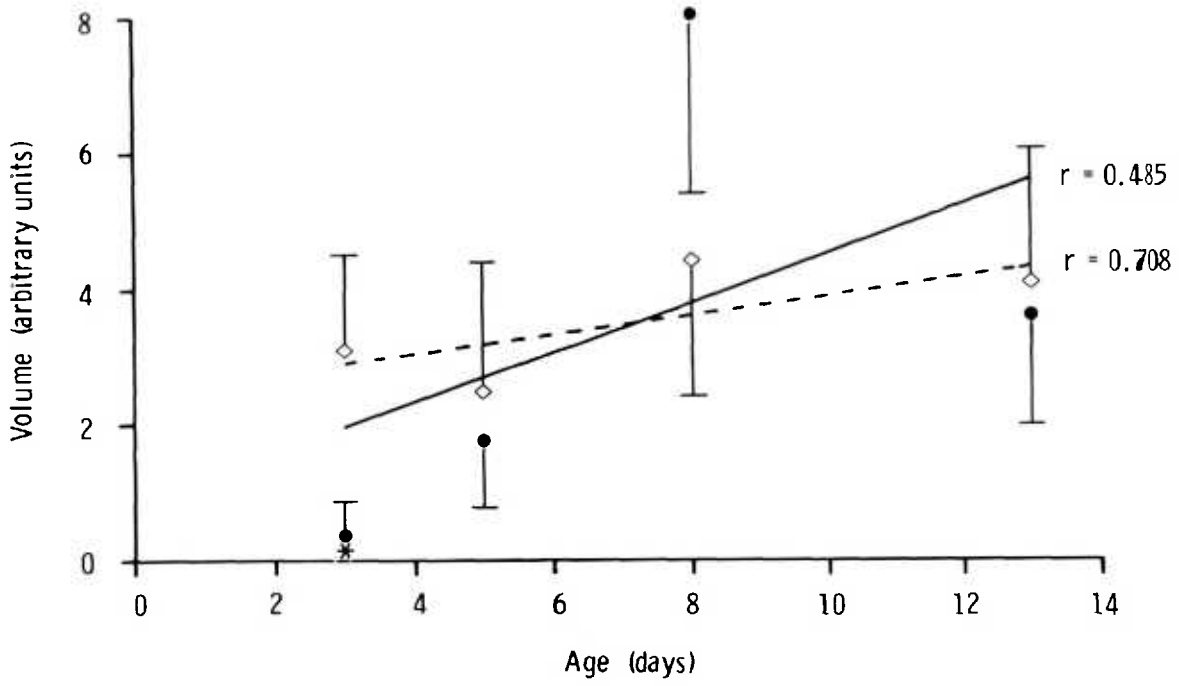


Fig. 54 Stereological comparison between the rounded and elongated cells of the external granular layer. (v) Golgi body volumes

The figure shows the mean and standard error for each age point. ●——● rounded cells; ◇——◇ elongated cells; * $p < 0.05$; r = coefficient of linear regression.

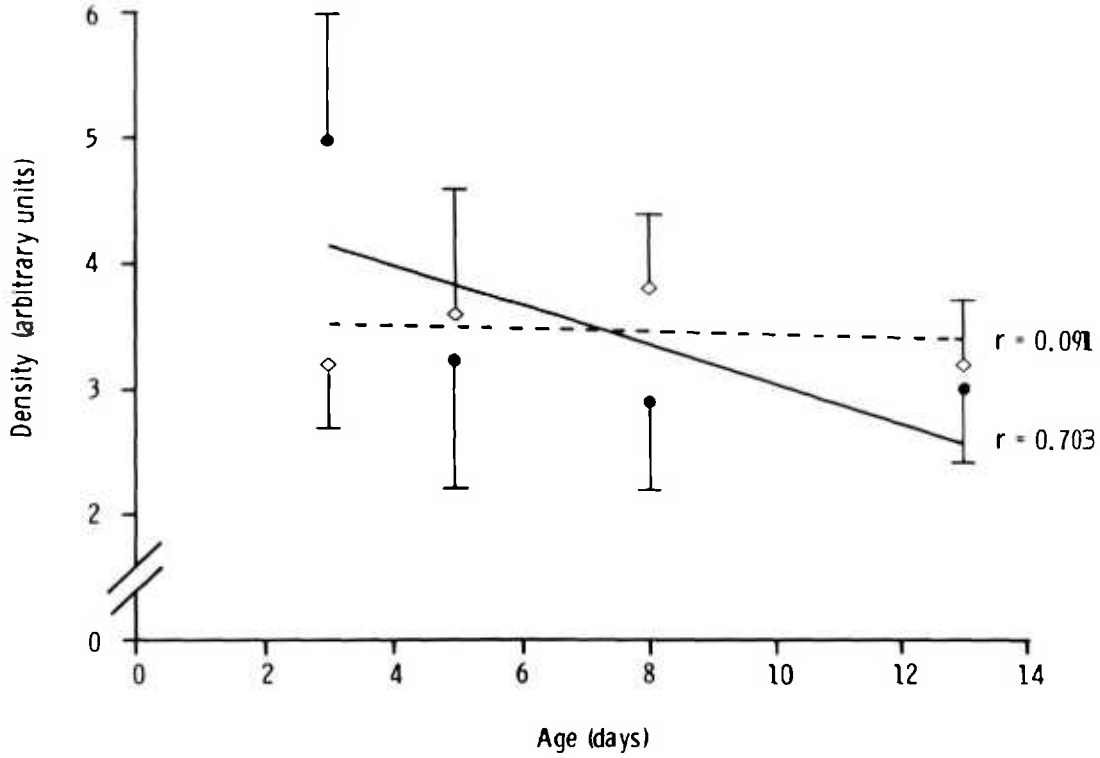


Fig. 55 Stereological comparison between the rounded and elongated cells of the external granular layer. (vi) Nuclear densities.
The figure shows the mean and standard error for each age point; ●——● rounded cells; ◇——◇ elongated cells; r = coefficient of linear regression.

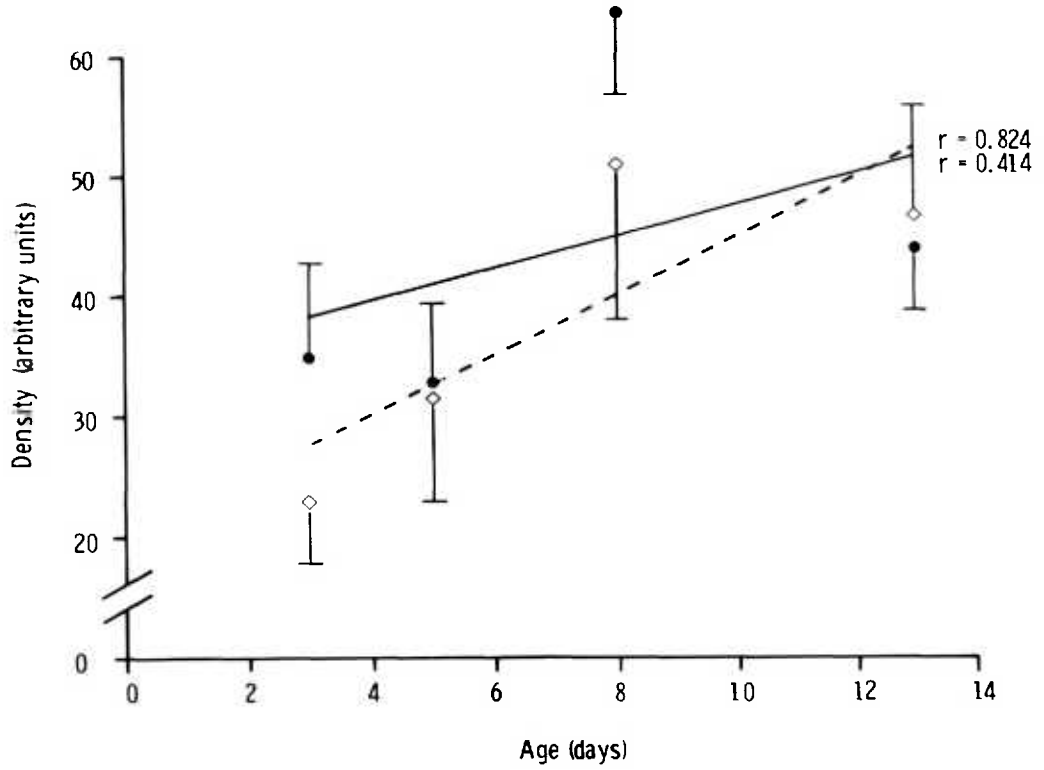


Fig. 56 Stereological comparison between the rounded and elongated cells of the external granular layer. (vii) Mitochondrial densities.

The figure shows the mean and standard error for each age point. ●——● rounded cells; ◇——◇ elongated cells; r = coefficient of linear regression.

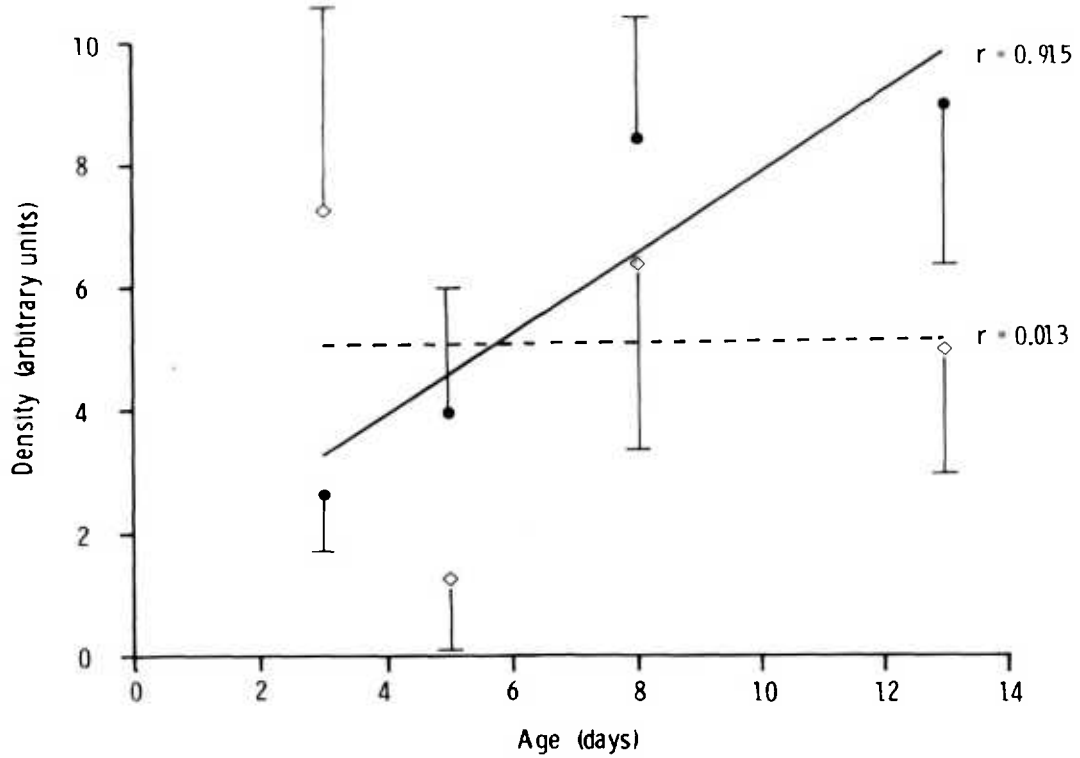


Fig. 57 Stereological comparison between the rounded and elongated cells of the external granular layer. (viii) Rough endoplasmic reticular densities. The figure shows the mean and standard error for each age point. ●——● rounded cells; ◇——◇ elongated cells; r = coefficient of linear regression.

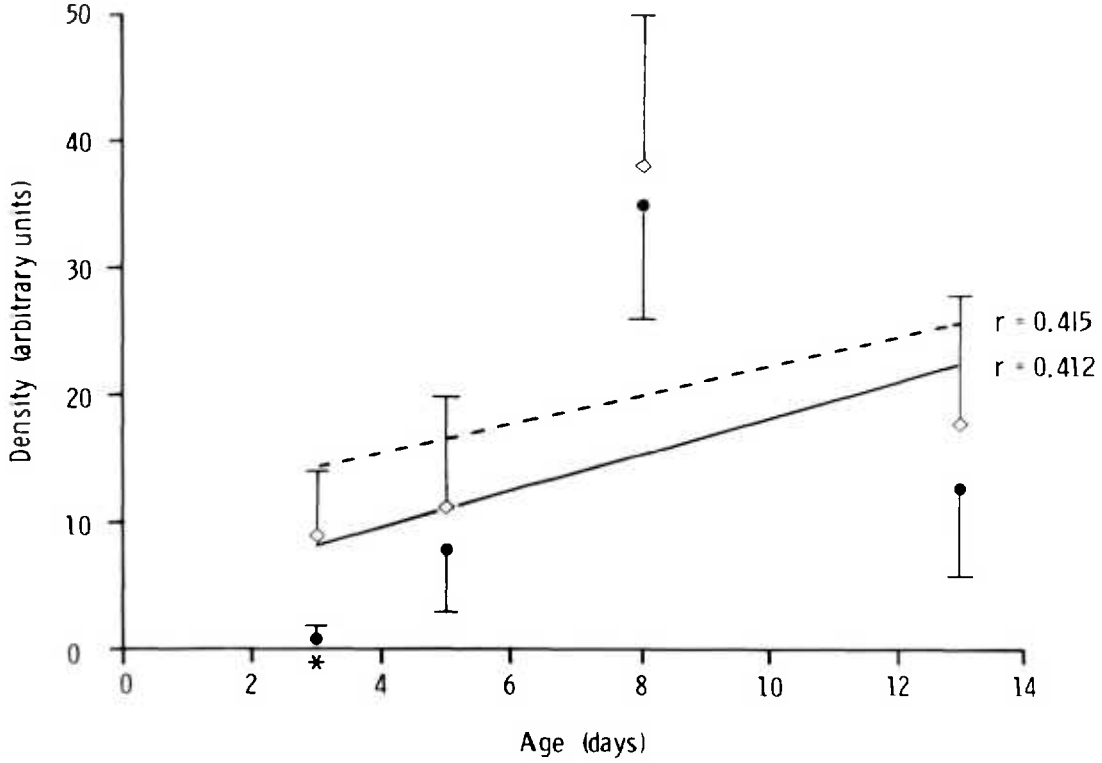


Fig. 58 Stereological comparison between the rounded and elongated cells of the external granular layer. (ix) Golgi body densities.
The figure shows the mean and standard error for each age point. ●——● rounded cells; ◇——◇ elongated cells; * $p<0.05$; r= coefficient of linear regression.

At postnatal day 3 the elongated cells possessed a significantly larger relative volume of Golgi apparatus than the round cells (Fig. 54). Otherwise, there was no statistically significant difference between the relative volumes of the organelles of the two groups (Figs. 50 - 54).

There were also no clear-cut changes in the relative volumes of the organelles with age (Figs. 50 - 54). There was no significant change in the relative volumes of cytoplasm (Fig. 50) but the relative nuclear volumes decreased in early postnatal life (Fig. 51). Mitochondria and rough endoplasmic reticulum increased with age (Figs. 52 and 53) and the relative volumes of the Golgi apparatus showed a peak at postnatal day 8 (Fig. 54) (Table VI).

Comparison of organelle density in round and elongated EGL cells : Only one statistically significant difference between the densities of organelles in the round and elongated EGL cells was detected. At postnatal day 3, the elongated cells contained a higher density of Golgi apparatus (Fig. 58). In all other cases the organelle densities in both groups of cells were statistically indistinguishable (Figs. 55 - 58). The nuclear density of the round cells showed a decrease for the first postnatal week, followed by a slight rise, while the nuclear density of the elongated cells remained statistically similar throughout (Fig. 55). The other organelles increased in density over the period examined, with the rough endoplasmic reticulum and Golgi apparatus showing a peak density at postnatal day 8 (Figs. 56 - 58) (Table VII).

iii. Comments

From the analyses of relative volumes it was determined that the IGL cells were larger overall than the EGL cells. With a greater volume of cytoplasm, it was not surprising that in the IGL cells the mean volumes for nucleus, mitochondria, RER and Golgi apparatus were greater than in the EGL. However, by calculating the organelle density and thus correcting for the larger cytoplasmic volume, the organelle density for the two populations at all ages was indistinguishable, with the exception of the RER. The difference in relative RER densities could well be accounted for by the fact that the IGL cells are functional neurons and thus require the apparatus for the synthesis of their neurotransmitter substance. The decrease in relative volume of the IGL cell nuclei has already been reported by Heinsen (1978), who described a rapid decrease in nuclear diameter between the first and third postnatal weeks in his studies on rats.

The densities of the mitochondria and Golgi apparatus in both the EGL and IGL cells increase linearly with age. It thus appears that the two populations are equally immature at birth, and mature at the same rate in situ.

The comparison between the two zones of the EGL seen in coronal section did not yield such a clear pattern. This was in part due to the very large variations in the size of the constituent cells. However, apart from the exceptions described above, the two populations of cells could not be differentiated on a statistical basis. This would indicate that the major, if not only, difference between the cells of the two zones, is their shape.

C. Cell proliferation in the external granular layer

i. Introduction

As has already been noted, since the description by Obersteiner (1883) of the presence of two zones in the EGL, attempts have been made to attach a functional significance to the morphological differences between them. Fujita (1967) reported [^{3}H] thymidine labelling among the cells of the outermost zone only, while the inner zone was said to comprise differentiated cells incapable of further DNA synthesis, i.e. "neuroblasts" in the process of migrating out of the EGL. Mareš et al (1970) reported mitoses among the deeper cells (confirming Cajal, 1911) but considered them sufficiently infrequent to confine their cell kinetic studies to the cells of the outer zone only. Altman (1972) proposed the term proliferative zone for the outer layer of round cells, and premigratory zone for the inner layer of spindle-shaped cells, and stated that there were no mitotic cells among the cells of the premigratory zone. Since these observations do not provide a comprehensive picture of cell proliferation within the EGL a quantitative study of the pattern of proliferation in the cells of this layer was undertaken (Koppel et al, 1978; Koppel and Lewis, 1978).

ii. Results

Mitotic figures were found at all levels of the EGL at every age from birth to postnatal day 21, when the EGL has virtually disappeared. Mitotic cells were most frequently seen in the outer reaches of the EGL, but were not uncommon amongst the elongated cells in the lower zone (Fig. 59).

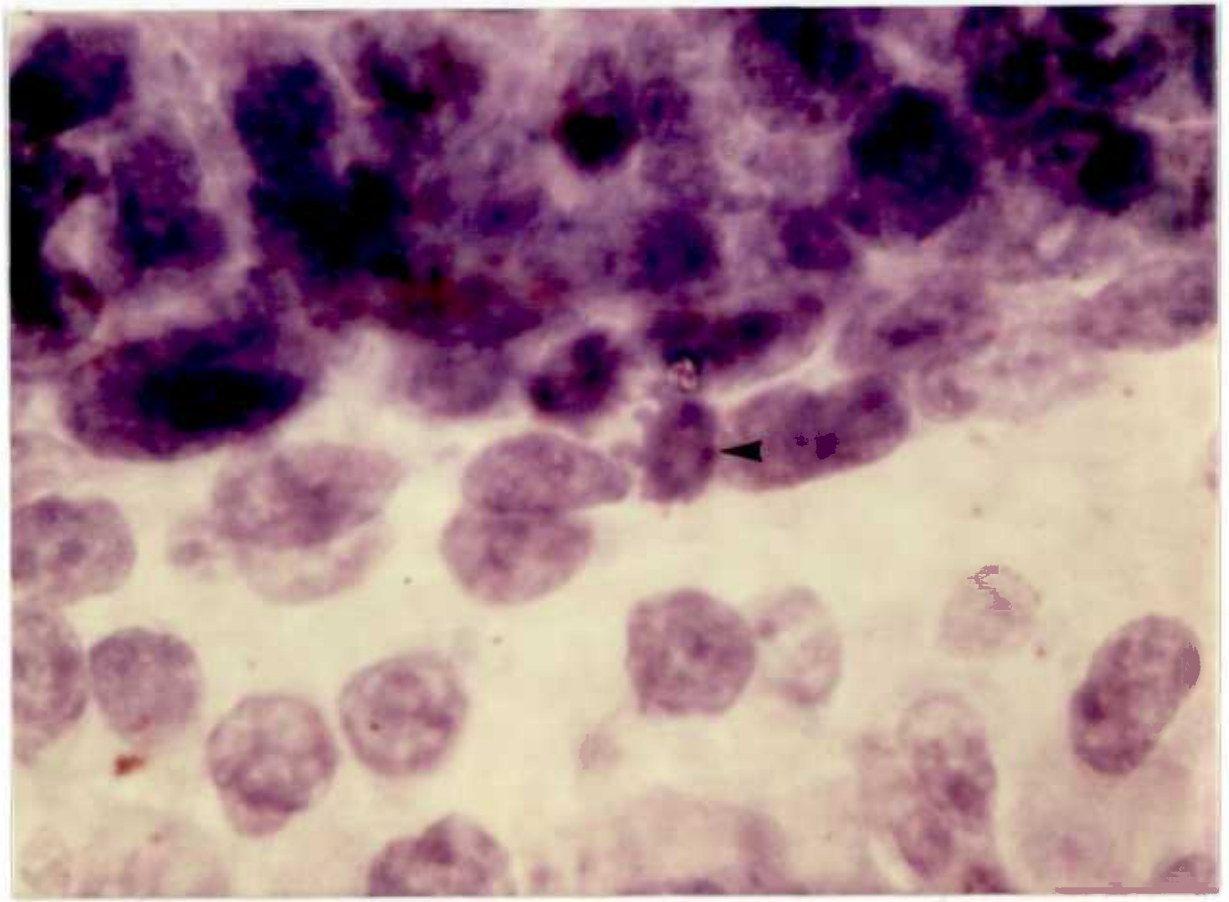


Fig. 59 Mitotic figure in the depths of the external granular layer of the 10 day old rat.

A late mitosis (arrow head) can clearly be seen at the bottom of the EGL. The clear zone with a low density of nuclei is the molecular layer while the pial surface is out of the field at the top of the picture.

Haemalum stained 7 μm coronal section (x 2,200)

The relative positions of mitotic figures in the EGL were plotted for postnatal days 9 and 12, the ages between which the EGL is at its maximum thickness (Fig. 7). The distribution of mitoses at these two ages was very similar, forming a skewed standard distribution (Fig. 60). There was a small amount of mitotic activity at the pial surface, the majority of mitoses being found in the rows of cells two- and three-fifths into the EGL and a small but significant number of mitoses occurring in the innermost depths of the EGL. At nine days, 2 per cent of all the mitotic cells were among the deepest rows of EGL cells, and this figure rose to 4 per cent in the 12 day old animals. While the distribution of mitotic figures showed little change between days 9 and 12 (Table VIII), the configuration of the EGL underwent a change, with the layer of rounded cells decreasing in thickness (Fig. 7). This means that while in the 9 day old animals only 26 per cent of the mitotic cells were among the elongated cells of the lower zone, by 12 days nearly 70 per cent of all the mitoses were found in the lower zone.

These measurements were repeated for a wider range of ages using the more sensitive technique of [^{3}H] thymidine uptake to detect proliferating cells in S phase. Examination revealed labelled nuclei at all depths of the EGL at all ages. As with mitotic cells, labelled nuclei were sometimes seen amongst the deepest elongated cells (Fig. 61). The relative positions of labelled nuclei in the EGL were plotted for postnatal days 0, 1, 5, 9 and 14. The results are shown in Figs. 62-63 and Table IX. At birth (beginning of postnatal day 0), there was only one zone of cells in the EGL, composed of round cells. At this time there was an even distribution of cells

Table VIII Distribution of mitoses in the external granular layer

Relative Depth	0-20 (pial surface)	20-40	40-60	60-80	80-100 (deep EGL)
mitoses (%)					
Day 9	21	39	28	10	2
Day 12	11	39	29	17	4

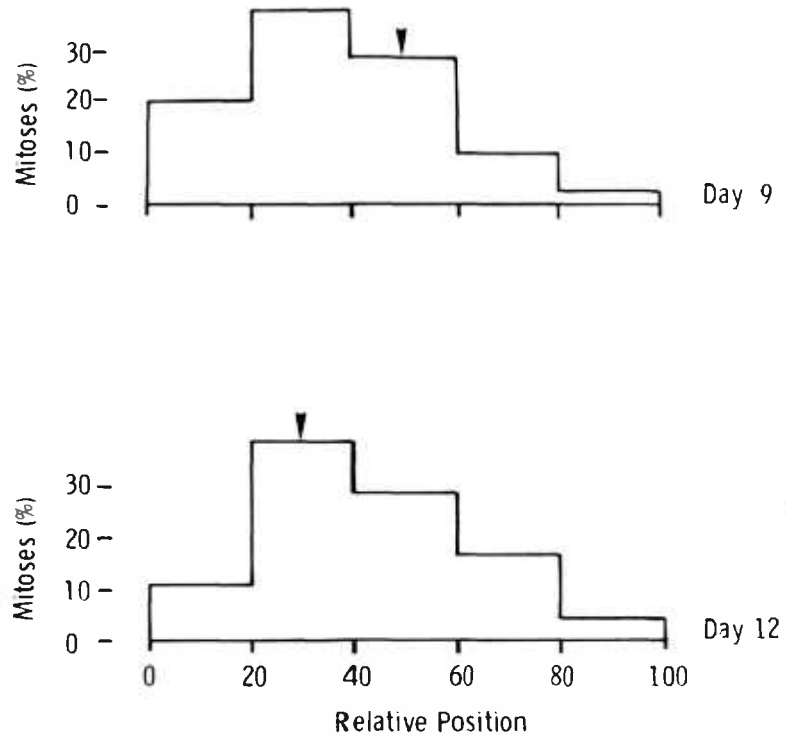


Fig. 60 Distribution of mitotic figures in the external granular layer of the 9 and 12 day old rat.

The figure shows the percentage of the total mitoses counted at each position within the EGL. On the horizontal axis 0 - 20 represents the first fifth of the EGL below the pial surface ; 80 - 100 the final fifth of the EGL above the molecular layer.

The arrowhead represents the boundary between (to its left) the zone of rounded cells and (to its right) the zone of elongated cells.

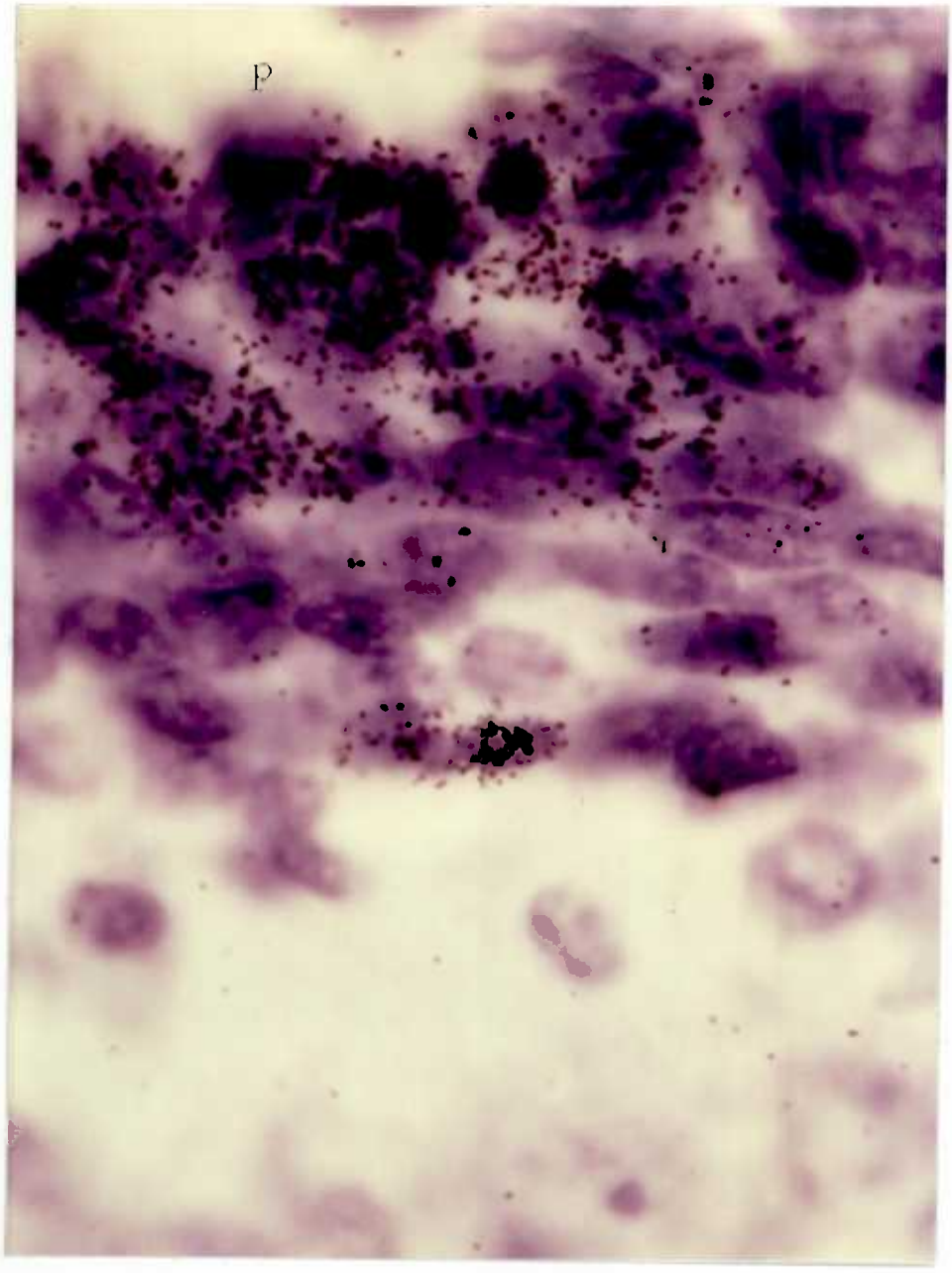


Fig. 61 [^{3}H] thymidine-labelled nuclei in the depths of the external granular layer of 10 day old rat one hour after labelling.

While most of the uptake of tritiated thymidine (as evidenced by silver grains over the nuclei) is by the rounded cells of the upper EGL, several elongated cells have 4 or more grains over their nuclei, and thus are synthesising DNA. Two labelled elongated nuclei can be seen at the very bottom of the EGL (p = pial surface).

Haemalum stained autoradiograph. 7 μm coronal section (x 2,200)

Table IX Distribution of labelled nuclei in the external granular layer 1 hour after the injection of (^{3}H) thymidine

Relative Depth	0-20 (pial surface)	20-40	40-60	60-80	80-100 (deep EGL)
labelled nuclei (%)					
Day 0	17	22	23	21	17
Day 1	28	23	24	16	9
Day 5	24.5	24.5	23	20	8
Day 9	38	34	19	6	3
Day 14	40	31	25	3	1

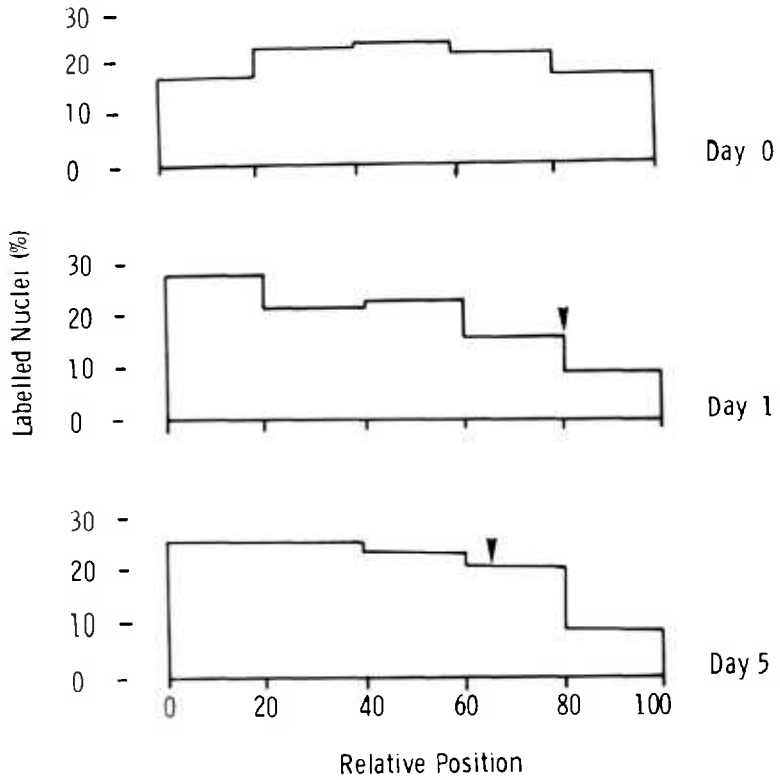


Fig. 62 Distribution of labelled nuclei within the external granular layer of the neonate, 1 and 5 day old rat one hour after labelling.

The figure shows the percentage of the total labelled nuclei at each position within the EGL. On the horizontal axis 0 - 20 represents the first fifth of the EGL below the pial surface, 80 - 100 the final fifth of the EGL above the molecular layer.

The arrowhead represents the boundary between (to its left) the zone of rounded cells and (to its right) the zone of elongated cells.

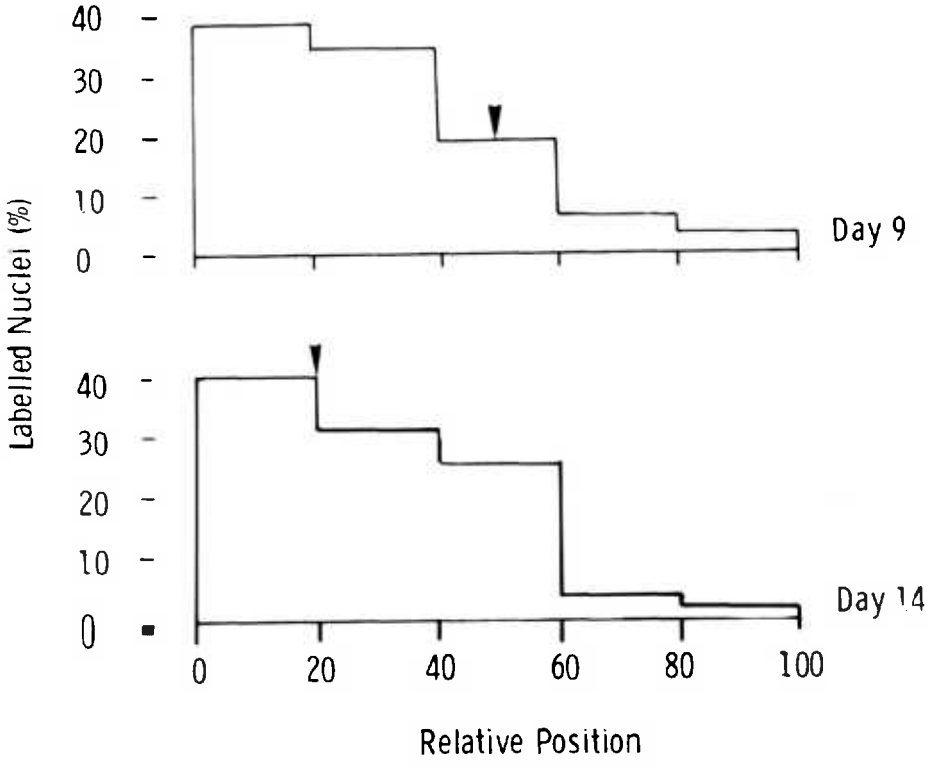


Fig. 63 Distribution of labelled nuclei within the external granular layer of the 9 and 14 day old rat one hour after labelling.

The figure shows the percentage of the total labelled nuclei at each position within the EGL. On the horizontal axis 0 - 20 represents the first fifth of the EGL below the pial surface, 80 - 100 the final fifth of the EGL above the molecular layer.

The arrowhead represents the boundary between (to its left) the zone of rounded cells and (to its right) the zone of elongated cells.

synthesising DNA throughout the entire EGL. Within 24 hours, there had been a slight shift in S phase cells so that there were somewhat more labelled nuclei to be found in the superficial EGL than in the deeper EGL. By postnatal day 1 there was a shallow layer of elongated cells comprising about 20 per cent of the entire thickness of the EGL. Of the cells in S phase, 10 per cent were to be found among the elongated cells. At postnatal day 5 the pattern of distribution of labelled nuclei in the EGL had not changed from the pattern in the 1 day old animal. The elongated cells now made up about 25 per cent of the thickness of the EGL. By postnatal day 9 the DNA synthesising cells were more closely concentrated in the outer half of the EGL, which contained just over 81 per cent of the total number. The elongated cells now comprised 50 per cent of the EGL. Postnatal day 14 is the latest age from which reliable data can be obtained; beyond this age the zone of round cells became too small to be evaluated accurately. At day 14 the pattern of distribution of labelled nuclei was very similar to that at day 9, with the majority of labelled cells being located in the outer EGL; however, even amongst the EGL cells adjacent to the molecular layer, a small number of labelled nuclei were found. The layer of rounded cells was almost absent by day 14 (see Altman's 1972 graph), so that at this age perhaps 60 per cent of the nuclei incorporating [^{3}H] thymidine were to be found among the elongated cells.

iii. Comments

These results suggest that the concept that the two morphologically

different cell populations of the EGL are functionally different must be reappraised. That both mitoses and uptake of [^{3}H] thymidine has been observed throughout the EGL is at odds with the statement by Altman (1972a) that mitoses are never seen among the elongated cells. These results show a gradient of proliferative activity across the EGL, with the peak shifting towards the pial surface with increasing age. However, at all ages examined, there was mitotic activity in the deepest parts of the EGL. Cajal (1911) and Mareš et al (1970) noted rare mitoses in the deeper EGL, and it is true that in any one microscope field the mitotic activity in the EGL is more apparent near the surface. However, taken as a whole, there were a significant number of cells in S phase in the lower zone.

With increasing age the ratio of elongated cells to round cells steadily increased. However, this had no apparent effect on pattern of proliferating cells, this phenomenon being most noticeable between days 9 and 14 (Fig. 63). These results suggest that there is no direct correlation between the shape of the EGL cells and their capacity to divide.

D. Vasculature of the developing cerebellum

i. Introduction

This part of the present work reviews the literature concerning the vascular supply of the cerebellum, and its development. Experimental studies on the vasculature of the rat cerebellar cortex are presented and the vascularisation of the EGL is analysed through its maturation and disappearance.

The blood supply to the adult vertebrate cerebellum has been well studied. Its basic pattern of arterial supply is similar to that of the cerebrum (Pape and Wigglesworth, 1979) : both consist in essence of a peripheral network of arteries which have radially orientated branches directed towards the ventricle, permitting a ventriculopetal blood flow. The ventriculopetal arteries of the cerebellum are derived from the leptomeningeal vascular plexus which gives rise to long perforating arteries which extend inwards towards the deep nuclei, and short cortical arteries. Complementing the ventriculopetal system but with no connection to it is a ventriculofugal blood flow. In the cerebellum this is provided by arteries entering the cerebellum via the cerebellar peduncles and giving off branches to the deep nuclei (van den Bergh and Vander Eecken, 1968). In primates there are three main arteries to the cerebellum. The most important are the superior cerebellar arteries (Lazorthes et al, 1951). They are a large pair of symmetrical vessels which arise from the basilar artery, and pass around the upper border of the pass, giving off branches which supply the pontine

tegmentum, the cerebellar cortex and central cerebellar structures. Two groups of branches supply areas outside the cerebellum, the first supplies the superior cerebellar peduncle, the lateral lemniscus, ventral spinothalamic tract and sometimes the tegmental bundle and ascending sensory tracts that comprise the lemniscal system, while the second group anastamose with the posterior cerebellar artery to form a plexus over the corpora quadrigemina from which arise arterioles penetrating the superior and inferior colliculi. The marginal artery arised from the superior cerebellar artery at the anterior margin of the cerebellar hemisphere giving off rami which supply the cerebellar cortex and the middle cerebellar peduncle. Near or at its termination the superior cerebellar artery gives rise to the superior paravermal artery which passes between the hemisphere and the vermis, anastomosing with an inferior paravermal branch of the inferior cerebellar artery. The superior artery to the dentate nucleus is an offshoot of the superior cerebellar artery arising near the lateral border of the superior cerebellar artery.

The second major pair of arteries are the posterior inferior cerebellar arteries. These are asymmetrical arteries arising from the vertebral artery. Branches arising in the first part of their length penetrate the lateral portion of the medulla oblongata and parallel the emerging ninth and tenth cranial nerves, supplying the lateral medullary zone. Cortical branches of the posterior inferior cerebellar artery supply the tonsil biventer and posterior inferior lobules. The main trunk of the posterior inferior cerebellar artery enters the fourth ventricle in the vicinity of the foramen of Magendie. The artery branches to supply the choroid plexus, the roof nuclei, and to form the inferior artery to the dentate nucleus and the

inferior paravermal arteries (which anastamose with the superior paravermal arteries). The anterior inferior cerebellar artery arises from the basilar artery. The first branches supply the lateral aspect of the inferior pontine tegmentum. The anterior inferior cerebellar artery usually supplies the flocculus and structures in the vicinity of the cerebellopontine angle, and also the choroid plexus protruding through the foramen of Luschka. A branch passes into the great horizontal fissure and other branches supply the cerebellar cortex in the vicinity. The middle cerebellar peduncle is also served by the anterior inferior cerebellar artery.

Arterioles penetrating the cerebellar cortex arise at right angles from the superficial arteries. They mainly supply the Purkinje cells and internal granular layer, ramifying to form a dense capillary bed. There is a less dense capillary bed in the white matter which derives from the dense cortical capillary bed and also from penetrating arterioles which extend below this. The molecular layer is poorly supplied with blood vessels, which form a thin wide-meshed rete (Gillilan, 1969). Craigie (1938) reviewed the literature pertaining to the capillary bed of the cerebellar cortex studied by Aby (1889), Conighi (1922), Fazzari (1924), Pfeifer (1928) and Cobb (1932). Summarising these studies he concluded that the meshes of the capillary bed were radially orientated in the molecular layer, and smaller and irregularly polygonal in the internal granular layer. Arterioles penetrated to various depths and branched, so that each layer was supplied with blood from all directions. Craigie's review highlighted the lack of agreement as to whether a special part of the net supplied the Purkinje cell

layer. This question has recently been re-examined and the existence of such a special net has been confirmed (Conradi et al, 1979). In the literature reviewed there was also doubt as to whether the blood in the cerebellar cortex all flowed from the surface into the deep veins (Craigie, 1938).

The venous drainage from the cerebellum complements the arterial supply. Intrinsic venules drain the dense capillary bed, some then passing upwards to join the superficial venules and veins, but most travelling in an inward direction to drain the capillaries in the white matter before going outward to join the superficial veins. The superior cerebellar veins drain the surfaces of the superior hemispheres and vermis and pass into the great cerebral vein of Galen or the straight sinus veins near the transverse sinus may empty into it. The lateral superior cerebellar veins drain into the superior petrosal sinus, as does the anterior cerebellar vein. The posterior inferior cerebellar veins empty into the transverse sinus. The veins of the cerebellum are fewer in number but larger than the arteries, and they do not run in parallel with them (Gillilan, 1969).

While the development of the superficial vascularisation of the cerebellum has been well studied (see Gillilan, 1973, for review), the development of its intrinsic vascular bed has received surprisingly little attention. Studies of the development of the intrinsic vasculature of the cerebral cortex have been made. In the rat, the intrinsic vasculature was found to develop during the first 10 postnatal days as solid cords of mesodermal cells (Caley and Maxwell, 1970) whose lumina became patent as a result

of internal blood pressure, and in which blood flow was initiated when communication was established with other patent vessels (Strong, 1961; Caley and Maxwell, 1970). Much of the investigation of the intrinsic vasculature of the cerebral cortex has been conducted with a view to examining the development of the blood-brain barrier. Dobbing (1968) suggested that what is observed during postnatal development is not a progressive establishment of a barrier between the intrinsic cerebral capillaries and the neural tissue. In early postnatal development there is a relatively rapid increase in brain weight (growth spurt). The rates of accumulation of brain constituents must represent (with certain reservations) the rates of their arrival (or the arrival of some of their precursors). In Dobbing's view, the apparent development of a blood-brain barrier was in fact a manifestation of diminishing demand on the blood supply once the brain growth spurt has passed (Dobbing, 1968). The capillaries of the cerebral cortex of the rat have been examined for ultrastructural evidence for the development of a blood-brain barrier, but few changes have been detected; a thickening of the basement membrane and a thinning of the endothelial cells were observed, but the relationship between neural tissue elements surrounding capillaries and the basement membrane was the same in immature animals as in adults (Donahue and Pappas, 1961). The views of Dobbing (1968) received support when a study of the capillary net in the rat cerebral cortex showed that its development reflected the brain growth spurt. It increased in the first two postnatal weeks, attaining its adult characertistics after a month. Subsequent capillary growth was mainly accomplished by endothelial cell elongation rather than division (Bar and

Wolff, 1973). A similar pattern was found in the spinal cord of the developing rat; blood vessel profiles increased between the first and third postnatal weeks. Active growth was confirmed by the presence of a dividing endothelial cell in a section from a two-week old animal (Hannah and Nathaniel, 1974).

Though the pattern of vascularisation of the cerebrum may be broadly similar to that of the cerebellum, studies of the former do not take into account the unique presence of the cerebellar external granular layer. It is thus surprising to find that the development of the intrinsic vasculature of the cerebellum has been rarely studied. Postnatal changes in the vascularisation of various regions of the rat brain have been quantified using stereological techniques (Craigie, 1924). At birth there was a low uniform level of vascularisation. By the fifth postnatal day there was a slight increase. By the tenth day a definite increase in vascularisation had occurred, and by the twenty first day the pattern had approached adult levels. However, there were some exceptions to this pattern the most striking of which was the paucity of vascularisation in the molecular and internal granular layers of the cerebellum. The vascularisation in the molecular layer actually decreased between the fifth and tenth postnatal days, and the adult pattern of vascularisation in the cerebellum did not reach adult levels until the ninetieth postnatal day.

The development of the vascular network of the chick metencephalon was described from three and a half to sixteen days of incubation (Feeney and Watterson, 1946). At three and a half days there were few blood vessels in the metencephalon. Ventrally penetrating, paired capillaries

from the basilar artery were enlarging into sinus-like spaces near the periphery of the ependymal surface. At 4 days the ventrally penetrating capillaries had diverged laterally and had formed antero-posterior connexions between them, ventral to the endoneural sinus, creating the endoneural plexus, which lay between the mantle and ependymal zones.

At 5 days the endoneural plexus had extended dorsally into the alar plates. The roof of the metencephalon was devoid of vessels. At 6 days there was peripheral spread of interconnections among penetrating capillaries, peripheral to the endoneural plexus. The mid-dorsal roof plate remained avascular. From 7 days the capillaries penetrating the alar plate became more numerous. By 12 days the pattern of the intraneural vessels of the cerebellar folia was emerging; the vascular bed was arising from two anlagen : the first from a plexus already formed in the centre of the mass of mantle zone cells which did not participate in the formation of the cerebellar cortex, the second from a superficial plexus which sent out capillaries into the walls of the invaginations made by the newly forming folia. These capillaries penetrated the cortex without branching and connected with the extensive plexus in the mantle zone. The inner plexus continued to develop as a dense capillary network spreading peripherally until it reached the internal granular layer. The penetrating vessels continued to increase in number until the cerebellum had achieved its characteristic layered structure (Feeney and Watterson, 1946).

Though the patterns of intrinsic vascularisation of all vertebrates are said to be the same (Gillilan, 1964), and the description given by Feeney

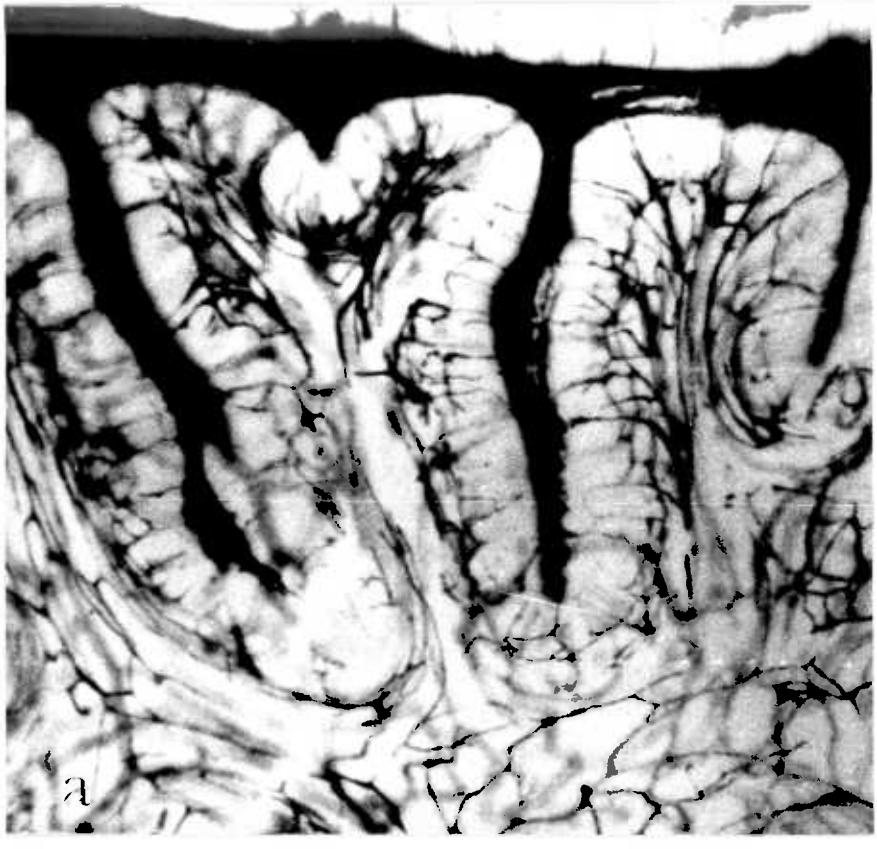
and Watterson (1946) for the chick was suggested as a model for the human (Gillilan, 1973) the differences between the development of the EGL in various species (see Table III) might invalidate interspecific comparisons. Furthermore, neither Craigie (1938) nor Feeney and Watterson (1946) gave an account of the vascular supply to the EGL, nor was there any mention of changes occurring as a result of its disappearance. Since the EGL consists of a large number of proliferating cells it must require a rich supply of metabolites and therefore its blood supply must be significant. Further, since it is a temporary feature of the cerebellum, it may be supposed that its blood supply is able to adapt itself to the absence of this layer. The present study was performed to investigate whether the EGL possessed an intrinsic blood supply and if not, as has been suggested (Craigie, 1938), to identify possible alternative sources of metabolic supply to the layer. This study was also designed so as to determine whether the changes in the vasculature were progressive, with the adult pattern of capillaries in the molecular layer appearing as this replaced the EGL.

ii. Results

During the first three postnatal weeks the EGL increases in thickness for approximately 10 days and then decreases, with the molecular layer replacing the EGL. By 21 days the EGL has virtually disappeared and the molecular layer extends from the Purkinje cell layer to the pial surface.

The perfusion of the cerebellum with indian ink and gelatin revealed the

changes in vasculature during this period. Some changes were gradual. In the very young animal the most densely vascularised region was the pial membrane overlying the cerebellum (Fig. 64). With increasing age the vascularity at this site was reduced, (Fig. 66) possibly as a result of a stretching or growth of the membranes without a comparable degree of angiogenesis to maintain the original capillary density. The rete of vessels in the white matter and IGL increased in density and complexity especially between the second and third postnatal weeks (Fig. 65 and 66). However, the vasculature of the molecular layer and EGL remained unchaged until the end of the third postnatal week. Before this time it was strikingly poor in intrinsic capillaries (Figs. 64 and 65). This is in confirmation of observations made by Craigie (1924) who noted a decrease in vascular density in the molecular layer between the fifth and tenth postnatal day. There were a few radial vessels spanning the EGL and molecular layer, but they were widely spaced and without branches so that it is difficult to imagine that they could make any major contribution to the vascular supply to the EGL. Towards the end of the third postnatal week the pattern of capillaries within the molecular layer and EGL changed. The radial capillaries begin to develop side branches. These were initially horizontal, i.e. parallel with the pial surface, but as they increased in length they became further branched and less regularly orientated (Fig. 66). By the time the EGL had completely disappeared, the network of side branches had become sufficently extensive as to make any differentiation between the molecular layer and the rest of the cerebellar cortex on the basis of vascular density impossible (Fig. 67).



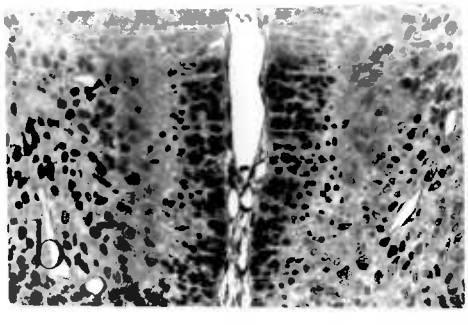
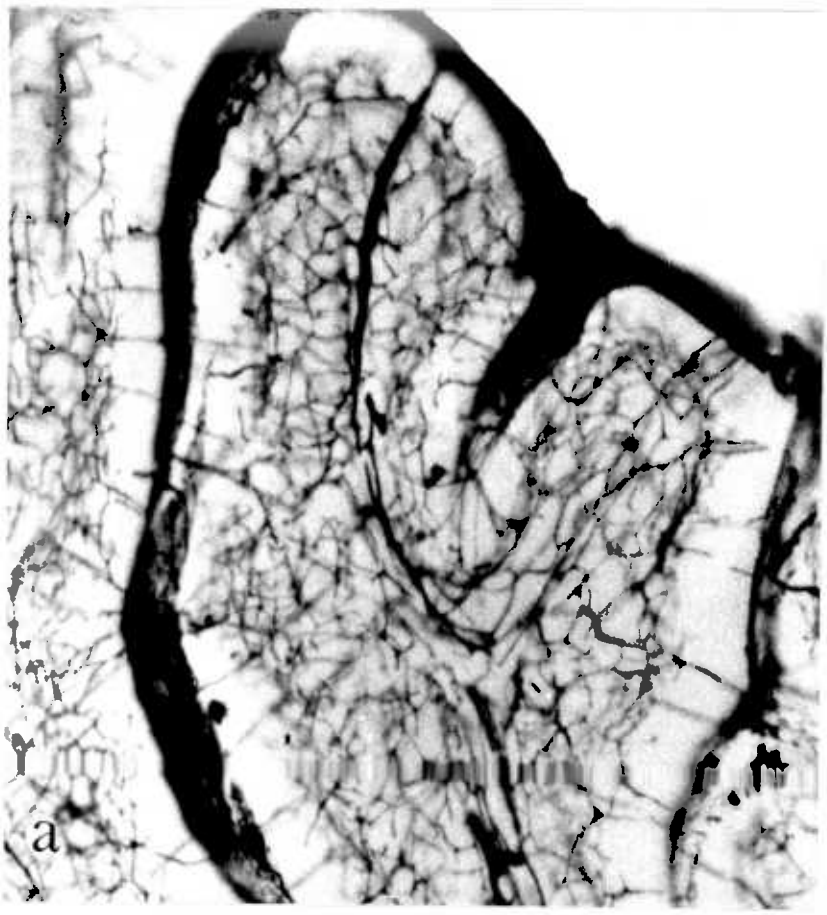


Fig. 64 Vasculature of the 2 day old rat cerebellum.

a. The major intrinsic cerebellar capillary bed lies within the white matter. Branches of vessels from within this network turn through 90° and penetrate the granule cell layer and, without forming side branches traverse the molecular and extemal granular layers. The pial membrane network is very dense. Gelatin-indian ink perfused, methyl salicylate cleared sagittal section (x117).

b. Toluidine blue stained 1 μm resin sagittal section (x 117)



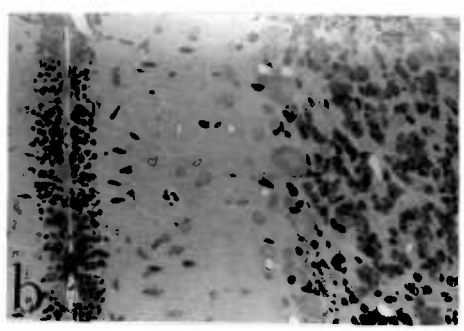


Fig. 65 Vasculature of the cerebellum of the 14 day old rat.

a. The vasculature in the white matter/granular layer is more dense, but the pattern of vessels in the molecular layer/EGL remains unchanged from that in the molecular layer/EGL of the 2 day old animal (see Fig. 64).
Gelatin-indian ink perfused, methyl salicylate cleared sagittal section (x 117)

b. Toluidine blue stained 1 μm resin sagittal section (x 117)

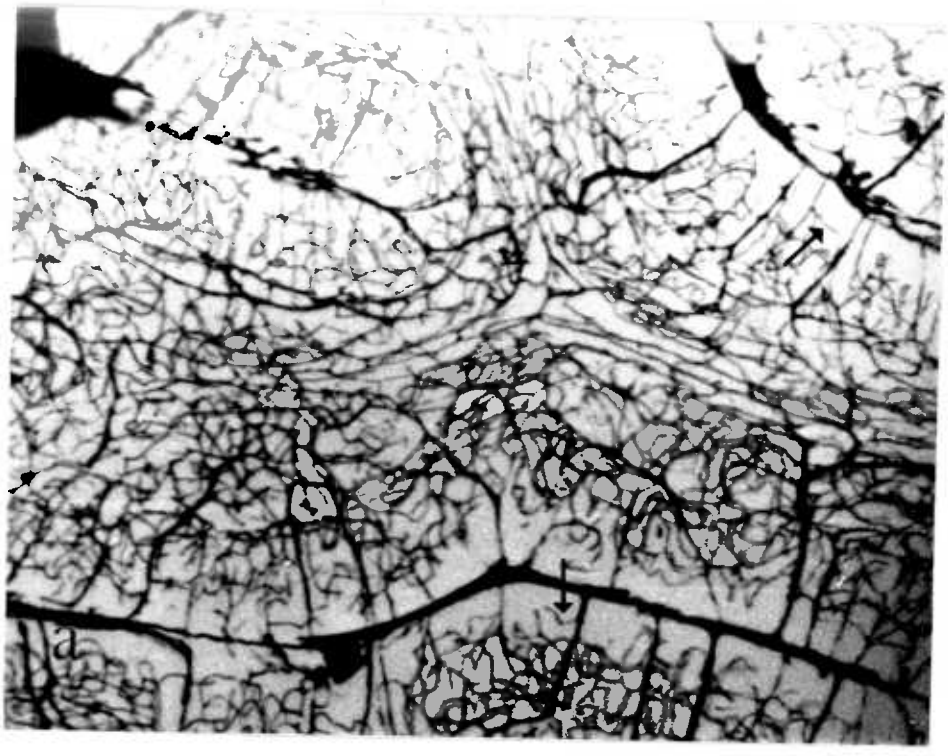
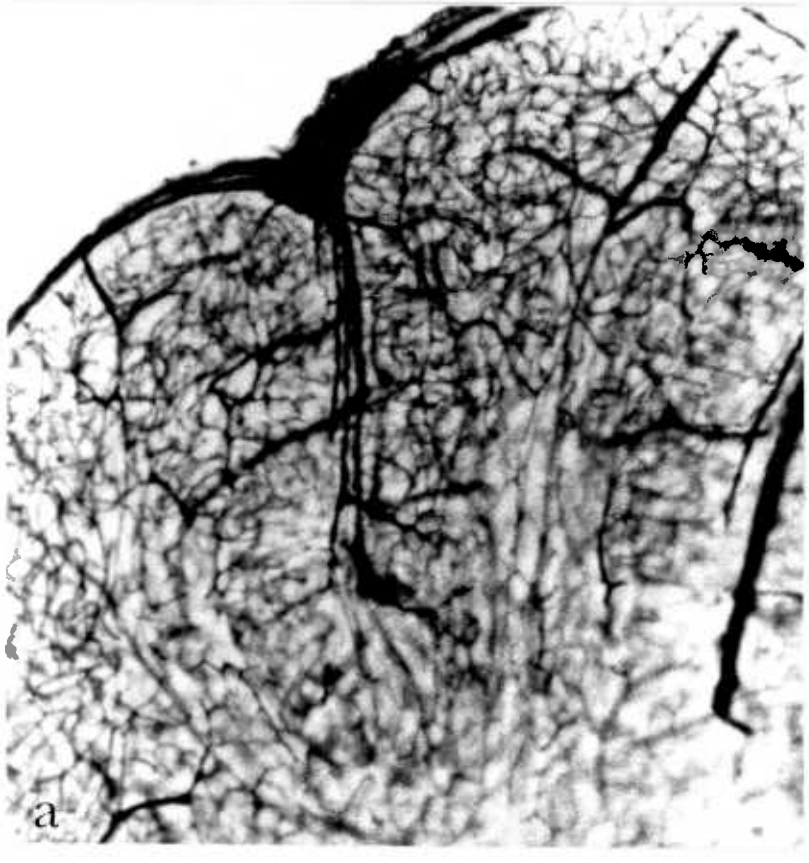


Fig. 66 Vasculature of the cerebellum of the 20 day old rat

a. The vascular bed in the white matter/internal granular layer is more complex still. The first appearance of a change in the capillary supply to the molecular layer/EGL is apparent at this age; the radial capillaries traversing these layers begin to generate side branches (arrows) Gelatin-indian ink perfused, methyl salicylate cleared sagittal section (x 117)

b. Toluidine blue stained 1 μm resin sagittal section (x 117)



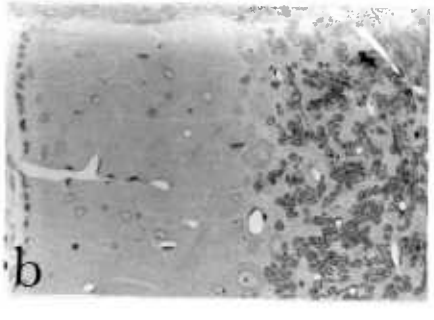


Fig. 67 Vasculature of the cerebellum of the 24 day old rat

a. By this age the EGL has completely disappeared. The vascular network in the molecular layer is now indistinguishable from that in the white matter/internal granular layer. Gelatin-indian ink perfused, methyl salicylate cleared sagittal section (x 117).

b. EGL - molecular layer - IGL of 21 day old rat. Note residual EGL and increased capillary penetration of molecular layer. Toluidine blue stained 1 μm resin sagittal section (x 117).

Note : While the micrographs of thin resin sections (Figs. 64b, 65b, 66b, 67b) allow the various layers of the cerebellar cortex to be identified, the difference in processing method invalidates any direct comparison with the micrographs of the indian ink-gelatin perfused material.

iii. Comments

The present study shows that during the existence of the EGL this layer receives, relative to the rest of the developing central nervous system, a poor supply of blood from within the cerebellum. There is no cortical network of vessels feeding it. This is not altogether surprising since such a permanent intrinsic network supplying the EGL would have to become redundant at the end of the third postnatal week, once the layer had disappeared. Craigie (1924) commented on the poor vascularisation of the cerebellum in the postnatal rat, relating it to its incomplete differentiation of structure and form at birth. He equated the increase in cerebellar vascularity after birth with the increase and elaboration of functional activity (Craigie, 1945). The present results showed that there was no progressive increase of vasculature in the molecular layer as it replaced the EGL. Rather, vascularisation of the molecular layer commenced towards the end of the third postnatal week, at a time when the EGL had almost disappeared.

Little is know about the metabolic requirements of the developing nervous system, or the role of the blood supply in providing them (for review see Friede, 1966). However, the EGL, as a population of actively dividing cells, must be dependent on a blood supply for the division and growth of its constituent cells. It has been demonstrated that interference with the

nutritional status of rats is reflected in alterations to the EGL (Balázs et al, 1975). Other blood-borne factors, notably thyroid hormone (Lauder, 1977 a, b; Patel et al, 1979) and possibly hydrocortisone (Bohn and Lauder, 1979) have been shown to have an important role in the normal development of the EGL. Since its intrinsic vasculature is so poorly developed it is necessary to propose an alternative capillary supply to the EGL and a route for the transfer of regulatory substances and essential metabolites from the supply to the EGL cells. A dense capillary bed in close proximity to the EGL is the pial capillary net. It is tempting to postulate that this is the major source of blood-borne factors required by the EGL. Apart from its proximity, another feature which makes the pial capillary net a suitable candidate is the way in which it is able to adapt to the disappearance of the EGL. If angiogenesis of the net does not parallel the growth of the cerebellum then the capillary density would decrease with age. There would therefore be no need to consider mechanisms for a major remodelling of vessels.

If the pial capillary bed supplies the EGL, the mechamism whereby substances are transferred from the vessels to the constituent cells of the EGL has to be considered. The means of metabolite supply to cells in the central nervous system prior to the establishment of an intrinsic vasculature has been examined in the rat foetus. Ultrastructural studies showed that endothelial cells had a high cell surface activity (Donahue, 1964). Similar studies of immature vessels in the rat spinal cord revealed the endothelial cells as having a high surface activity with many pseudopod projections, as well as containing pinocytotic vesicles (Hannah and

Nathaniel, 1974). In the neonatal rat cerebral cortex large numbers of intercellular spaces containing floccular material, possibly polysaccharide, were observed (Caley and Maxwell, 1970). The combination of active cell surfaces and polysaccharide-containing intercellular spaces lends itself to the suggestion that prior to the establishment of patent intrinsic capillaries cells acquire their metabolic requirements by pinocytosis from the intercellular matrix (Hannah and Nathaniel, 1974). This hypothesis holds for the conditions found in the EGL. As shown in this study, the EGL cells have active surfaces, possessing similar pseudopod projections to those found in the endothelial cells of the rat spinal cord (Hannah and Nathaniel, 1974). The EGL cells also contain vesicles, said to be related to the absorption of protein from extracellular spaces (Mugnaini and Forströnen, 1967). Throughout the existence of the EGL there are spaces between the EGL, though they decrease in size and frequency in the older animals (this study). Whether they contain carbohydrate or any other metabolite is not known, but it seems reasonable to suggest that the route of substances to the EGL cells is from the pial capillary bed by diffusion to the extracellular spaces and from there by active and/or passive transport into the cells.

The EGL is often considered to be the source of medulloblastoma (Rubinstein, 1975) and in studying the changes in its blood vessels occurring in the course of postnatal development it is of interest to consider general aspects of vascularisation of neoplasms during their growth. The vascularisation of medulloblastomas is usually rather poorly developed (Bhandarkar et al, 1978), but some other neoplasms show a change in their pattern of blood

supply which has some features in common with those observed in the present study. Like the EGL, many tumours initially pass through an avascular phase but this is only possible while the tumour remains below a threshold diameter of a few centimetres (Folkman, 1975). Once this upper size limit has been reached, the growth of a solid neoplasm is dependent on its capacity continuously to elicit new capillary endothelium (Algire et al, 1945; Folkman, 1975). It has been suggested that neoplasms produce a substance called tumour angiogenesis factor (TAF). This would act to stimulate vascular proliferation once the tumour has exceeded the maximum size for its avascular stage (Folkman et al, 1971). However, this concept of tumour vascularisation with growth provides only a very superficial parallel to cerebellar angiogenesis, for here the most peripheral region remains relatively avascular until the EGL has disappeared.

E. Immunocytochemical localisation of neuron- and glial-specific markers in the cerebellar cortex

i. Introduction

The concept of the EGL cell as a bipotential cell, capable of producing both neurons and glia, was introduced by Schaper (1894). Since that time it became widely supported, despite the assertion by Cajal (1911) that the EGL was strictly a source of neurons. Only recently has any evidence which confirms Cajal's (1911) opinions been given (see Table I). Elsewhere in this thesis it has been proposed that the EGL cells are a homogeneous population of neurons. This hypothesis precludes the possibility that the EGL is a source of glia. For it to be tested, it was necessary to examine the EGL for the presence of astrocytes and oligodendrocytes in order to determine whether these cell types were being generated there.

A range of techiques exist for the classification of cells in the central nervous system. In order of increasing sensitivity these include differential staining of sections for light microscopy, electron microscopy and protein chemical characterisation techniques. Electron microscopy has already been used to investigate the question of whether glial cells originate from the mouse EGL (Swarz and del Cerro, 1977), but since no evidence of gliogenesis was obtained, more sensitive techniques are required to resolve this problem. Protein chemical characterisation techniques include :

: the identification of structural components important in the operation of the nervous system, for example microtubules or myelin,

: the use of high affinity markers, for example α neurotoxins which specifically bind to nicotinic acetylcholine receptors (Kuhar, 1978),

: the recognition of enzymes involved in specific functions such as metabolic pathways,

: the detection of nervous system-specific proteins localised by immunochemical techniques (Bock, 1978).

To examine the EGL for the production of glial cells, immunohistochemical localisation of the following neuron- and glial-specific proteins was determined (Koppel and Lewis, 1979) :

Glial fibrillary acidic protein (GFAP) : This was first isolated by Eng et al, (1971) from tissues rich in fibrous astrocytes. These were obtained from human brains with pathological conditions giving rise to gliosis, for example, multiple sclerosis plaques. Antisera to GFAP react only with astrocytes in the central nervous system (Uyeda et al, 1972).

Glutamine synthetase : Purified antibodies raised to this enzyme and localised in rat brain by the peroxidase labelling technique showed that glutamine synthetase was localised to glia. This indicated that glia have a crucial role in glutamic acid, GABA and ammonia metabolism in the brain (Martinez-Hernandez et al, 1977).

Galactocerebroside : This is the major glycolipid in myelin (Norton and Autilio, 1966), and was used successfully as a cell surface marker for rat oligodendrocytes in dish and suspension culture (Raff et al, 1978; Lisak et al, 1979).

Enolases : When whole brain extracts were fractionated by DEAE cellulose ion-exchange chromatography, the total enolase activity was

resolved into three peaks, the most acidic peak being a neuron-specific isoenzyme, the least acidic peak a neuron non-specific isoenzyme of enolase, and the third peak a hybrid. Using immunocytochemical techniques the first peak of enolase activity (NSE) was found to be restricted to neurons while the second (NNE) was detected in glial and epithelial elements. NNE also stained liver and therefore was considered not to be specific to the nervous system (Schmechel et al, 1978a). NSE has also been reported to be a specific marker for peripheral and central neuro-endocrine cells (Schmechel et al, 1979).

D2 : This brain specific membrane protein was identified by means of an antiserum raised against rat synaptosomal membranes (Jørgensen and Bock, 1974). The level of D2 in developing mouse brain was found to decrease with age, reflecting the rate of synapse formation rather than the number of formed synapses (Jacque et al, 1976).

Myelin basic protein (MBP) : This constitutes approximately 30% of the total protein of the myelin membrane (Kies et al, 1965, Eng et al, 1968) which itself is composed of 30 per cent protein and 70 per cent myelin. Antibodies raised to MBP have been shown specifically to stain oligodendrocytes and myelin sheaths in newborn rat brainstem and spinal cord (Sternberger et al, 1978a).

ii. Results

Immunocytochemical staining

Glial fibrillary acidic protein (GFAP) : a) Light microscopic observations The cerebellum of rats aged one, 6 and 10 days was examined. Sections

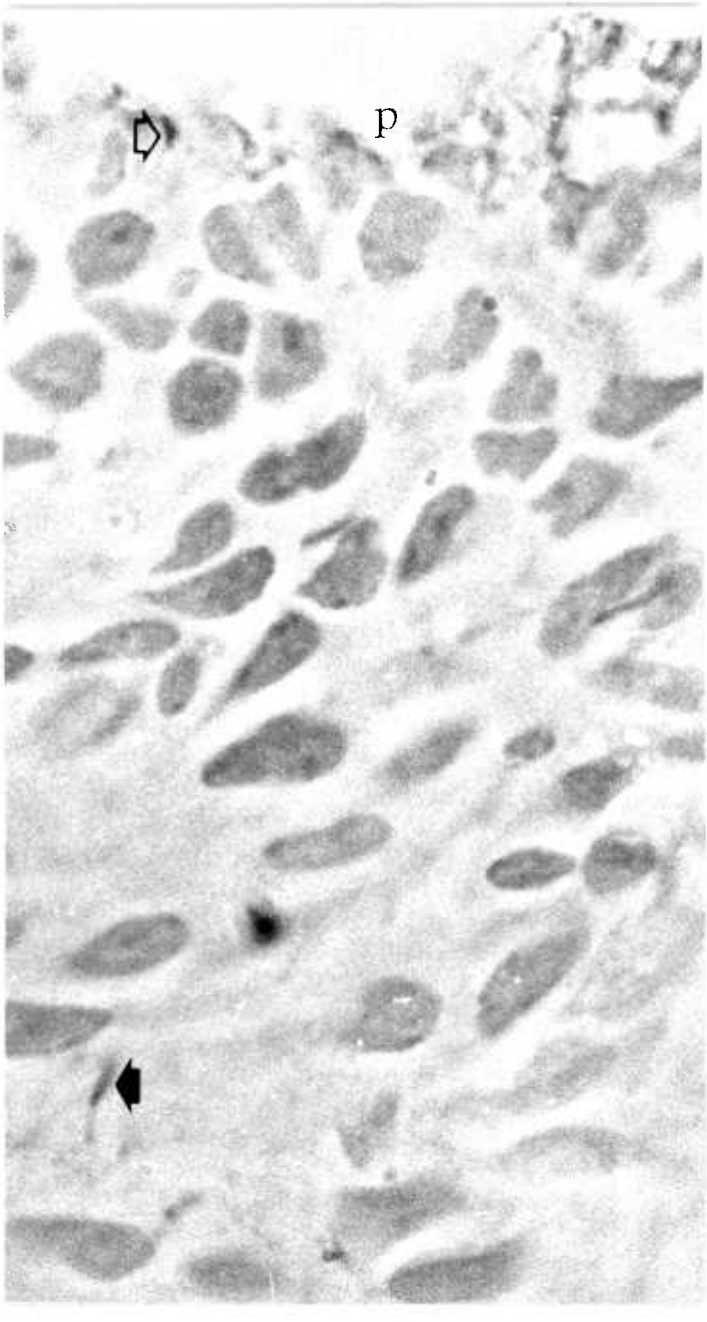


Fig 68 Immunocytochemical localisation of GFAP in the external granular layer of the 1 day old rat.

Although not strongly reactive at this age Bergmann glial endfeet and fibres (open and closed arrows respectively) can be detected. The perikarya of the EGL cells do not contain reaction product (p = pial surface).
Antiserum diluted 1 : 500.
Toluidine blue stained 1 μm resin coronal section (x 1,700)

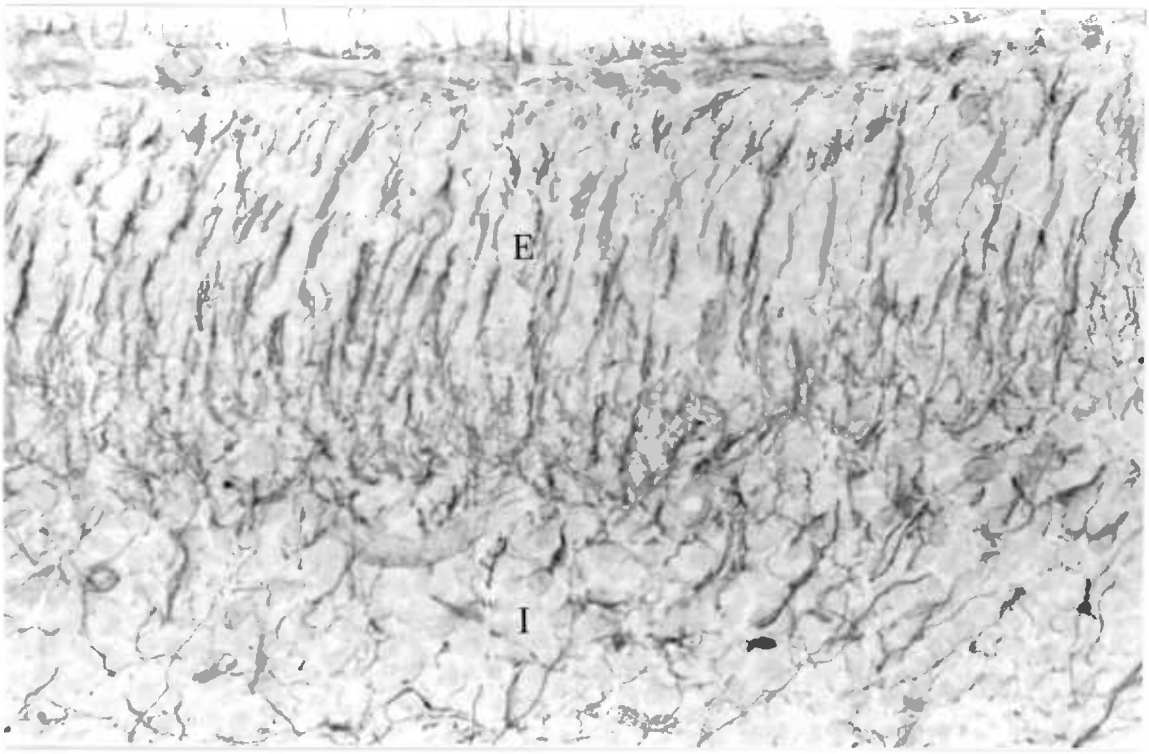


Fig. 69 Immunocytochemical localisation of GFAP in the cerebellum of the 6 day old rat.

Radial Bergmann glial fibres traversing the molecular and external granular layers show intense positive staining, but there is no reaction product in the perikarya of cells in these layers. In the internal granular layer astrocytic processes and perikarya containing reaction product can be seen.
(I = internal granular layer; E = external granular layer)
Antiserum diluted 1 : 500.
Unstained 10 µm frozen sagittal section (x 440).

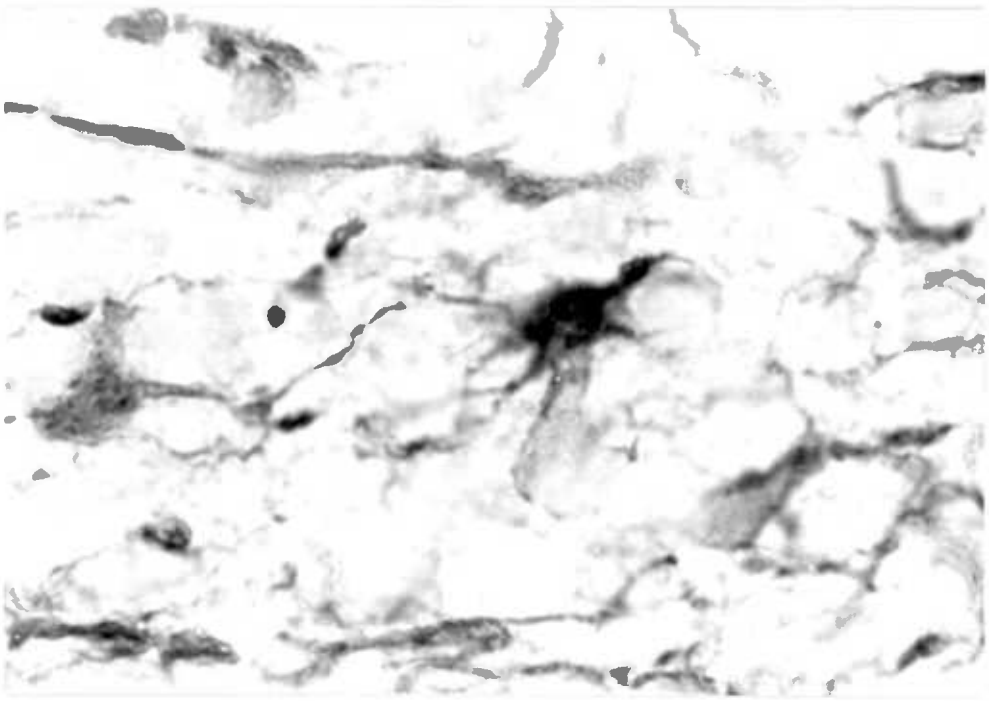


Fig. 70 Immunocytochemical localisation of GFAP in the cerebellum of the 6 day old rat.

Astrocyte in the internal granular layer.

Antiserum diluted 1 : 500
Unstained 10 µm frozen sagittal section (x 1,700)

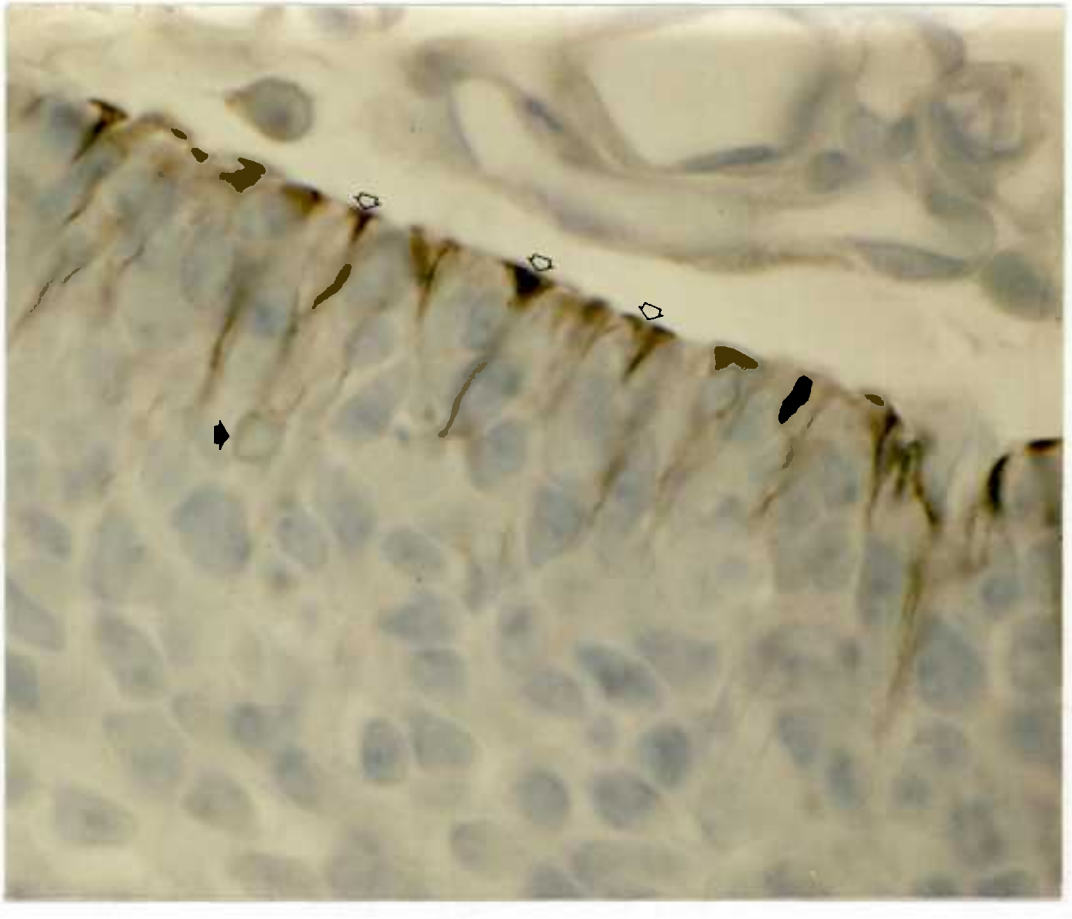


Fig. 71 Immunocytochemical localisation of GFAP in the cerebellum of the 10 day old rat.

The cell which appears to have reaction product in its perikaryon (closed arrow) is in fact not stained but in close proximity to 2 glial fibres, one on either side of the nucleus. Endfeet (open arrows) are clearly visible at the pial surface.

Antiserum diluted 1 : 500

Toluidine blue stained 1 μm resin coronal section (x 880)

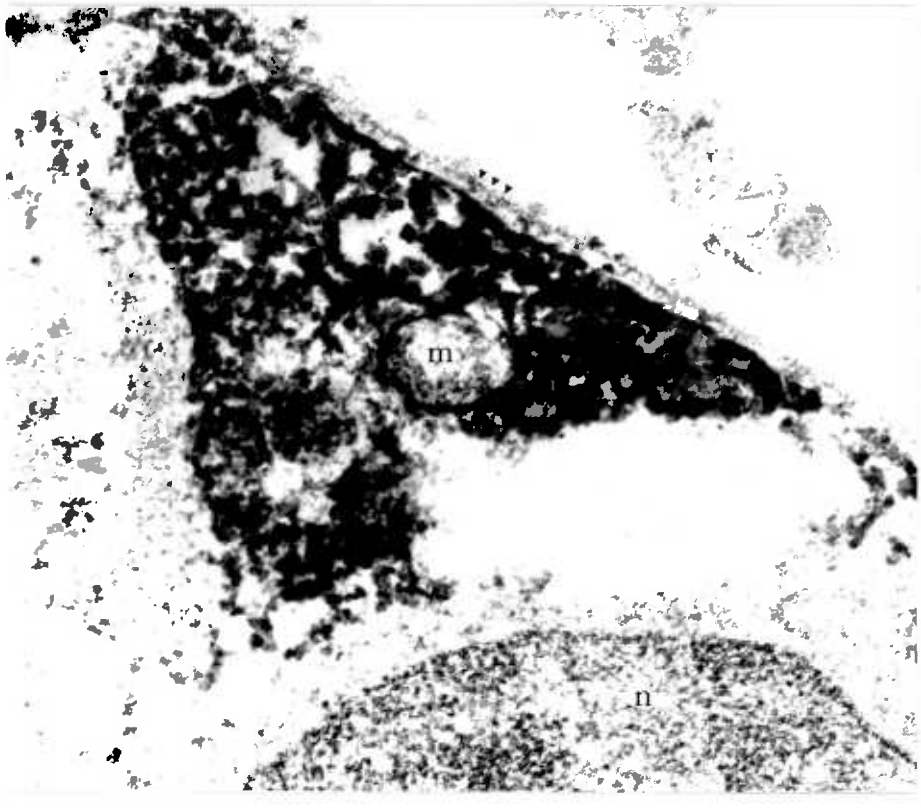


Fig. 72 Immunocytochemical localisation of GFAP in the cerebellum of the 10 day old rat.

The Bergmann glial endfeet just beneath the basement membrane (arrowheads) contain reaction product in granular form. This does not appear to be associated with any particular sub-cellular structure, and does not appear within organelles such as the mitochondrion (m). The nucleus of an EGL cell (n) is also visible.

Antiserum diluted 1 : 500

Uranyl acetate/lead citrate stained coronal section (x 46,700)

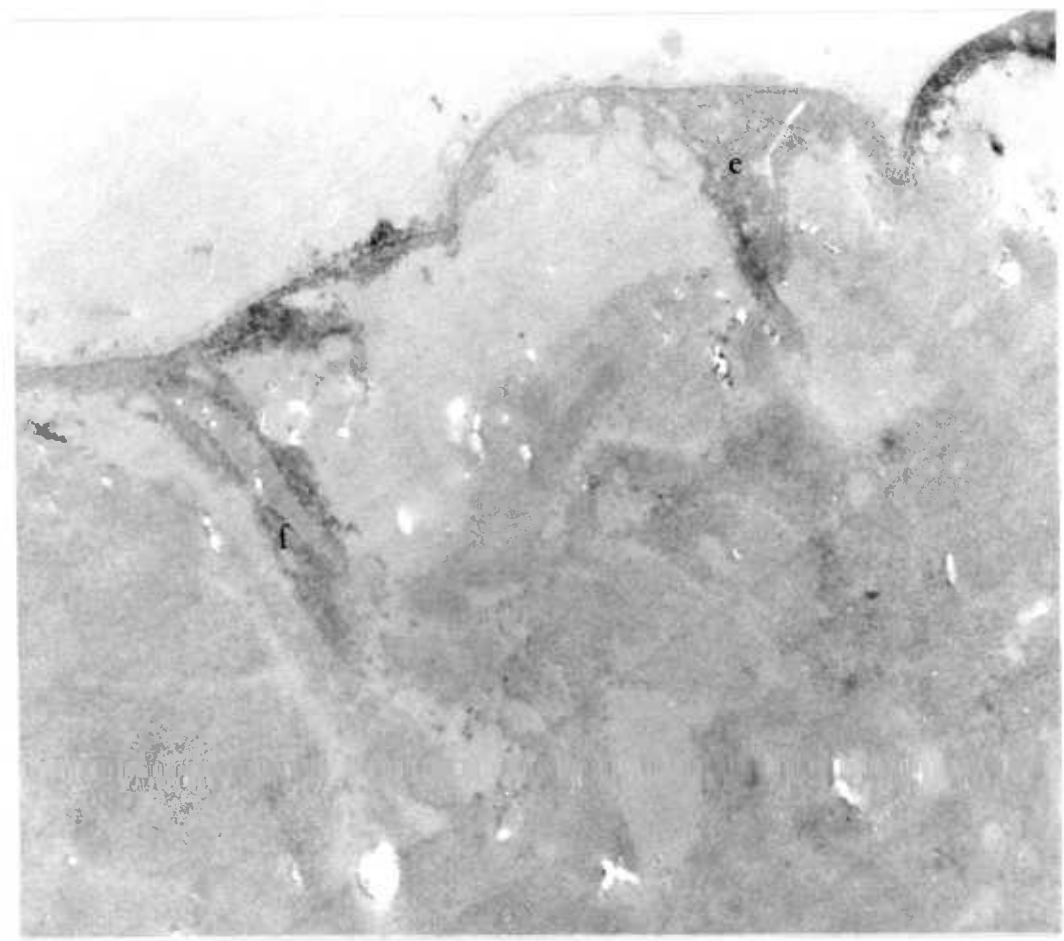


Fig 73 Immunocytochemical localisation of GFAP in the cerebellum of the 10 day old rat.

In the external granular layer the reaction product is confined to endfeet (e) at the pial surface and fibres (f) of Bergmann glia. The EGL cells contain no reaction product.

Antiserum diluted 1 : 500

Unstained coronal section (x 19,300)

at all three ages treated with anti-GFAP and visualised with DAB revealed reaction product confined to astrocytes situated in the IGL (Fig. 70), and to Bergmann glial cells (Figs. 68 and 69). In all the material examined there was no evidence of reaction product within the EGL, other than in the Bergmann glial processes and endfeet, and none was found in the perikarya of EGL cells (Figs. 68, 69 and 71).

Compared with 6 and 10 day old tissue sections, there was somewhat less reaction product in the cerebellum of one day old animals (Fig. 68). This may not have been an age-related phenomenon since the reduction was within the limits of experimental variation.

In some of the material a non-specific reaction was detectable at the edges of the slices but this was clearly distinguishable from the specific reaction which showed across the width (about 100 μm) of the sections (Fig. 69). Control sections treated with non-immune serum showed a complete absence of reaction product.

b) Electron microscopic observations

At the ultrastructural level no trace of reaction product could be detected in control sections or in cells which appeared GFAP-free at the light microscope level. The only cells displaying a positive reaction were astrocytes in the IGL (Fig. 74) and, in the EGL, Bergmann glial processes and endfeet (Figs. 72, 74 and 75). This was true for all three ages examined. In sections not stained with lead citrate, the reaction product appeared finely granular, evenly distributed throughout positively-reacting structures, with an electron-density not much greater than osmiophilic

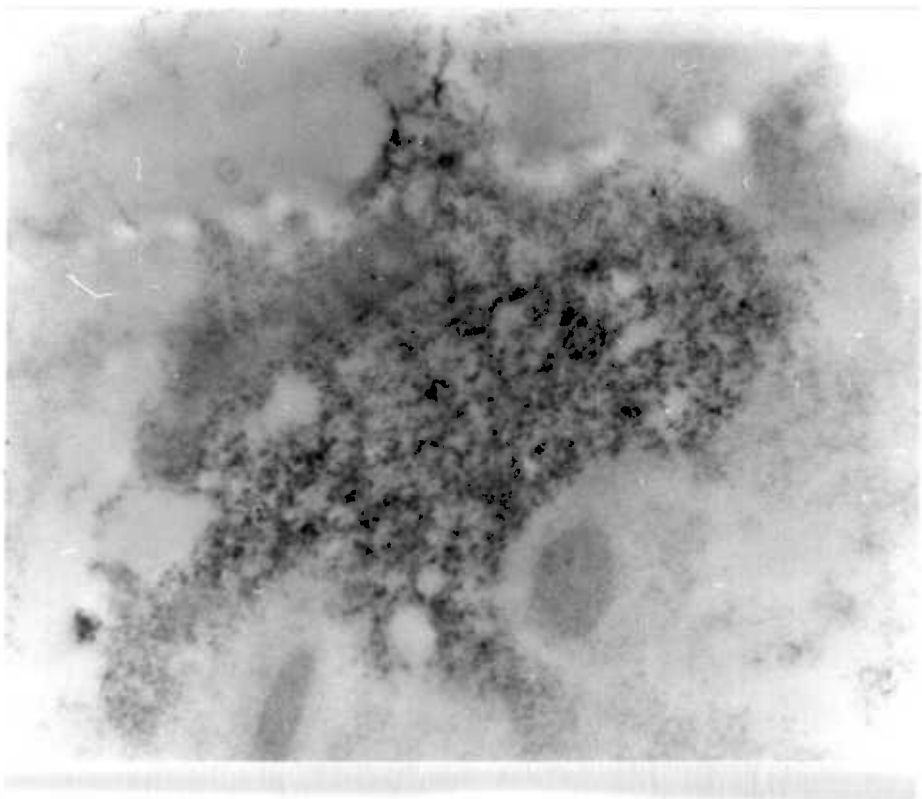


Fig. 74 Immunocytochemical localisation of GFAP in the cerebellum of the 1 day old rat.

An astrocyte containing reaction product in the internal granular layer. The preservation of the material is poor and it is not possible to identify the association of the reaction product with specific organelles.

Antiserum diluted 1 : 500

Unstained coronal section (x 29,000)

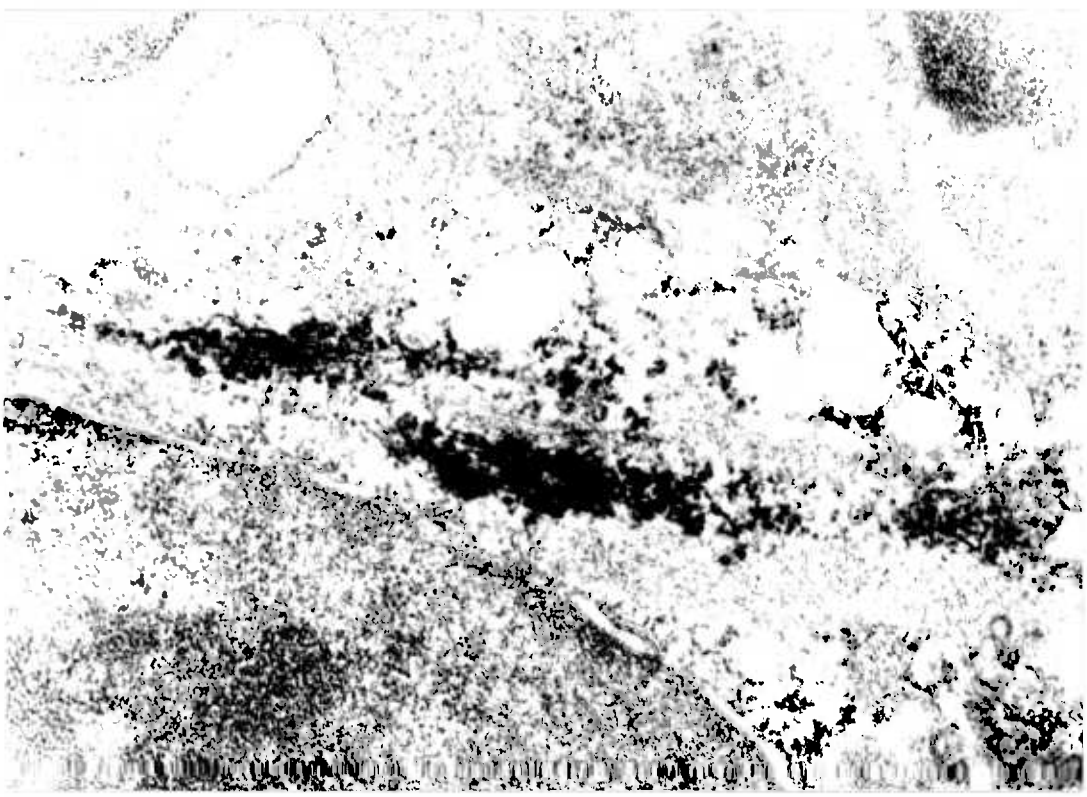


Fig. 75 Immunocytochemical localisation of GFAP in the cerebellum of the 10 day old rat.

Bergmann glial fibre in the external granular layer. Reaction product is associated with the glial filaments, though some filaments are only poorly stained.

Antiserum diluted 1 : 500

Uranyl acetate/lead citrate stained coronal section (x 29,000)

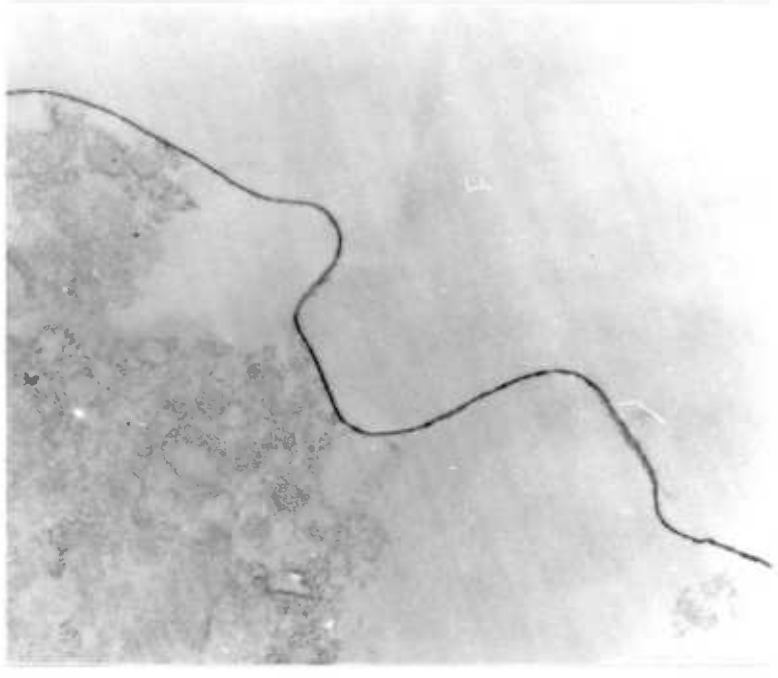


Fig. 76 Immunocytochemical localisation of GFAP in the cerebellum of the 10 day old rat.

At the edges of the edges of the vibratome section there is usually a dense deposition of reaction product. This is non-specific staining.

Antiserum diluted 1 : 500

Unstained coronal section (x 18,400)

structures such as nuclei (Figs. 73 and 74). When stained with lead citrate, the reaction product appeared as discrete round bodies, with a much more uneven distribution. In the Bergmann glial fibres and end-feet there was no association of the anti-GFAP positive material with glial filaments (Fig. 72), but in the processes, globules of reaction product were associated with and aligned along the glial filaments (Fig. 75). The non-specific reaction product deposited on the cut edges of the section was clearly distinguishable in the electron microscope (Fig. 76).

Glutamine synthetase (GS)

a) Light microscopic observations

The cerebellum of rats of postnatal days 0, 3 and 7 was examined. The result were similar for those obtained by staining for GFAP.

At the light microscope level the EGL cells were free from reaction product at all ages. The only positive-staining elements were Bergmann glia, both end-feet and processes (Fig. 77) and astrocytes in the IGL (Fig. 78). As with the material treated with anti-GFAP, sections stained for GS showed some non-specific reaction at the edge of the slice. Control sections treated with non-immune serum showed complete absence of any reaction product.

b) Electron microscopic observations

High power examination revealed an absence of low levels of reaction product in the control sections, and also in cells which were GS-negative on light microscopic examination. The only positive cells were astrocytes

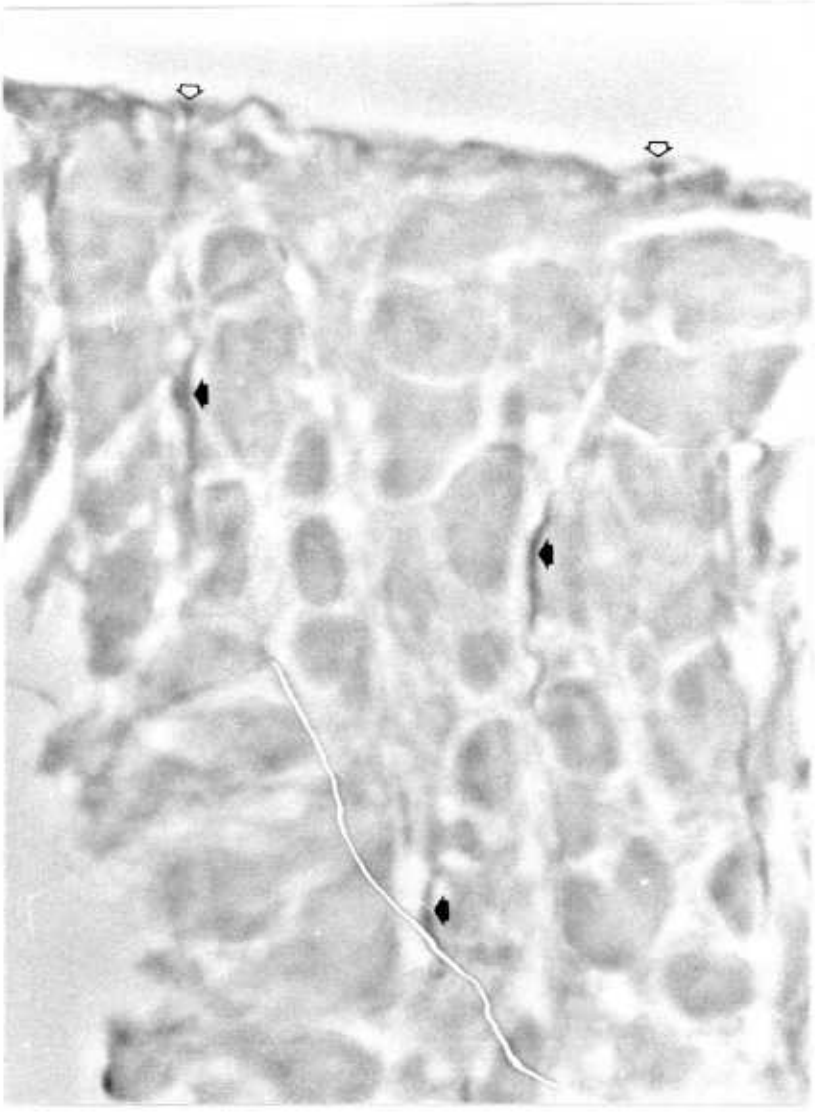


Fig. 77 Immunocytochemical localisation of glutamine synthetase in the cerebellum of the 7 day old rat.

Reaction product in the external granular layer is confined to the processes (closed arrows) and end-feet (open arrows) at the pial surface of the Bergmann glia. There is no reaction product in the perikarya of the EGL cells.

Antiserum diluted 1 : 200

Toluidine blue stained 1 μm resin coronal section phase contrast (x 1,600).

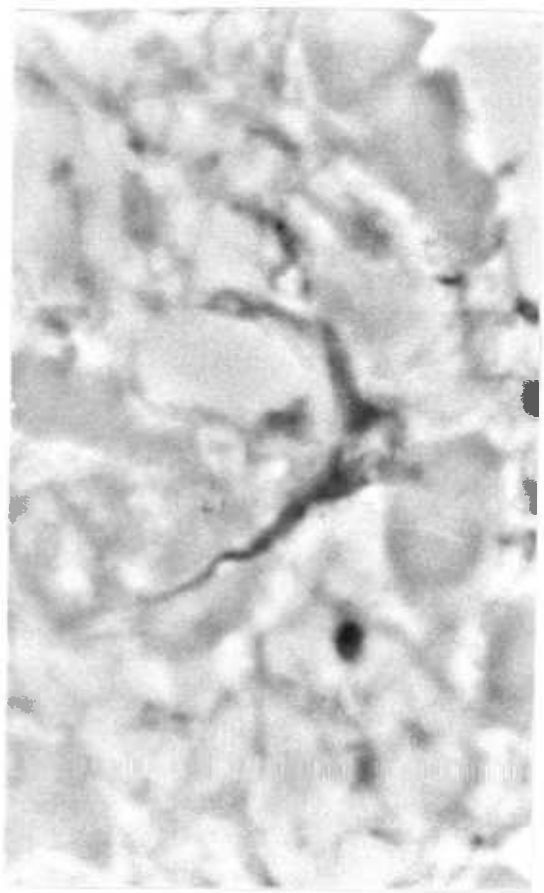


Fig. 78 Immunocytochemical localisation of glutamine synthetase in the cerebellum of the 7 day old rat.

Astrocytic processes containing reaction product in the internal granular layer.

Antiserum diluted 1 : 200

Toluidine blue stained 1 µm resin coronal section phase contrast (x 3,500)

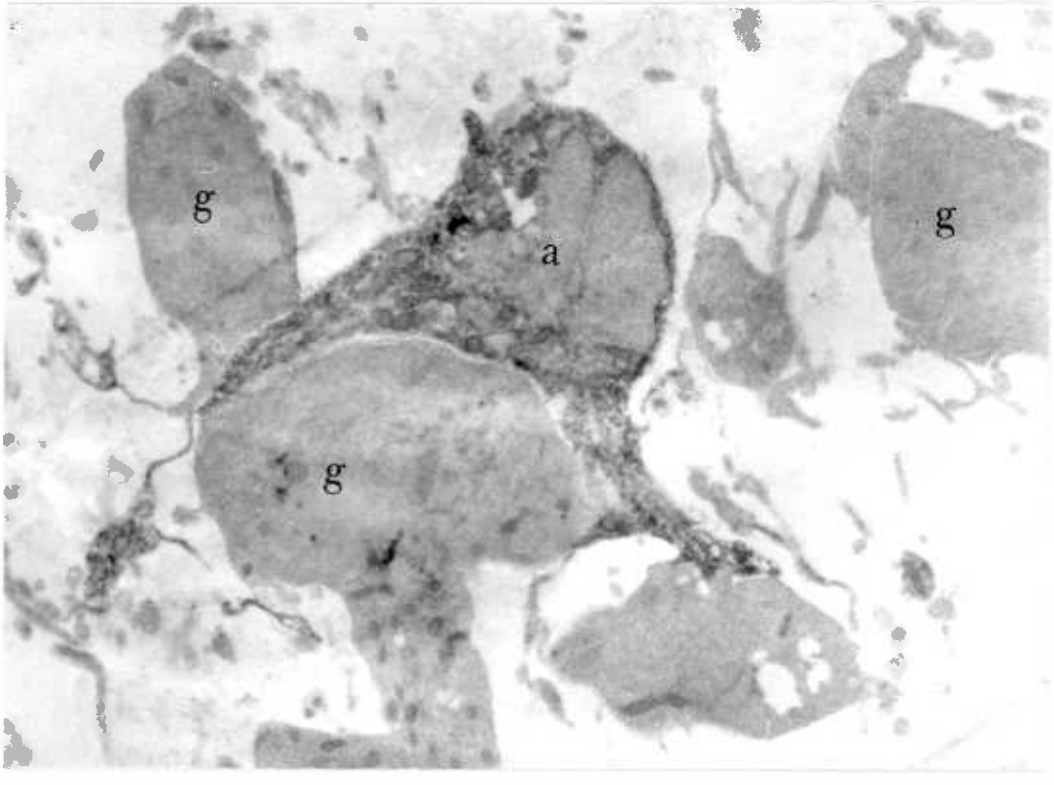


Fig. 79 Immunocytochemical localisation of glutamine synthetase in the cerebellum of the 7 day old rat.

Astrocyte (a) with cytoplasm containing reaction product in the internal granular layer. The granule cells (g) are free of reaction product.

Antiserum diluted 1 : 200

Uranyl acetate/lead citrate stained coronal section (x 7,500)

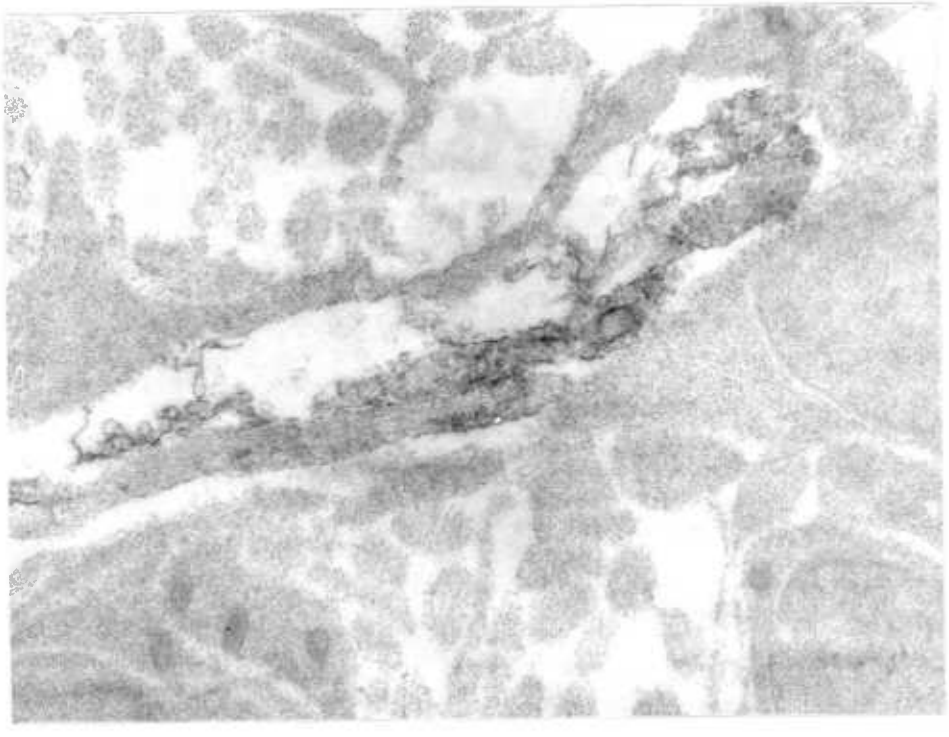


Fig. 80 Immunocytochemical localisation of glutamine synthetase in the cerebellum of the 7 day old rat.

Reaction product is associated with the external membranes of the Bergmann glial fibres traversing the external granular layer.

Antiserum diluted 1 : 200

Uranyl acetate/lead citrate stained coronal section (x 22,000)

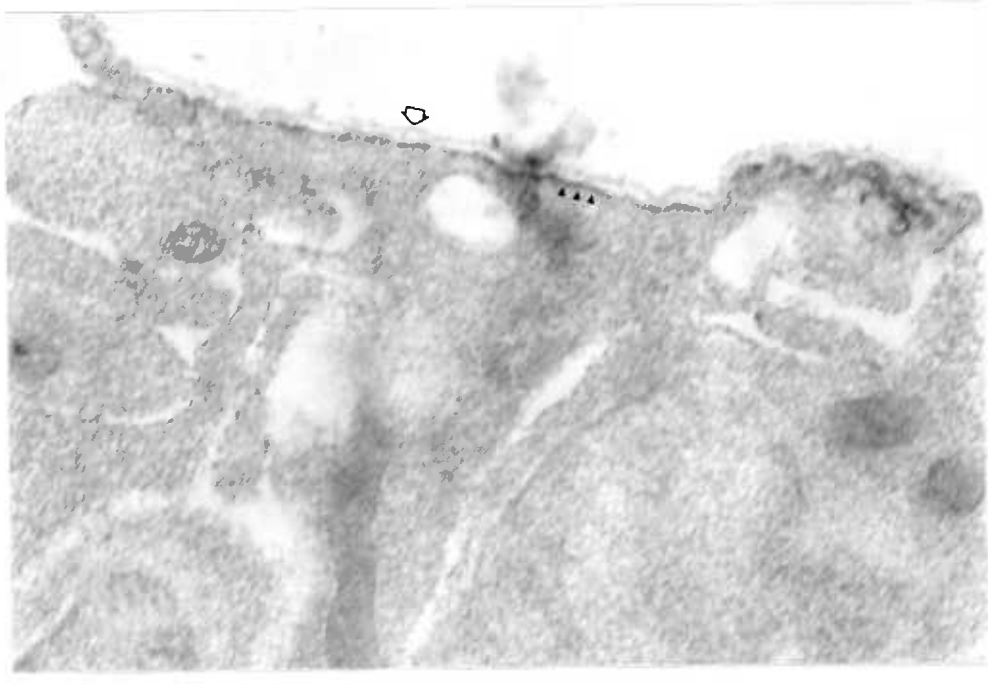


Fig. 81 Immunocytochemical localisation of glutamine synthetase in the cerebellum of the neonate rat.

A Bergmann glial end-foot at the pial surface. Reaction product is visible on the membrane surface (arrowheads), just below the basement membrane (open arrow).

Antiserum diluted 1 : 40

Uranyl acetate/lead citrate stained coronal section (x 40,000)

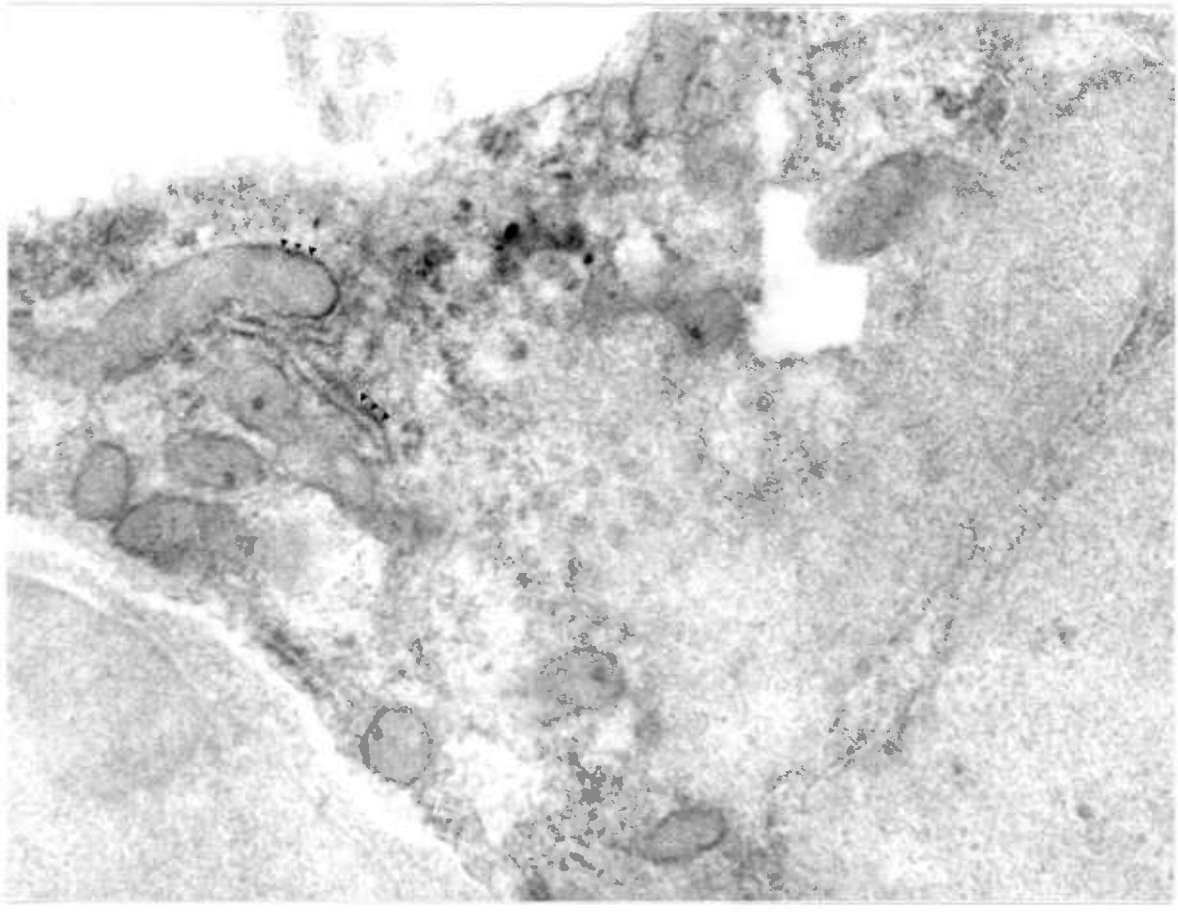


Fig. 82 Immunocytochemical localisation of glutamine synthetase in the cerebellum of the 7 day old rat.

Reaction product in astrocytes in the internal granular layer was mainly confined to the membranes of the mitochondria and the endoplasmic reticulum (arrowheads).

Antiserum diluted 1 : 200

Uranyl acetate/lead citrate stained coronal section (x 80,000)

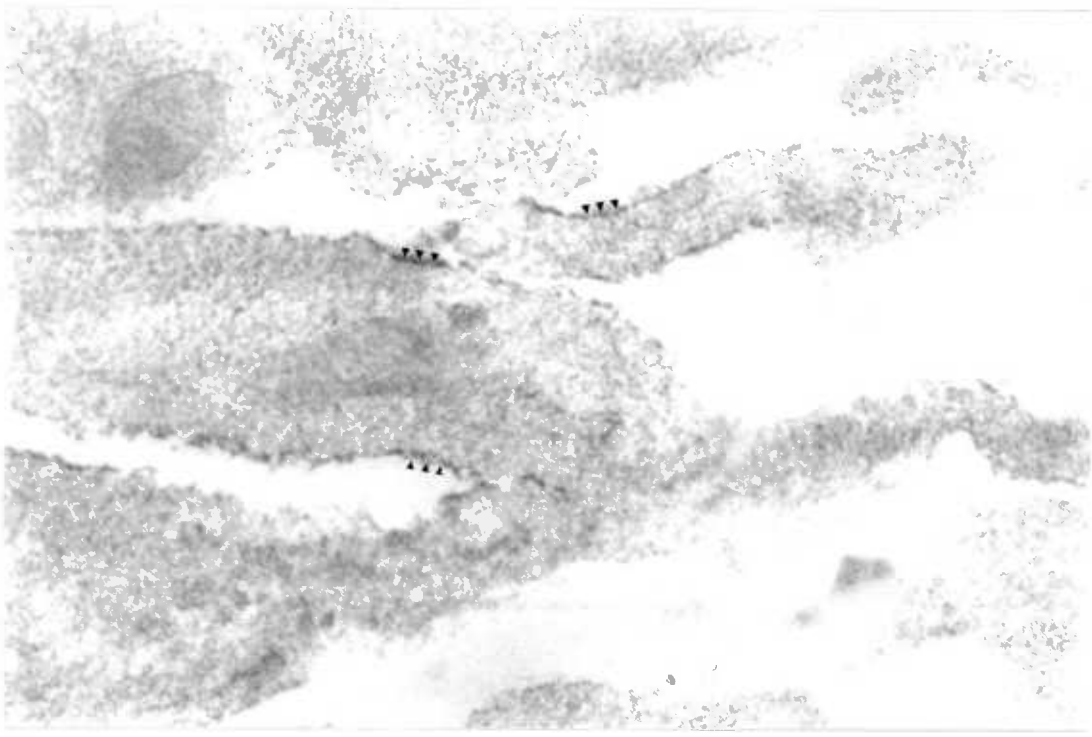


Fig. 83 Immunocytochemical localisation of glutamine synthetase in the neonate rat cerebellum.

The Bergmann fibres in the EGL show a light deposition of reaction product on the surface membrane (arrowheads).

Antiserum diluted 1 : 40

Uranyl acetate/lead citrate stained coronal section (x 63,000).

in the IGL (Fig. 79), and Bergmann glia (both fibres and end-feet (Figs. 80 and 81). In the astrocytes the reaction product was mainly associated with the mitochondrial membranes and the endoplasmic reticulum (Fig. 82). Bergmann glia showed reaction product associated with the pial surface of the end-feet (Fig. 81) and in the processes containing mitochondrial/membrane and endoplasmic reticulum (Fig. 83). These results confirm that glutamine synthetase is restricted to astrocytes (Norenberg and Martinez-Hernandez, 1979).

Galactocerebroside (Gal)

a) Light microscopic observations

The cerebellum of 0, 3 and 7 day old rats was examined. There was very little reaction product detectable in any specimen at the light microscope level. The EGL was completely unstained (Figs. 84 and 85). The cut edges showed some non-specific staining; in one case the whole perikaryon of a cell was stained (Fig. 86); but this could well be due to the leakage of reagent into the cytoplasm through a damaged membrane.

b) Electron microscopic observations

The ultrastructural examination of material stained for galactocerebrocide showed only traces of reaction product confined to the white matter (Figs. 87 and 88). It was difficult to relate it to specific structures owing to the poor preservation of the material. The EGL was completely free of even low levels of reaction product (Fig. 89), as were the control sections.

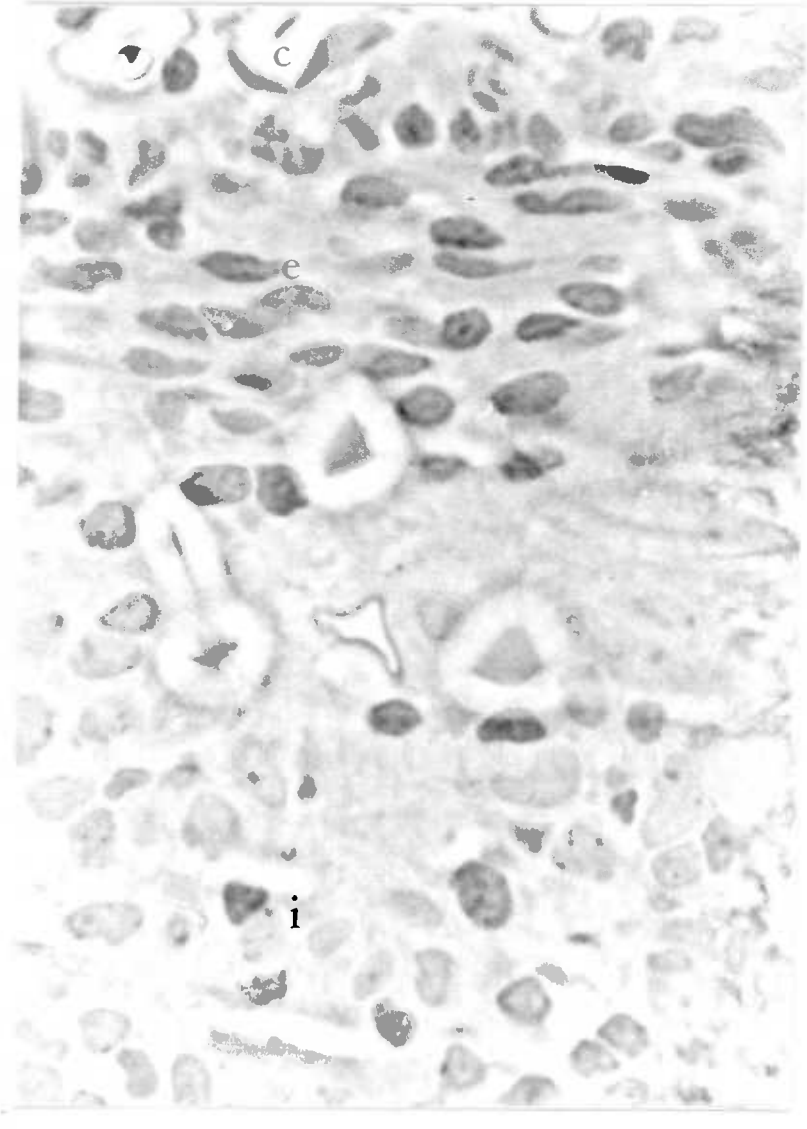


Fig. 84 Immunocytochemical localisation of galactocerebroside in the cerebellum of the neonate rat.

Whole vibratome-cut strip of cerebellum showing lack of obvious concentrations of reaction product.
c = pial capillary ; e = external granular layer;
i = internal granular layer.

Antiserum diluted 1 : 100

Toluidine blue stained 1 μm coronal resin section (x 100)

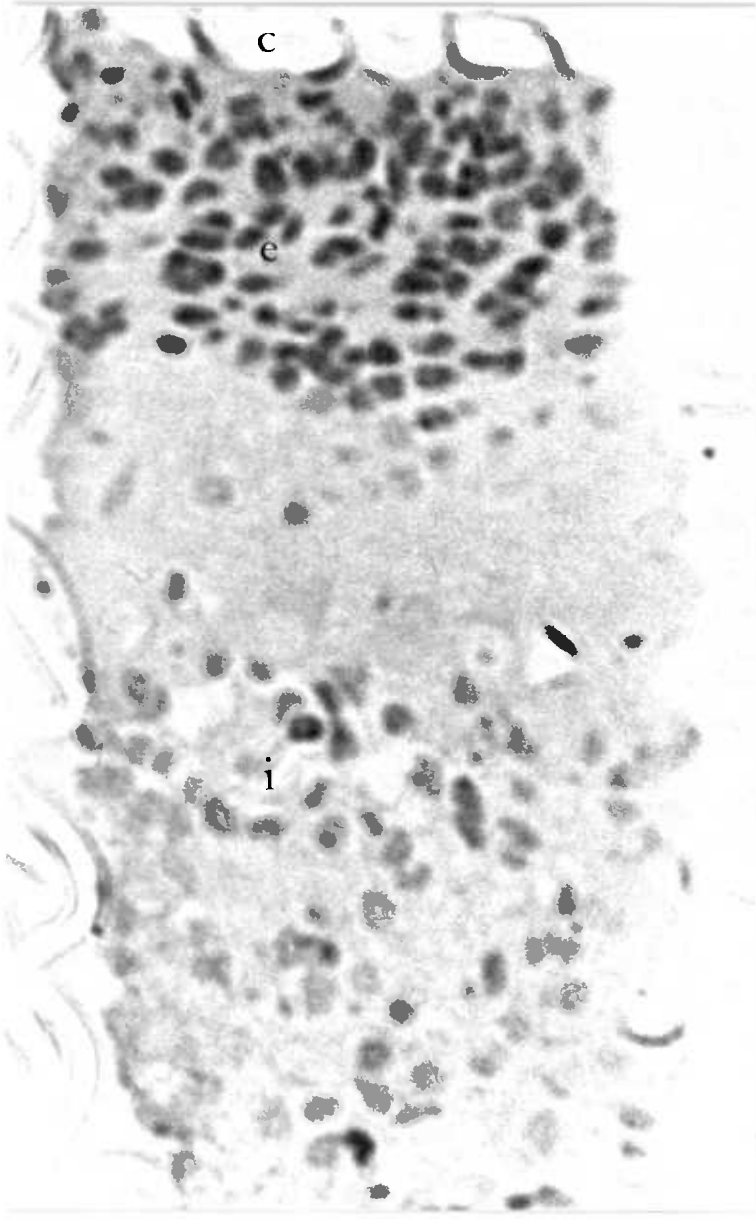


Fig. 85 Immunocytochemical localisation of galactocerebroside in the cerebellum of the 7 day old rat.

Whole vibratome-cut strip of cerebellum showing lack of obvious concentrations of reaction product.
c = pial capillary; e = external granular layer;
i = internal granular layer.

Antiserum diluted 1 : 500

Toluidine blue stained 1 μm coronal resin section (x 100)

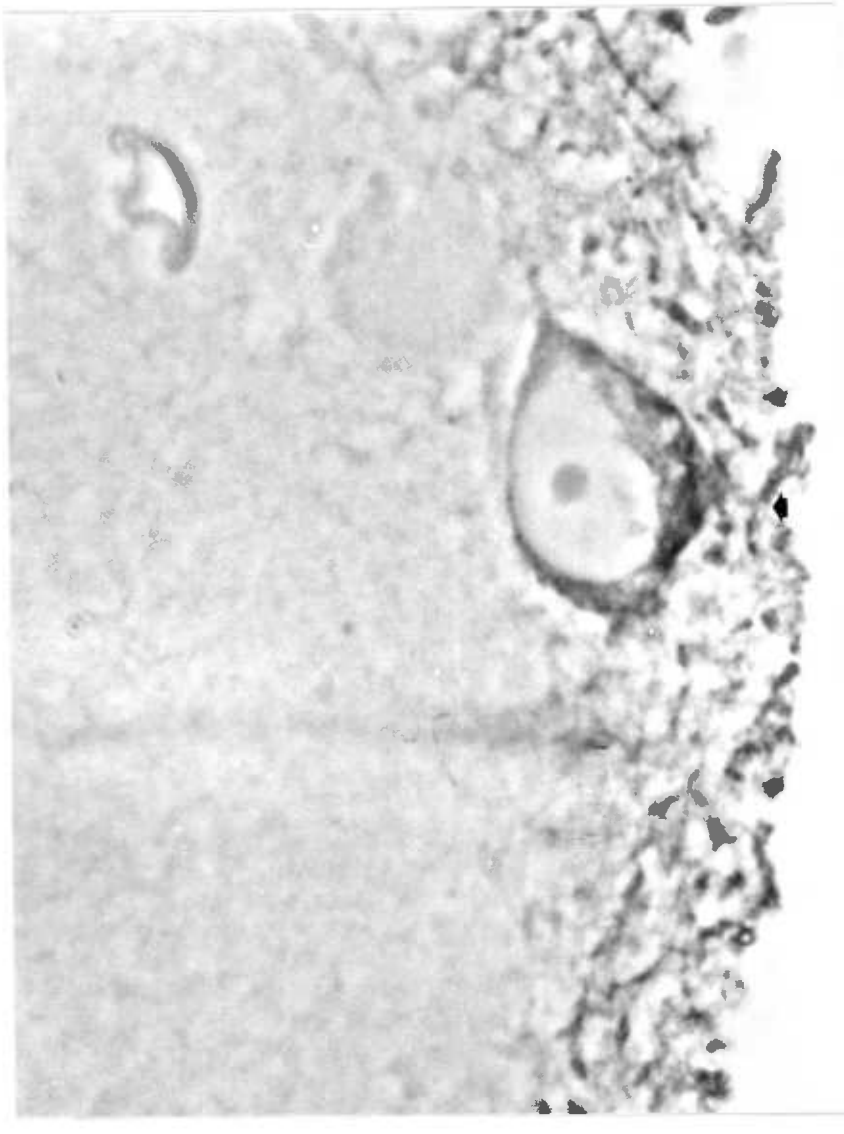


Fig. 86 Immunocytochemical localisation of galactocerebroside in the cerebellum of the neonate rat.

Reaction product in the perikaryon of a cell in the internal granular layer. This is probably non-specific leakage into the cytoplasm from a damaged process at the vibratome-cut edge (arrow).

Antiserum diluted 1 : 100

Toluidine blue stained 1 μm coronal resin section (x 3,500)

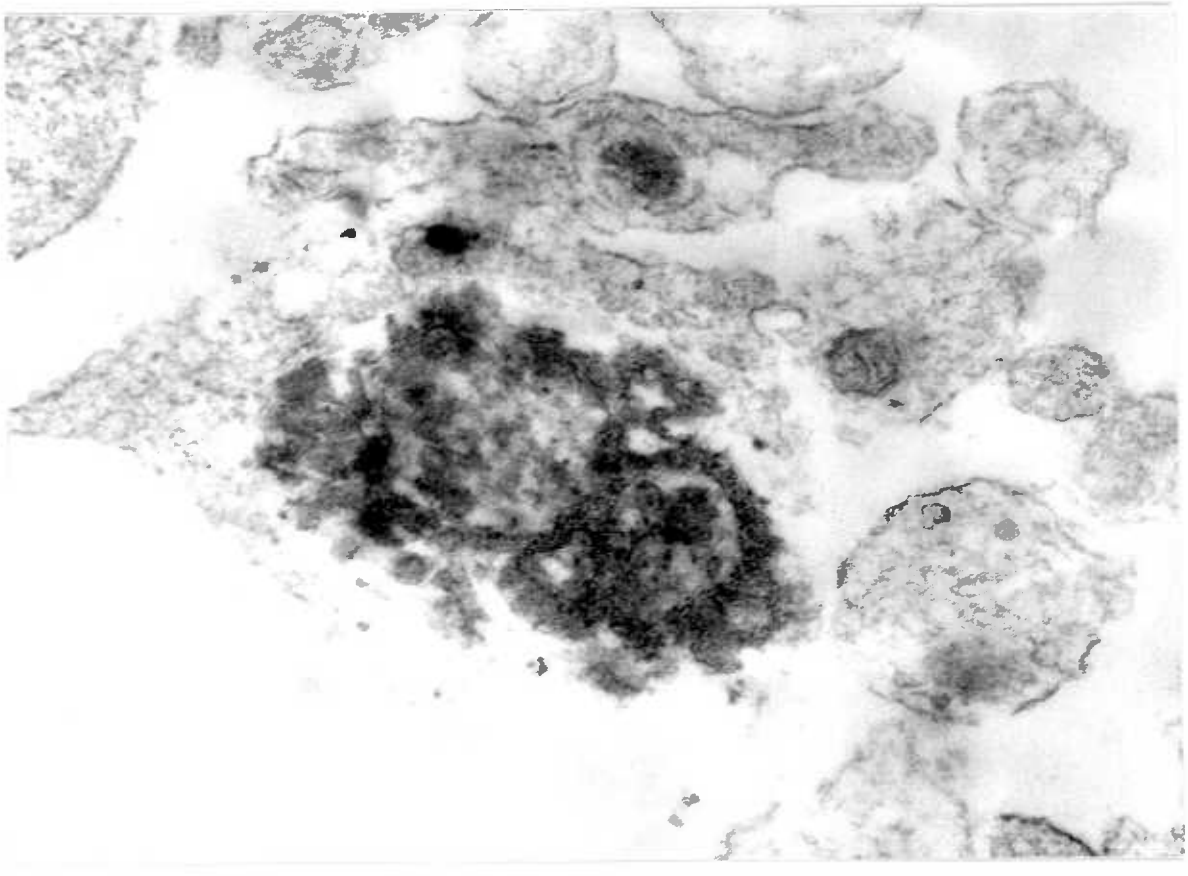


Fig 87 Immunocytochemical localisation of galactocerebroside in the cerebellum of the 7 day old rat.

Reaction in a process (possibly oligodendroglial) in the white matter.

Antiserum diluted 1 : 100

Uranyl acetate/lead citrate stained coronal section (x 62, 500)

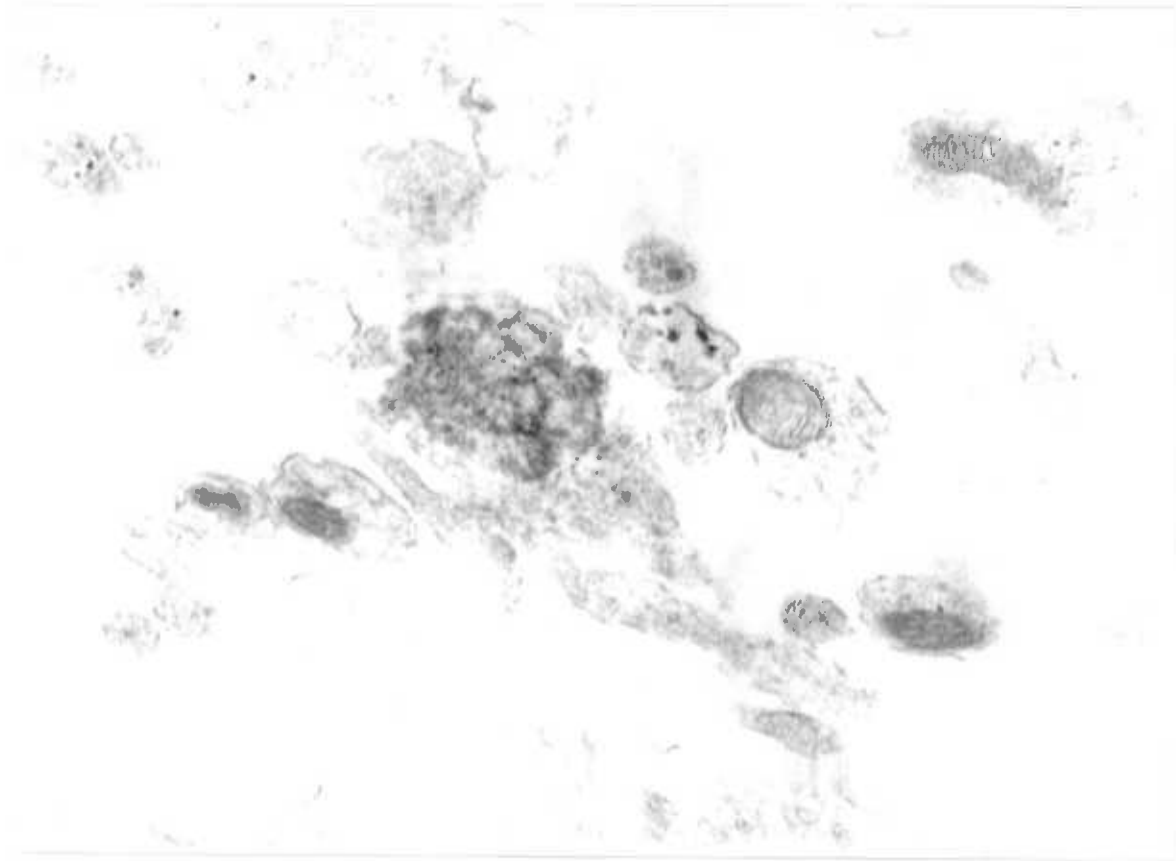


Fig. 88 Immunocytochemical localisation of galactocerebroside in the cerebellum of the neonate rat.

Reaction product in a process (possibly oligodendroglial) in the white matter.

Antiserum diluted 1 : 100

Uranyl acetate/lead citrate stained coronal section (x 32,000)

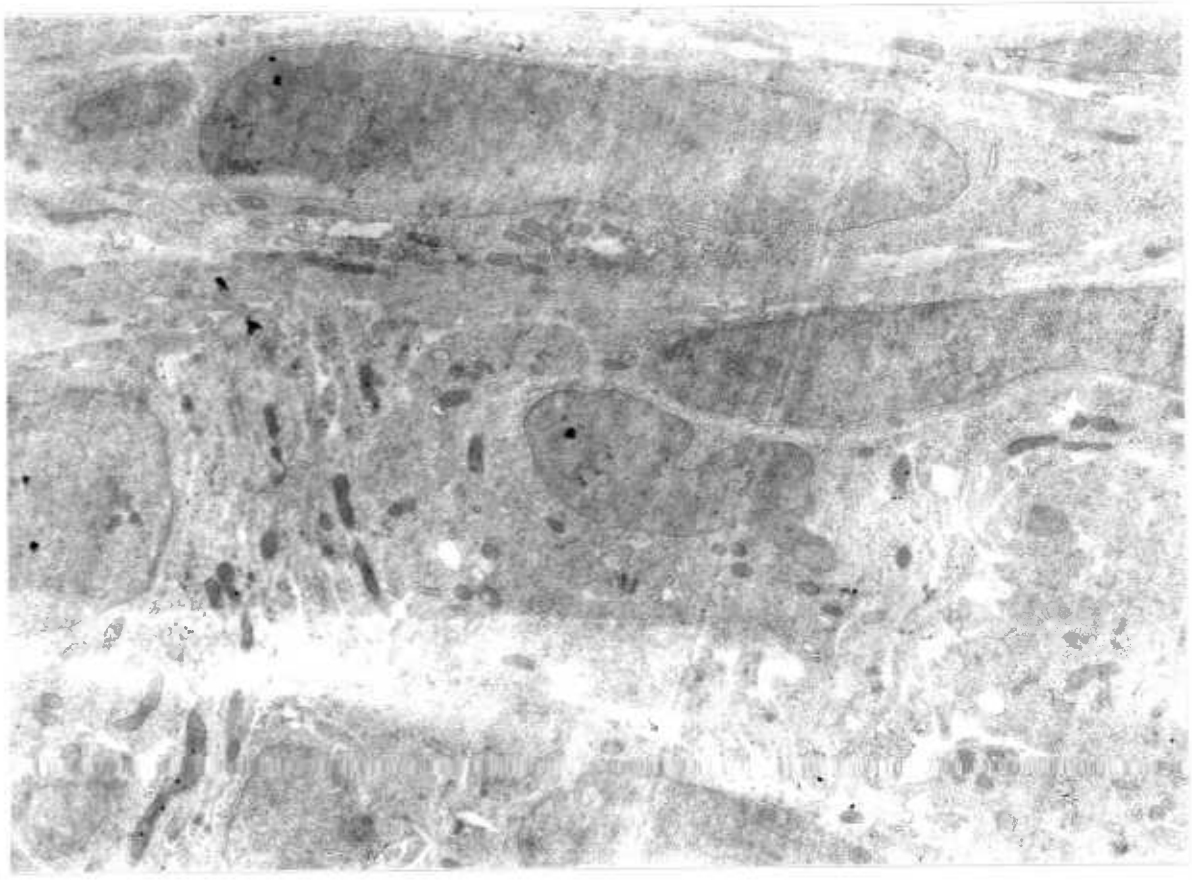


Fig. 89 Ultrastructural localisation of galactocerebroside in the cerebellum of the 7 day old rat.

The cells of the external granular layer are completely free from reaction product.

Antiserum diluted 1 : 500

Uranyl acetate/lead citrate stained coronal section (x 8,000)

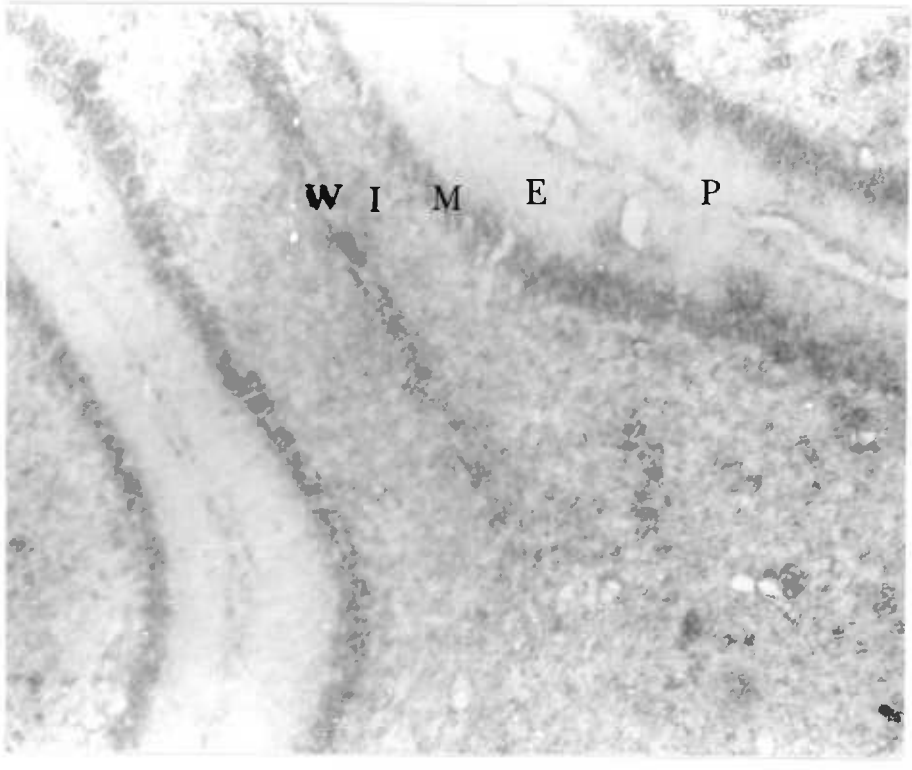


Fig. 90 Immunocytochemical localisation of NSE in the cerebellum of the 8 day old rat.

The staining pattern for the neuron specific enolase is similar to that of the non-specific enolase.

W = white matter
I = internal granule layer
M = molecular layer
E = external granule layer
P = pial surface.

Antiserum diluted 1 : 100

Unstained 10 μ frozen sagittal section (x 170)

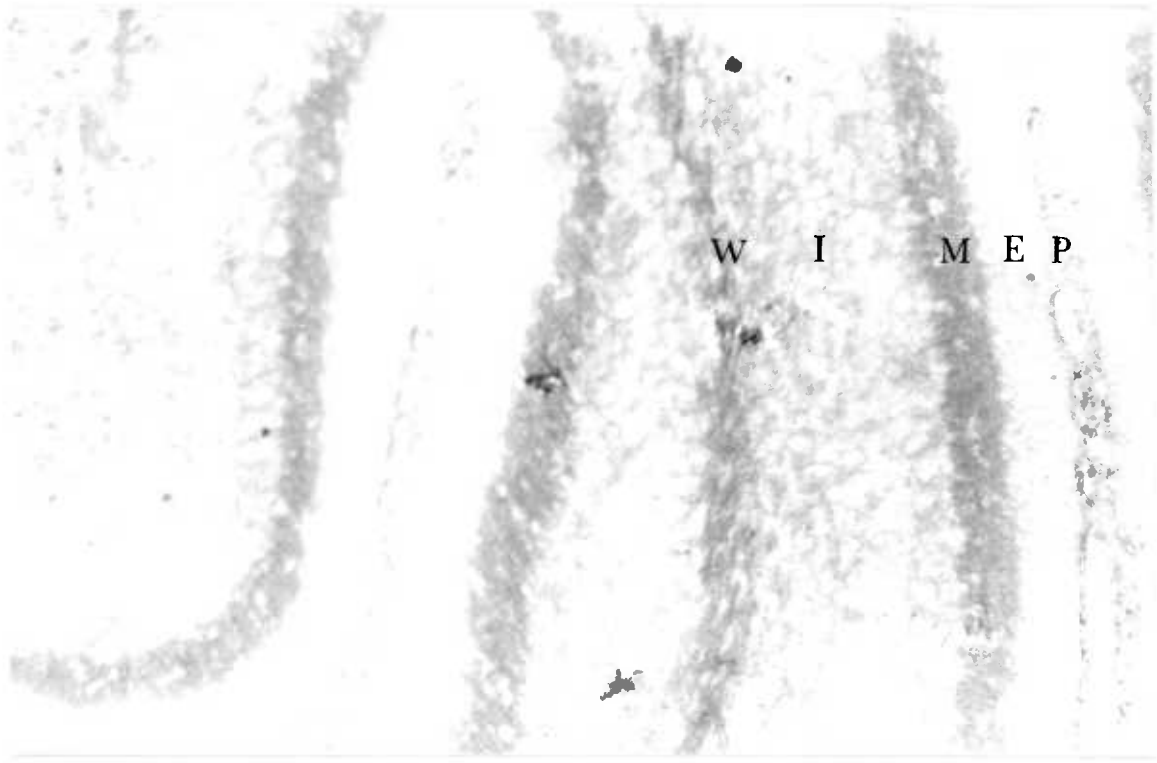


Fig. 91 Immunocytochemical localisation of NNE in the cerebellum of the 7 day old rat.

No specificity is apparent. The molecular layer (M) and white matter is darker than the internal granular layer (I), itself darker than the external granular layer (E).
p = pial surface.

Antiserum diluted 1 : 1000

Unstained 10 µm frozen sagittal section (x 170)

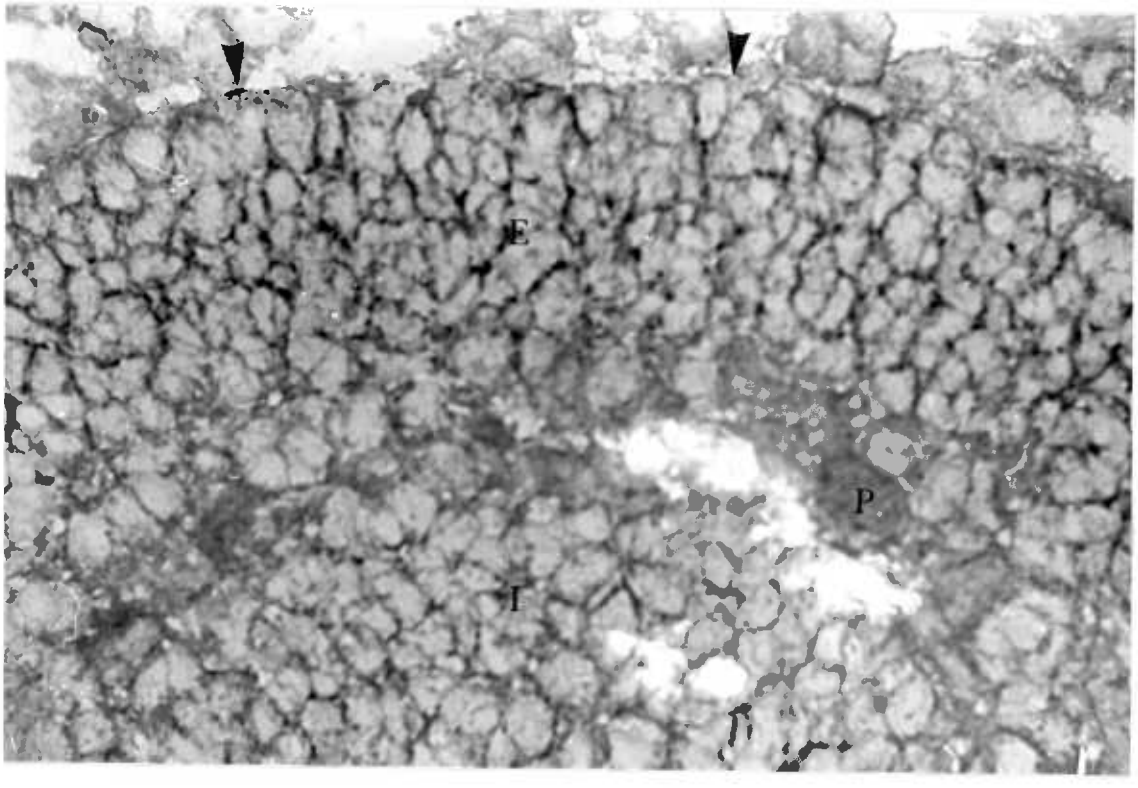


Fig. 92 Immunocytochemical localisation of D2 protein in the cerebellum of the 7 day old rat.

Non-specific reaction product is visible between the cells of the external granular layer (E) and the cells of the internal granular layer (I). Arrowheads = pial surface; P = Purkinje cell.

Antiserum diluted 1 : 100

Unstained 10 um frozen sagittal section (x 700)

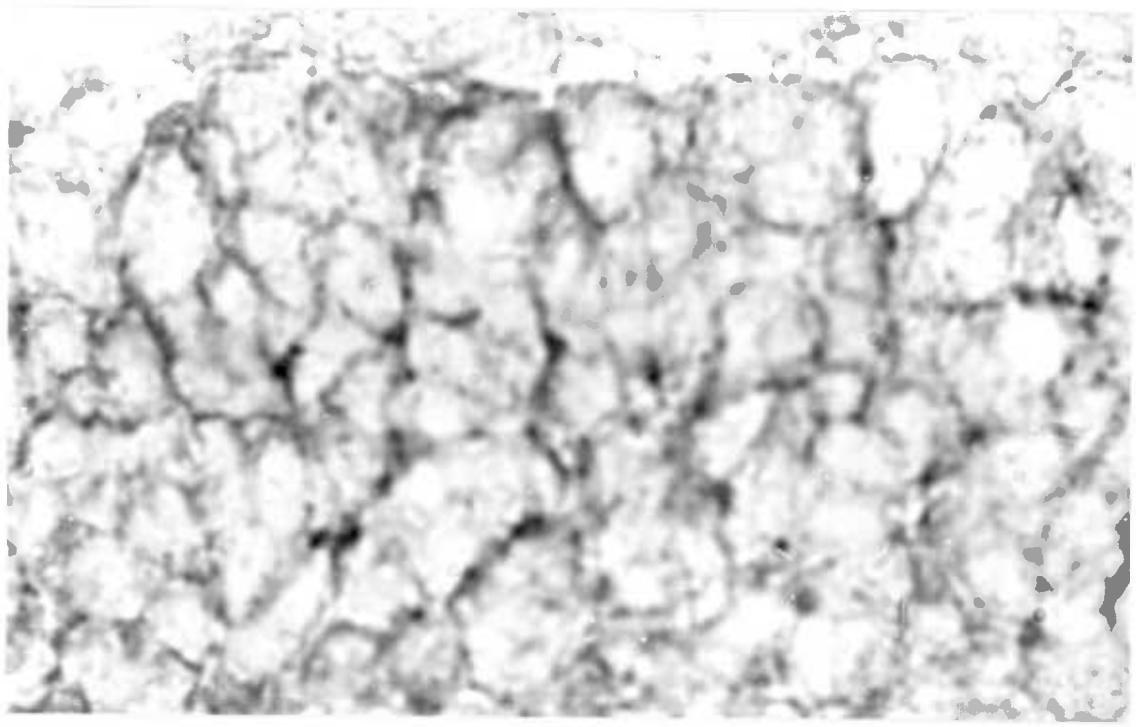


Fig. 93 Immunocytochemical localisation of D2 protein in the cerebellum of the 7 day old rat.

Reaction product visible only in the intracellular spaces in the external granular layer.

Antiserum diluted 1 : 100

Unstained 10 µm frozen sagittal section (x 1700)

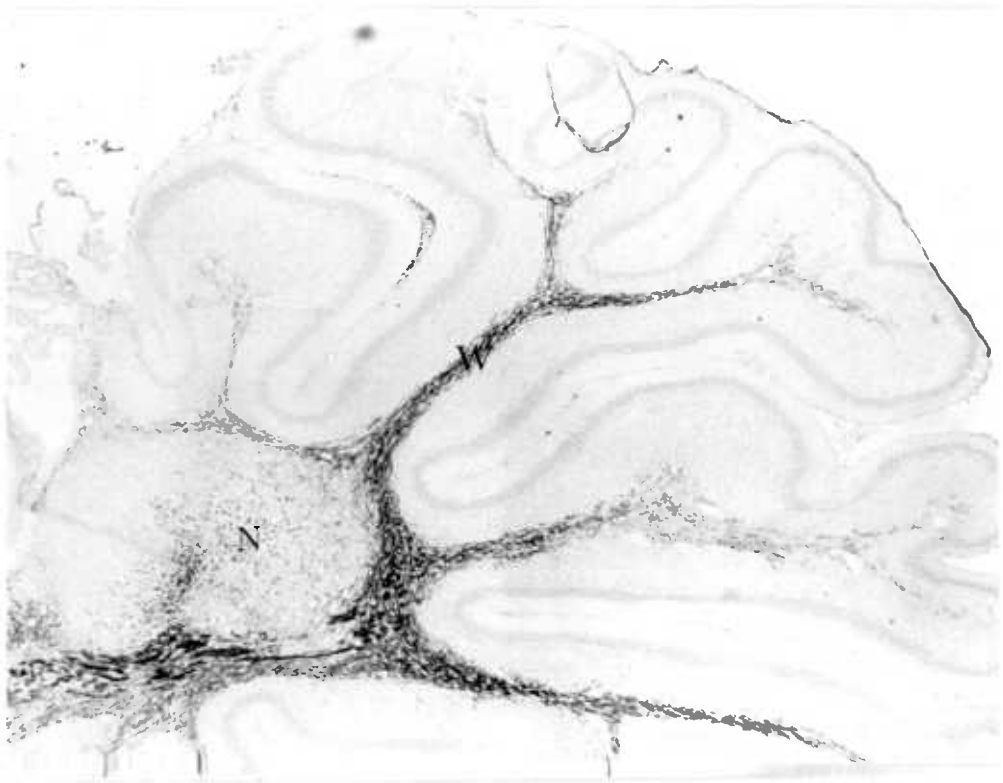


Fig. 94 Immunocytochemical localisation of myelin basic protein in the cerebellum of the 8 day old rat.

Low power view showing specific staining of white matter (W) and deep cerebellar nuclei (N).

Antiserum diluted 1 : 100

Unstained 10 μm frozen sagittal section (x 44)

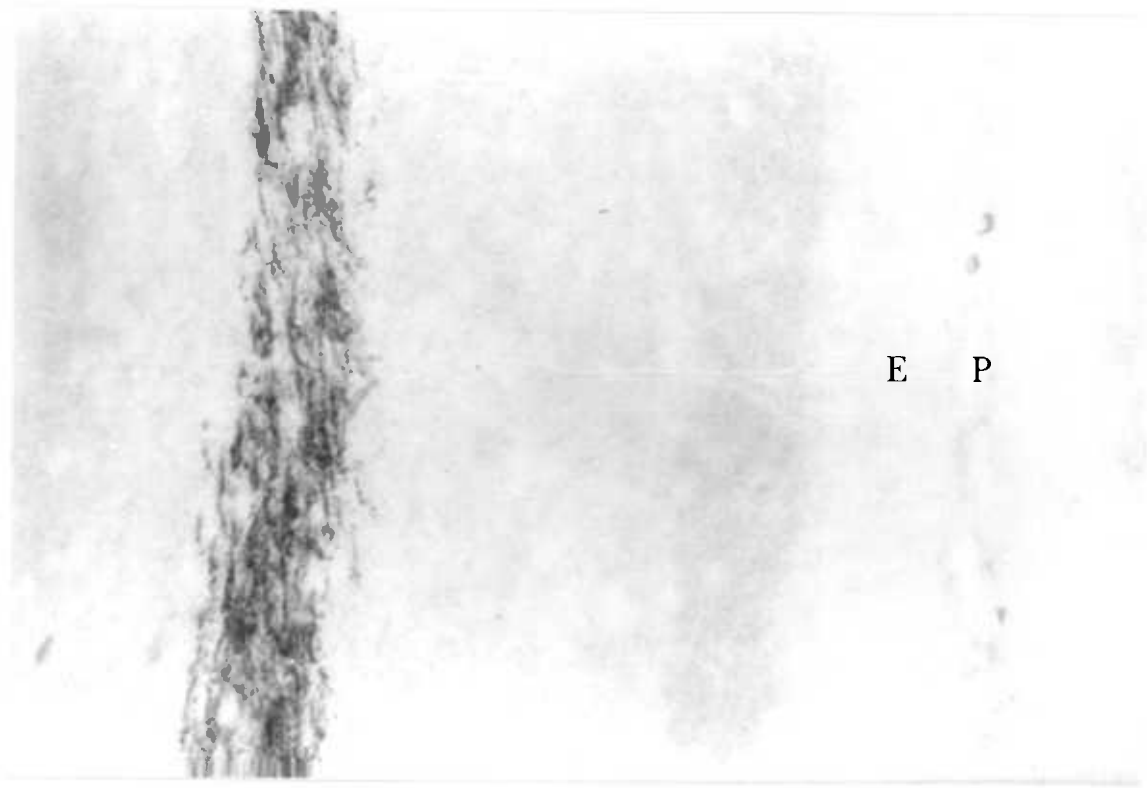


Fig. 95 Immunocytochemical localisation of myelin basic protein in the cerebellum of the 8 day old rat.

Only the white matter shows a reaction to the antiserum. The external granular layer (E) is unstained. P = pial surface.

Antiserum diluted 1 : 100

Unstained 10 μm frozen section sagittal section (x 440)

Enolases (NNE and NSE)

Despite published reports and careful adherence to prescribed methods, antisera to these two isoenzymes did not yield specific results. To establish optimal dilutions, various concentrations of antisera were reacted with 10 μ thick frozen sections of cerebellum of 7 and 9 day old rats. There was no difference between the neuron specific and neuron non-specific enolase distribution at this age (Figs. 90 and 91).

D2

This antiserum, raised against rat synaptosomal membranes, did not show a specific reaction with 10 μ frozen sections of 7 day old rats. It was not known whether this was due to insufficient purification in the preparation of this batch of antiserum. The staining pattern at the light microscope level is shown in Figs 92 and 93.

Myelin basic protein

Several antisera to this oligodendrocyte-specific protein were tested against 10 μ frozen sections of the vermal cerebellum of 1, 3, 8 and 11 day old rats. In all cases there was no positive reaction in the EGL though strong and highly specific staining of white matter was obtained (Figs. 94 - 95).

iii Comments

Of the neuron- and glial-specific proteins currently available for

neuropathological research, GFAP has been the most extensively studied. Antibodies to this protein have been shown to react specifically with astrocytes. Positive staining has been reported for astrocytes in vivo in brain of man and other animals (Bignami et al, 1972; Schachner et al, 1976; Antanitus et al, 1976) in astrocytes in culture (Antanitus et al, 1975; Gilden et al, 1976; Cohen et al, 1979; Manthorpe et al, 1979) and in tumours of the central nervous system (e.g. Jacque et al, 1978; Van der Meulen et al, 1978). The filamentous nature of GFAP has provoked speculation about the identity of the extracted material against which antisera are raised. The original material (Eng et al, 1971) has been identified with tubulin (Liem and Shelanski, 1978; Eng, 1979) but this view has been challenged (Bock et al, 1977; Bignami and Dahl, 1979). A probe for possible cross-reactivity between GFAP and neurofilament protein established the antigenic specificity of these two proteins (Schachner et al, 1978). In the publications describing GFAP as a specific astrocyte marker (of which only a selection are cited above), the restriction of positive staining to astrocytes has repeatedly been confirmed. It is important to establish this point, since the validity of the results presented in this thesis is dependent upon the specificity of the reaction of the anti-GFAP with astrocytes. In the present study there was a complete absence of reaction product in the cytoplasm of EGL cells at all ages examined. Positively-staining cytoplasm was found in astrocytes in the IGL. There was also positively-staining cytoplasm in the EGL, but this was found to be confined to the processes and end-feet of the Bergmann glia. The distribution and staining pattern at the electron

microscopic level was similar to that described by Schachner et al (1977). Using an immunofluorescent technique, Bignami and Dahl (1973) were unable to detect astrocytic protein in the rat cerebellar cortex for the first three postnatal days. The one exception to this negative result was the positive staining of a few fibres overlying the fourth ventricle and in the medulla in the one day old rat. However, the same authors were later satisfied that GFAP staining was at least as sensitive as Weigert's stain when used to study the differentiation of fibrous neuroglia in the developing rat neocortex and hippocampus (Bignami and Dahl, 1974). It should be recalled that ultrastructural evidence is presented elsewhere in this thesis for the existence from the day of birth at least, of cerebellar radial glia, while in the mouse they have been shown to be present from E13 - E15 (Swarz and Oster-Granite, 1978). In the human foetal cerebellum, GFAP positive radial fibres have been detected as early as 7 - 10 weeks of gestation (Choi and Lapham, 1978). These observations suggest that the failure to detect radial glial fibres at the earliest postnatal stages of rat cerebellar development (Bignami and Dahl, 1973) may be related to the techniques used. The advantage of the immunoperoxidase method over immunofluorescence is that the former permits ultrastructural examination. Since there was found to be positive staining in the EGL in the present study it was essential to discover whether this was restricted to the pre-existing Bergmann glia, or whether it could also be found in EGL cells. The results showed that anti-GFAP reaction was indeed absent from EGL cells.

In the light of the above mentioned challenges to the specificity of GFAP, observations using the antibody raised to glutamine synthetase are important. This enzyme has been confirmed as glial specific (Martinez-Hernandez et al, 1977) and the results presented in this thesis show that the pattern of staining in the postnatal rat cerebellum for glutamine synthetase was similar to that for GFAP. In the adult cerebellum, glutamine synthetase was only found in astrocytes, with none present in neurons, synaptic endings, oligodendrocytes, microglia, pericytes or endothelial cells and other mesenchymal vascular elements (Norenberg and Martinez-Hernandez, 1979). In the same publication, these authors describe the ultrastructural localisation of the reaction product as being in a diffuse pattern throughout the cytoplasm, with no reaction product found within the mitochondria, Golgi bodies or nuclei. The results presented here confirm that finding. Since astrocytes show positive staining for GFAP early in the development of the brain (Bignami and Dahl, 1973; Choi and Lapham, 1978) it might be expected that if the EGL was a source of astroglia, some EGL cells might show positive reaction product in their perikarya. However, the results presented in this thesis show that for the two astrocyte-specific antibodies tested, the only positive-staining material in the EGL at any age was the Bergmann fibres. This may be interpreted as further evidence that the EGL is not a source of astrocytes.

The use of anti-galactocerebroside to locate oligodendrocytes within the cerebellum had only a limited success. While some reaction product was found within the white matter and none within the EGL, the results were not clear-cut. This may be related to the fact that galactocerebroside is a surface marker, and while it shows a very strong reaction with

oligodendrocytes in culture (Raff et al, 1978), the reaction with the same cell type in tissue sections is likely to be rather less easy to interpret. For this reason a second oligodendrocyte marker was examined, namely anti-MBP. The results for this stain were much clearer, with the white matter staining intensely, and an absence of stain in the IGL, Purkinje cell layer, molecular layer and EGL. MBP has been demonstrated by immunocytochemical staining to be present in oligodendrocytes prior to myelin sheath formation (Sternberger et al, 1978b). This suggests that if the EGL were producing any oligodendroglia these might well be detectable by anti-MBP. As the EGL showed a completely negative reaction, it may be taken as further evidence that the EGL does not produce oligodendrocytes.

Since the present findings raise questions about the specificity of the anti-D2 preparation used, the lack of neuron-specific staining in the EGL is not entirely surprising. The results of the attempts to localise the enolase isoenzymes were considerably more disappointing. It has been reported that the developmental profile of the two isoenzymes in the rat brain shows a change between birth and 25 days postnatally; the neuron-specific form (NSE) increases over this period, while the non-neuron specific form (NNE) decreases. The acquisition of NSE activity may be correlated with development of functional activity. The decrease in NNE levels suggests that this isoenzyme is present in immature neurons and acquisition of functional activity correlates with a switch to NSE (Marangos et al, 1978). In the rat and the monkey cerebellum the EGL is said to contain

NNE while migrating granule cells, having reached the molecular layer, are said to contain NSE rather than NNE. It has been stated that a switch from NNE to NSE occurs during migration (Schmechel et al, 1978b). However, the published details behind these statements do not indicate whether more than one age was examined, or whether there were any age-related changes in isoenzyme constitution of the EGL. It was hoped that a more complete repetition of this published report might provide data to correlate with the findings of the stereological analysis presented here; namely an in situ increase in maturity of both the EGL and IGL cells with increasing age.

The neuronal specificity of NSE has recently been confirmed in vitro (Hooghe-Peters et al, 1979). However, no further light can be cast on the possible reasons for the failure of the NNE and NSE antisera to stain specifically in the present experiments. Alpha-bungarotoxin (αBuTX) a nicotine antagonist, has been shown to have specific, if limited, binding sites in the granular layer of the adult rat cerebellum (Hunt and Schmidt, 1978). However, a recent study of αBuTX binding sites in the chick cerebellum has shown that neither cells in the EGL or IGL have receptors for this substance (Hirokawa, 1979); this initially promising ligand seems therefore to have no part to play in the study of cell specificity in the EGL.

F. Cell death in the external granular layer

i. Introduction

Degenerate cells in the tissue of healthy developing animals were first noted at the turn of the century. It was not easy for some workers to accept the concept that actively growing tissues were producing cells which were destined to die; so that what were in fact pyknotic cells were initially described as mitotic metabolites (Stoffwechsel Produckte) (Rabl, 1900; Jokl, 1918; Jokl, 1920). By the time the subject of cell death during development was reviewed by Glücksmann (1951), the theory of mitotic metabolites had been totally discredited. Glücksmann discussed 74 different sites in which cell death during development had been observed, and as a result classified cell death according to its developmental function in those cases where a functional significance could be ascribed. The group of so-called morphogenetic degenerations includes the cell deaths related to the changes in form of organs, to ingrowth of tissue, to fusion or separation and to the formation of lumina in solid or partly occluded organs. The histogenetic degenerations include cell deaths related to the differentiation of tissues and organs, and to the formation of matrices and fibres. Phylogenetic degenerations include cell deaths in vestigial organs and those occurring during the regression of larval organs. Glücksmann (1951) was not able to suggest a cause for the localised cell deaths; vascular or nutritional disturbances were ruled out since the dying cells were located in the same environment as the surviving cells. He did not describe a sequence of morphological changes in dying

cells and gave a wide range of estimates for the time taken for a cell to die. These varied from less than one hour to seven hours when only a few cells were involved, and to as much as several days if many cells were involved (for a comprehensive review of the literature describing developmental cell death see Pexieder, 1975).

In a review of cell death in embryonic systems Saunders (1966) emphasised and extended the argument that some cell deaths in development are functional. He introduced the idea that the death of cells and the destruction of tissues, organs and organ systems are programmed as normal events in development. The use of the term "programmed" later provoked critisism since it was considered that it should be reserved for those instances in which a sequence of steps had been clearly demonstrated. Where cells died in the absence of an obvious toxic stimulus the term "physiological cell death" was deemed more appropriate (Lockshin and Beaulaton, 1974).

Cell death caused by noxious stimuli has been extensively studied (Judah et al, 1965, Trump and Ginn, 1969). This type of cell death falls, from the morphological viewpoint, into the category described as coagulative necrosis, (Curran and Codling, 1972) and is probably the result of irreversible disturbances of cellular homeostatic mechanisms (Trump et al, 1973). Detailed accounts of the morphological changes in such dying cells have been given (Trump et al, 1973; LaVia and Hill, 1975).

The morphology of physiological cell death has received less attention, owing in part to the difficulty of devising suitable experimental models.

However, a morphologically distinct form of cell death has been reported as occurring primarily in healthy tissues. While also produced by toxic agents such as zinc (Elmes, 1977) and cancer chemotherapeutic agents (Searle et al, 1974), it has been noted in a variety of undamaged tissues in which there is a high turnover of cells, for example the intestinal mucosa (Potten and Allen, 1977). It is of interest that this type of cell death also occurs in developing tissue, such as the posterior necrotic zone of the chick wing bud (Hurle and Hinchliffe, 1978) and the the rat otocyst (Marowitz et al, 1976). There are several important differences between this variety of cell death and coagulative necrosis, and the term "apoptosis" has been suggested to describe it (Kerr et al, 1972). The main differences between apoptosis and coagulative necrosis are that in apoptosis only scattered, single cells are affected, and they shrink away from their neighbours with concomitant condensation of nucleus and cytoplasm which contains well preserved organelles; there is no accompanying inflammation, and the debris (apoptotic bodies) are digested by epithelial cells as well as by macrophages. In contrast coagulative necrosis usually affects groups of cells which show nuclear margination or progressive loss of chromatin; the cytoplasm swells and the organelles disintegrate, in particular the mitochondria, which balloon and develop flocculent densities. There is usually associated inflammation, and only macrophages have been observed as being responsible for the phagocytosis of necrotic cells (Searle et al, 1974).

Cell death in the developing nervous system of vertebrates falls into the three categories defined by Glücksmann (1951). The death of nerve cells

in the caudal end of the spinal cord is an example of phylogenetic cell death. The cell death that occurs during the invagination of the mammalian eye rudiment (see Silver, 1978) is an example of morphogenetic cell death. Histogenetic cell death in the nervous system has the primary function of matching the number of presynaptic neurons with that of their postsynaptic targets (Cowan, 1973). This is particularly well illustrated in the isthmo-optic nucleus (ION) of the chick, axons from which extend to the contralateral retina. At 13 days of incubation there are about 22,00 neurons in the ION, but over the next 5 days 60 per cent of them die, leaving about 9,500 neurons in the ION at hatching (Cowan and Wenger, 1968). If the target (the eye or the retina) is removed, there is a hundred per cent reduction of ION neurons (Cowan and Wenger, 1968). The ION axons reach the eye by the tenth to eleventh day of incubation, as can be shown by retrograde transport of horseradish peroxidase (HRP) label; nearly all the neurons in the ION were labelled when HRP was injected into the contralateral vitreous (Clarke, 1975). Eye removal after 2 days of incubation increases cell death in the ipsilateral ION to 80 per cent and in monocular embryos both IONs are labelled if the remaining eye is injected on the thirteenth day of incubation (Clarke et al, 1976). This suggests that the additional cell death in the ION on the side of the eye removal is caused by the competition between the ION neurons fro the remaining targets and that the neurons need to make and receive specific connexions in order to survive.

In the ciliary ganglion, synapses are made before cell death occurs (Landmesser and Pilar, 1974b), but it is possible that in this case the dying neurons make insufficient numbers of synapses for survival.

In the cerebellum, cell death has been studied in rats which have been undernourished (Lewis, 1975) or made thyroid deficient in early postnatal life (Lewis et al, 1976; Rabié et al, 1977) and in mutant mice (Sotelo and Changeux, 1974). In undernutrition the numbers of degenerating cells in the EGL were found to be increased above the control level (Lewis, 1975). In the absence of information on the clearance of debris from the layer this result may have signified a delayed removal of the remains of dying cells rather than an increase in the number of cells that die. In the hypothyroid animal the mechanism of cell death may have features in common with that causing the elevated level of nuclear pyknosis in the developing granule cell layer of the staggerer mutant mouse (Lewis, 1979). In the latter the dendritic spines of the Purkinje cells are defective . The result is that that the granule cell axons (the parallel fibres) do not find their synaptic targets, resulting in the death of the granule cells (Sotelo and Changeux, 1974).

In the EGL of normal rats cell death has been observed. Since it occurs prior to synaptogenesis the failure of those cells to reach their appropriate targets cannot account for their death (del Cerro and Snider, 1972a). Numbers of pyknotic nuclei in the EGL of normal rats have been quantified (Lewis, 1975). Though the level was highest at 12 days after birth, regional variations were not investigated, and only four age points were examined.

To study cell death in the EGL further, two separate approaches were adopted. In the first, the distribution and morphology of degenerating cells in the normal EGL was studied by light and electron microscopy . In the second, an experimental system for inducing a brief wave of cell death was used, in order to permit the calculation of rates of cell loss from the EGL. Various agents have been employed to induce cell death in the developing brain. These have included gamma irradiation (Phemister et al, 1969a, 1969b), x-irradiation (Altman et al, 1968a,b, 1969; Altman and Nicholson, 1971; Ebels, 1972; Ebels and Peters, 1974). 5-Fluorodeoxyuridine (Shimada and Langman, 1970; Langman and Shimada, 1971; Langman and Cardell, 1977), N-ethyl-N-nitrosourea (Knowles, 1976; Das and Pfaffenroth, 1977), 5-Bromodeoxyuridine (Yu, 1977) and hydroxyurea (Ebels et al, 1975).

In most cases the agents were selected for the reason that their cytotoxic effect was directed specifically against proliferating cells. Hydroxyurea (HU) in particular has lethal effect only on cells which are committed to DNA synthesis (Philips et al, 1967; Sinclair, 1967). It causes cell death by inducing an immediate inhibition of thymidine incorporation into DNA. Since HU is rapidly cleared (Rajewsky, 1970), it is particularly suitable for causing a short-lived wave of cell deaths in a population of actively dividing cells, such as is found in the EGL. In order to determine whether the cell death in the EGL makes a significant contribution to the cerebellar cell acquisition, the temporal and spatial distribution of normal cell deaths was determined. Using HU to induce a wave of cell deaths, the mean duration of pyknotic cells was calculated. From these data the rates of cell loss by cell death could be compared with the rates of cell

acquisition by proliferation.

ii. Results

a) Cell death in the normal postnatal EGL

In the three regions of the cerebellar EGL in which cell death was quantitated, the pattern of cell death was similar (Table X, Fig. 96). The pooled data from the three regions studied show that in the first 8 postnatal days the pyknotic index remained at a fairly constant level, between 0.56 per cent and 0.8 per cent. By postnatal day 12, however, there was a marked rise in the pyknotic index which doubled to 1.35 per cent. Within 4 days it had returned to the base level, where it remained until the EGL had all but disappeared at postnatal day 20. The pyknotic index for the three groups of lobules counted showed a similar overall pattern, but several statistically significant differences were observed (Table X, Fig. 96).

At P0 the incidence of cell death in lobules I and II was significantly lower than in either lobule VI or lobules IX and X. At P4 and P8, lobules IX and X had a significantly lower level of cell death. When the pyknotic indices reached their maximum level at P12, all three regions were significantly different from each other; the greatest number of cell deaths were found in lobule VI, the smallest number in lobules IX and X. By P20 the pyknotic index in lobules IX and X had risen to be statistically indistinguishable from that of lobule VI, and the lowest level of cell death was found in lobules I and II.

Table X Pyknotic indices (P.I.) in lobules I and II; VI; IX and X of the rat cerebellum from birth to postnatal day 20

Day	P.I. in lobules: I + II	VI	IX + X	Mean P.I.
0	1.05 ± 0.086†	(NS)	0.89 ± 0.087	0.803 ± 0.061
	***		***	
	0.047 ± 0.042			(NS)
4	0.66 ± 0.076	**	0.37 ± 0.035	0.617 ± 0.050
	(NS)		***	
	0.82 ± 0.077			(NS)
8	0.64 ± 0.083	**	0.37 ± 0.050	0.557 ± 0.046
	(NS)		***	
	0.66 ± 0.080			***
12	1.35 ± 0.092	**	0.98 ± 0.083	1.359 ± 0.075
	***		***	
	1.72 ± 0.073			***
16	0.58 ± 0.064	(NS)	0.62 ± 0.075	0.677 ± 0.042
	**		*	
	0.83 ± 0.063			(NS)
20	0.53 ± 0.067	(NS)	0.81 ± 0.034	0.683 ± 0.043
	*		**	
	0.71 ± 0.085			

† mean of 10 data values ± standard error

* $p < .05$ probability values calculated from student's test

** $p < .01$ for independent t

*** $p < .001$

(NS) not statistically significant ($p > .05$)

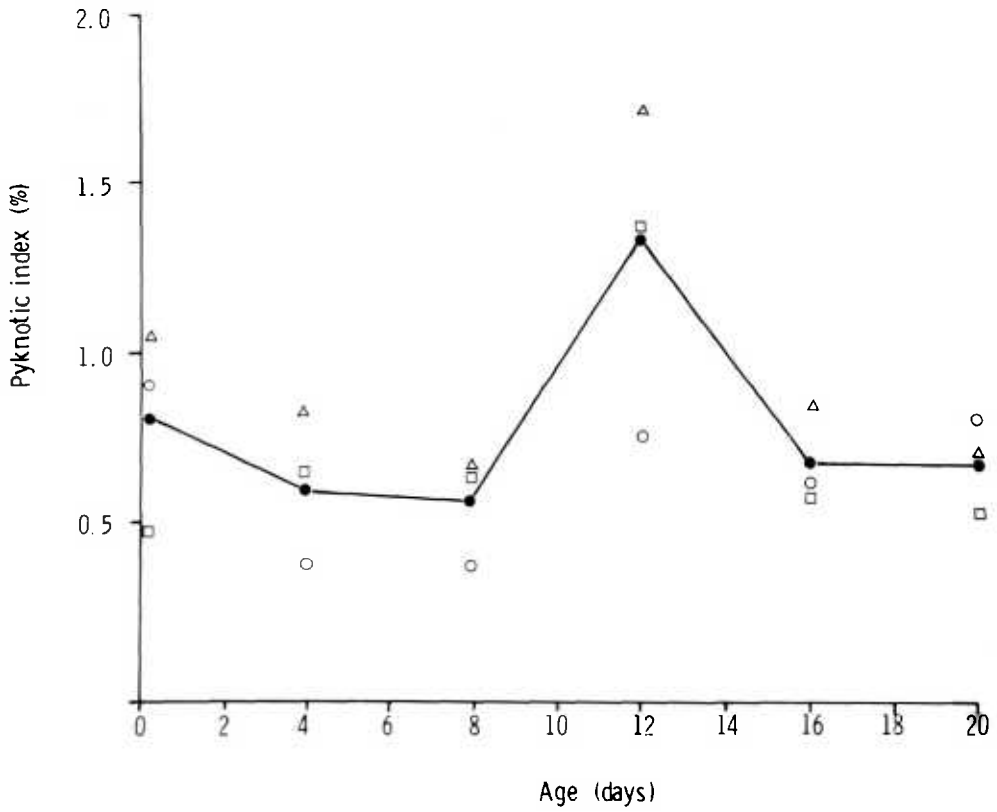


Fig 96 Pyknotic indices in the normal external granular layer.

The figure shows the mean pyknotic index for each region at each age point and the mean pyknotic index of all three regions at each age point.

□ lobules I and I; △ lobule VI; ○ lobules IX and X; ●——● mean of lobules I and II, VI and IX and X

b. Ultrastructure of cell death in the normal postnatal EGL

The occurrence of cell death in the material studied was not frequent enough to allow a systematic analysis of the morphology of dying cells in the absence of specially developed techniques enabling thin sections to be cut from 1 μm sections previously examined by light microscopy (e.g. Kaplan and Hinds, 1977). However, sufficient numbers of dying cells were seen to permit description of the possible sequence of events taking place in normal cell death in the rat EGL.

The first detectable sign of incipient cell death was an increase in the density of the chromatin at the periphery of the nucleus (Fig. 97). At this stage the affected cell appeared normal in all other respects. In particular its organelles appeared unchanged and its contacts with neighbouring cells did not seem disturbed. Following the progressive condensation of the chromatin, cytoplasmic condensation was seen (Fig. 98). The organelles, though more difficult to detect in the dark cytoplasm, still appeared normal, as did the relationship of the dying cell to its neighbours. In the final stages the cell appeared to collapse inwards, detaching itself from the cells surrounding it. The chromatin was highly condensed and strongly osmiophilic, and the nucleus by this time was often fragmented (Fig. 99). The cytoplasm was sometimes darker than that of the unaffected cells, and usually less granular in appearance, probably as a result of the aggregation of ribosomes. Usually the organelles seemed more dense; sometimes vacuoles were seen, suggestive of ruptured mitochondria (Fig. 99). Ultimately the full picture of a dying cell was seen, very dense and detached from its neighbours, though still with some evidence of organelles in the compacted cytoplasm (Fig. 100).

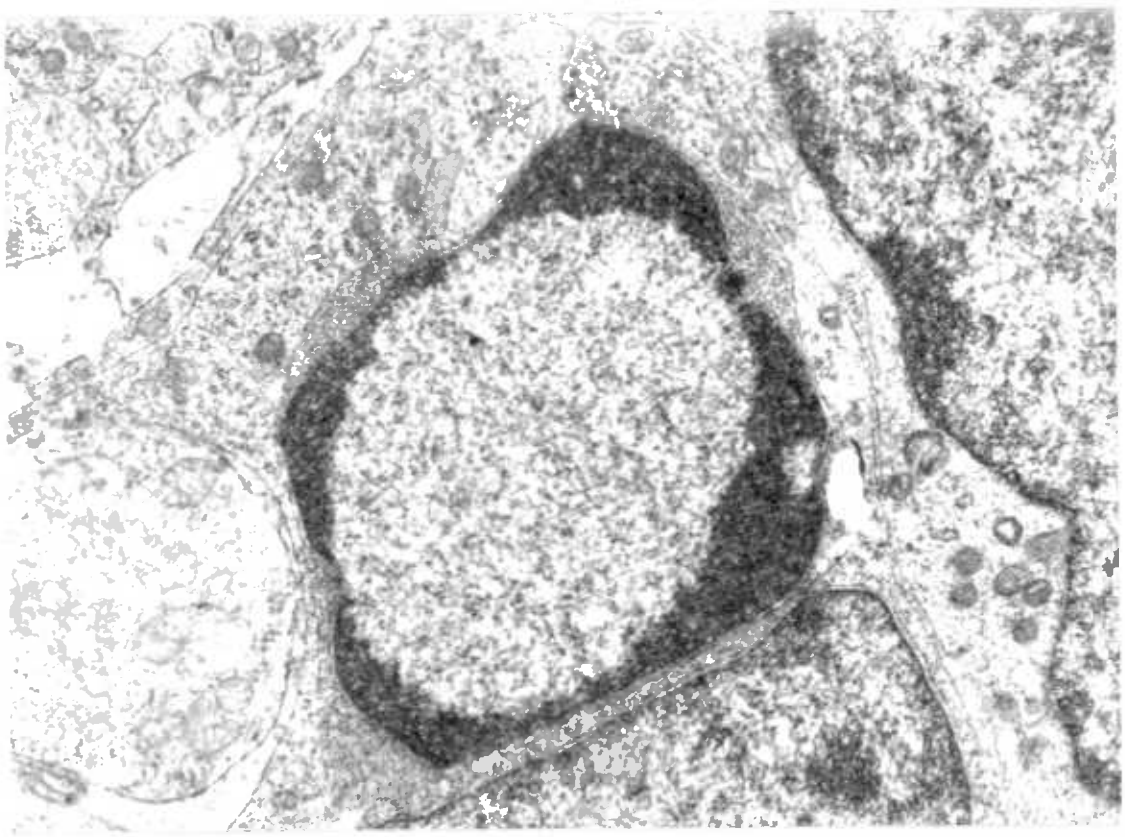


Fig. 97 Ultrastructure of cell death in the normal external granular layer.

First sign of change : there is increase in the density of chromatin around the edge of the nucleus - 2 day old rat.

Uranyl acetate/lead citrate stained sagittal section (x 20,000)

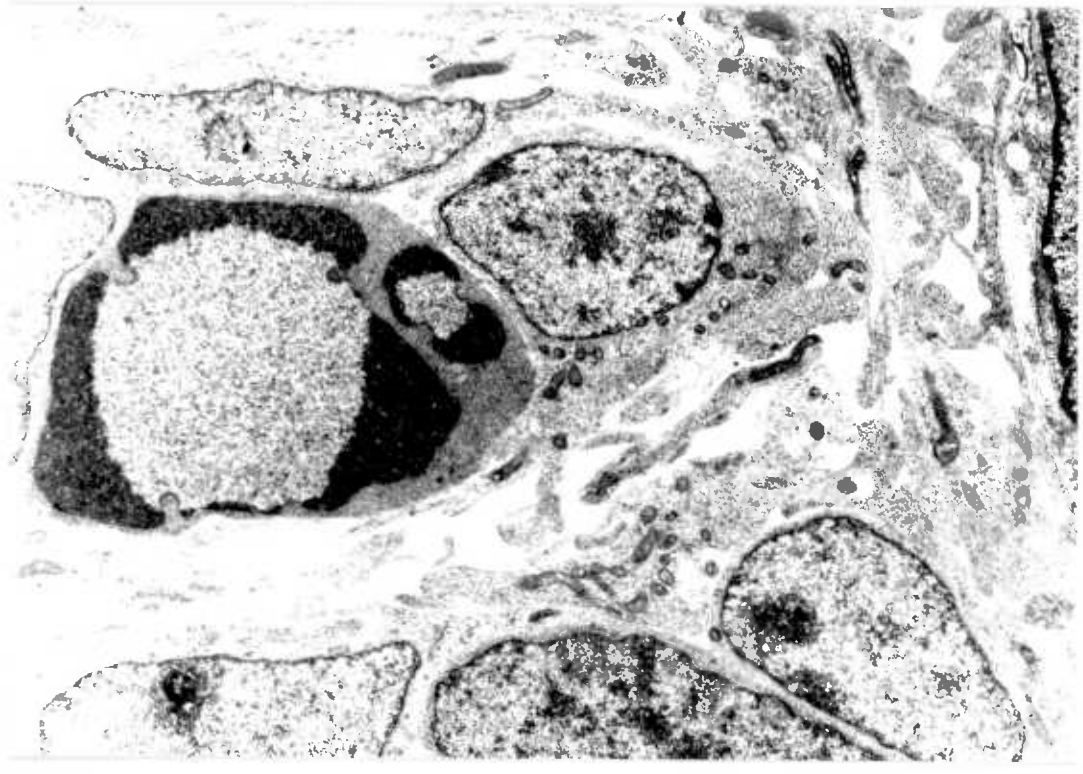


Fig. 98 Ultrastructure of cell death in the normal external granular layer. At this stage the cytoplasm has become more dense but the cell remains in contact with its neighbours - 12 day old rat.

Uranyl acetate/lead citrate stained sagittal section (x 8,400)

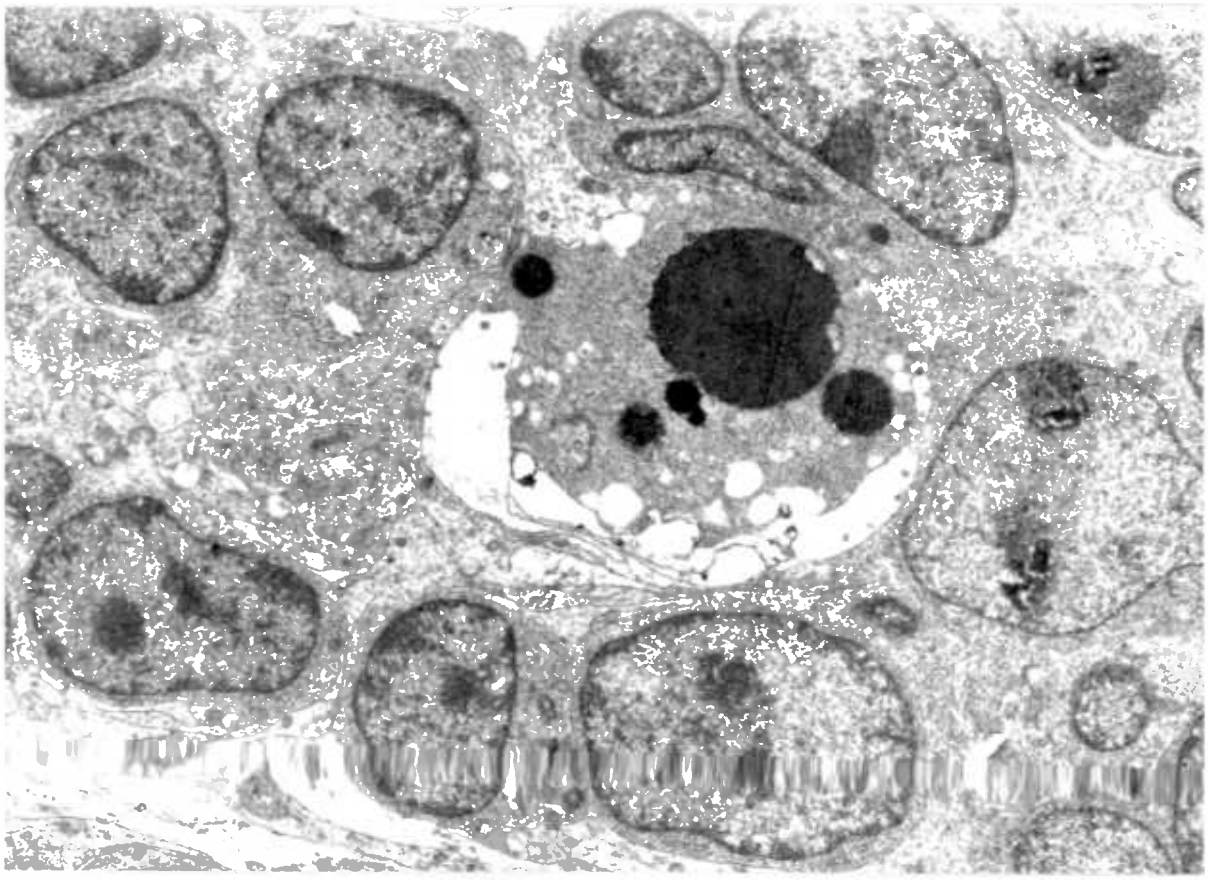


Fig. 99 Ultrastructure of cell death in the normal external granular layer.

The dying cell has a dense fragmenting nucleus, and dense vacuolated cytoplasm. It still remains partially in contact with adjacent cells - 12 day old rat.

Uranyl acetate/lead citrate stained sagittal section (x 8,000)

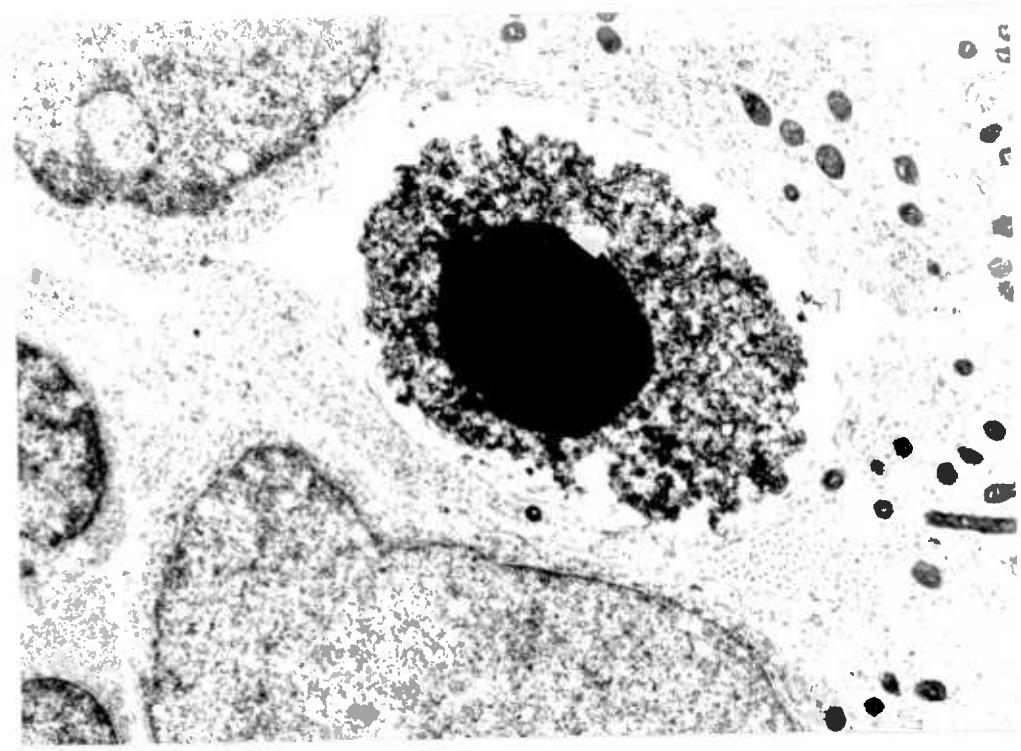


Fig 100 Ultrastructure of cell death in the normal external granular layer.

Final stages before phagocytosis. This cell still appears to possess intact endoplasmic reticulum (arrow) - 12 day old rat.

Uranyl acetate/lead citrate stained sagittal section (x 12,500)

The ultrastructural appearance of the dying cells, and the suggested sequence of events closely follows that described as apoptosis (Kerr et al, 1972; Wyllie, personal communication). Major points of similarity are the shrinkage of the affected cell away from its unaffected neighbours, the usual lack of vacuolation and the persistence of normal morphology of the organelles. A further feature in common with apoptosis is the lack of inflammatory response, the remains of the dead cell possibly being engulfed by one of the cells adjacent to it (see Fig. 11). The final similarity with apoptosis is the fact that dying cells were only found singly, never in groups.

c. Cell death induced in the postnatal EGL by hydroxyurea (HU)

The injection of 0.5 mg/g HU (low dose) or 2.0 mg/g (high dose) caused a wave of cell death in the EGL (Fig. 101, Table 11). The pyknotic index increased in a linear fashion to a peak, and then decreased exponentially. The peak was reached at 12 hours with high dose treatment, two hours earlier than in the low-dose animals. The higher dose of HU also slightly increased the peak pyknotic index, though the difference was not statistically significant ($p > 0.1$). The exponential decrease in pyknotic indices in the EGL represented the rate at which the dead cells were cleared from the EGL. Logarithmic plots of the clearance rates (Fig. 102) show that the low-dose treated animals eliminated dead cells at almost the same rate as the high-dose treated animals, while the pyknotic index in the EGL of the low-dose treated group returned to normal only a few hours before the high-dose treated group. From the clearance rate plots the half-time of

Table XI Cell death in the EGL after HU injection

Time after injection (hours)	Pyknotic Indices ± SEM 0.5 mg/g *	log	2.0 mg/g *	log
4	0.2 ± 0.23		0.6 ± 0.02	
6	7.2 ± 0.72	r=0.99	3.0 ± 0.56	
8	r = 0.980 13.8 ± 0.96		14.3 ± 0.66	
12	19.1 ± 1.31		33.1 ± 1.90	1.5198
14	29.5 ± 1.04	1.4698	30.9 ± 2.44	1.4899
16	17.2 ± 0.75	1.2355	30.3 ± 1.56	1.4814
18	17.6 ± 1.36	1.2455	16.9 ± 2.66	1.2279
20	9.9 ± 0.87	0.9956	15.1 ± 1.67	1.1790
24	4.1 ± 0.85	0.6128	11.4 ± 2.68	1.0569
28	1.4 ± 0.22	0.1461	--	--
36	0	0	0	0
coefficient of linear regression (r)		0.969		0.978
slope		-0.072		-0.064
intercept		2.42		2.42
half time		4.25 hr		4.75 hr
mean duration		6.12 hr		6.84 hr

* Each figure represents the mean of the percentage of pyknotic nuclei per 1000 cells for 10 separate counts made in vermal lobule VI

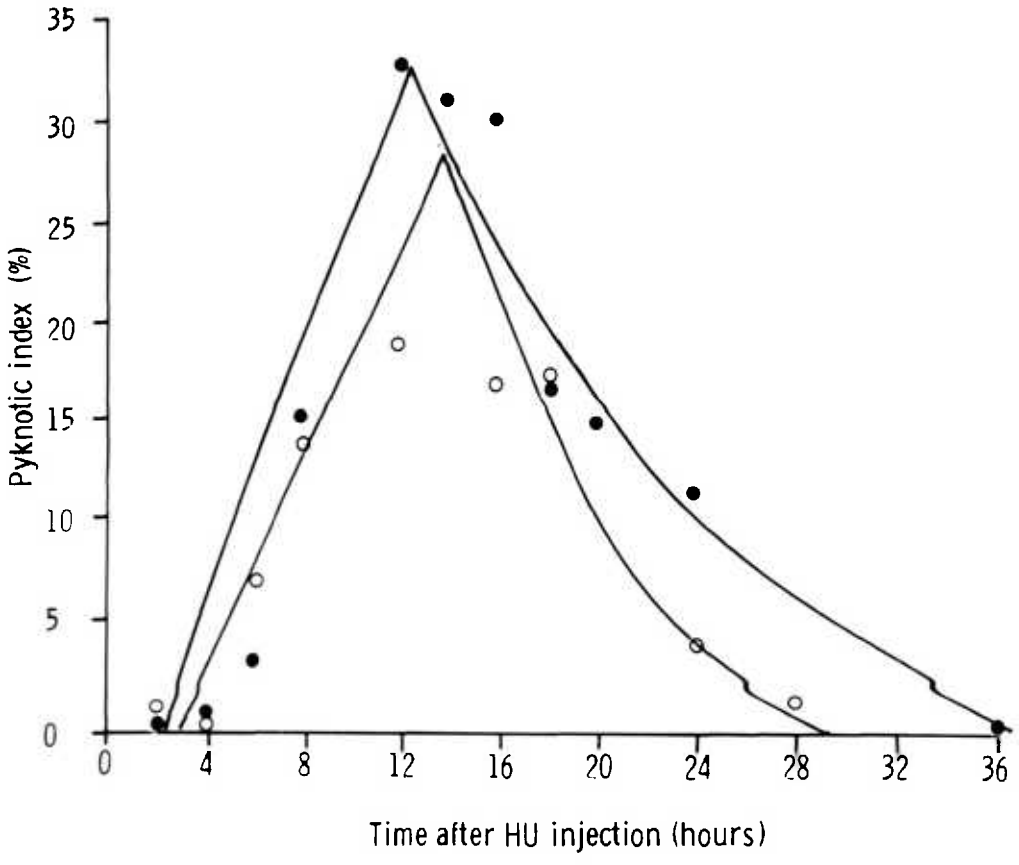


Fig. 101 Pyknotic indices in the external granular layer of the 9 day old rat following the injection of hydroxyurea.

The figure shows the mean pyknotic index for each time point. • 2 mg/g hydroxyurea ; o 0·5mg/g hydroxyurea.

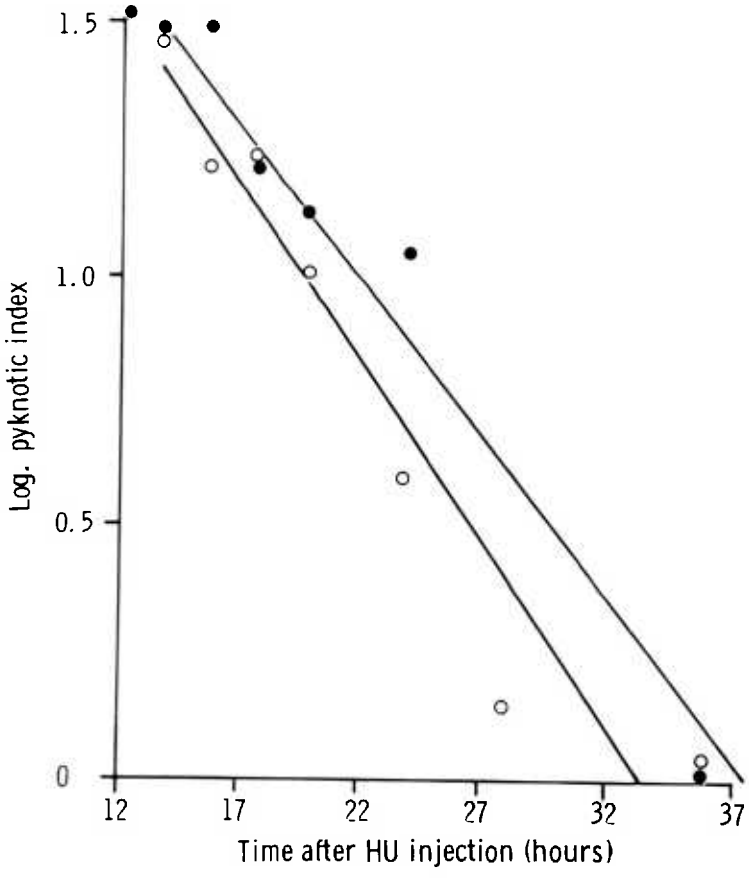


Fig. 102 Rates of clearance of degenerating cells from the external granular layer of the 9 day old rat following the injection of hydroxyurea

● 2 mg/g hydroxyurea; o 0.5 mg/g hydroxyurea

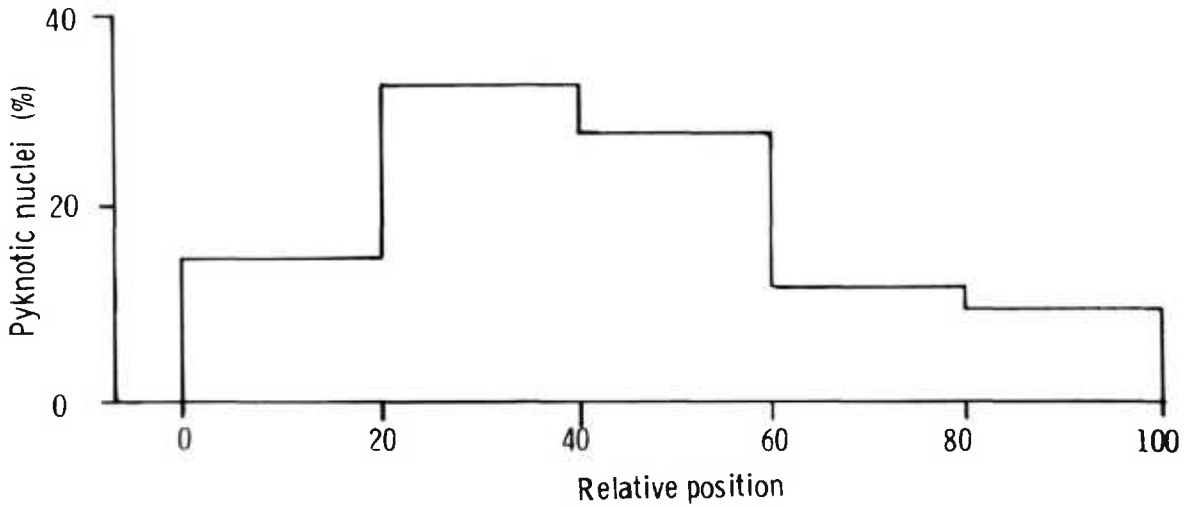


Fig. 103 Disturbance of pyknotic cells in the external granular layer of the 9 day old rat following the injection of hydroxyurea.

The figure shows the percentage of total mitoses counted at each position within the EGL. On the horizontal axis 0 - 20 represents the first fifth of the EGL below the pial surface, 80 - 100 the final fifth of the EGL above the molecular layer.

a pyknosis could be calculated, and thence the mean duration of a pyknosis (mean duration = half-time x 1.44; Johns, 1964). Thus the mean durations of a pyknosis was 6.12 hours and 6.84 hours for the low and high doses respectively, giving an average time of about 6.5 hours.

The wave of cell death induced by the high dose of HU was first detectable at 6 hours (Fig. 101). At this time the distribution of pyknoses within the EGL, and their ultrastructure was examined. As has been noted, HU kills cells that enter mitosis so it was not surprising to find that the distribution of dying cells within the EGL (Fig. 103) was very similar to the distribution of mitoses and labelled nuclei. The ultrastructural appearance of the affected cells was very similar to that of dying cells in the untreated EGL (Figs. 97 - 100). The typical appearance of the EGL 6 hours after the injection of 2 mg/g HU is shown in Figure 104. It can be seen that the damaged cells are separated from each other by what appear to be normal healthy cells, that is, there are no foci of necrosis. More detailed examination of the degenerating cells revealed all the features of apoptosis. These included condensed nuclei and dense cytoplasm containing organelles which, though compacted, were otherwise well preserved (Fig. 105). There never appeared to be an inflammatory response. There were never any cells in the EGL that could be identified as invading macrophages or microglia. The degenerating cells appeared to be engulfed by neighbouring EGL cells (Figs. 104, 107). In the case of cells dying at the pial surface, the phagosomes might have been Bergmann glia, specifically the end-feet of these cells (Fig. 103).

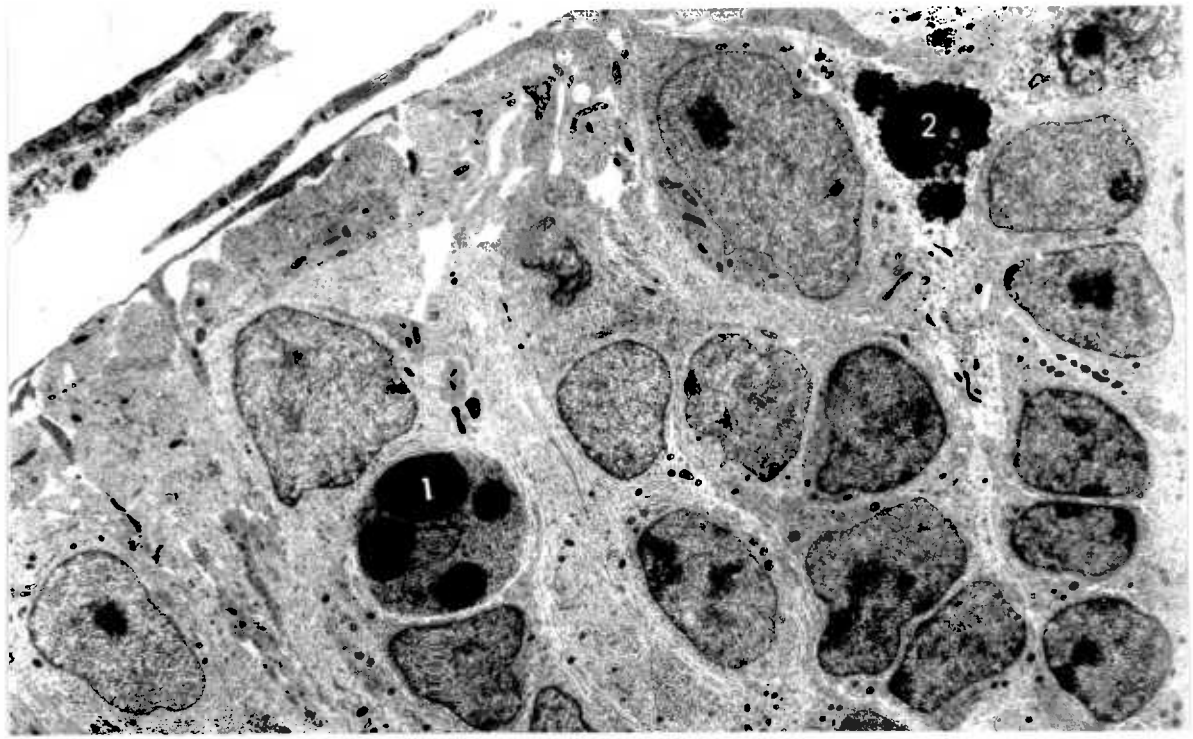


Fig. 104 Ultrastructure of cell death in the external granular layer of the 9 day old rat 6 hours after the injection of 2.0 mg/g hydroxyurea.

Two dying cells, 1 and 2, can be seen. Cell 2 is at a considerably more advanced stage of degeneration than cell 1. Both cells appear to be condensing; there is no evidence of cytoplasmic or organelle swelling.

Uranyl acetate/lead citrate stained sagittal section (x 4,800)

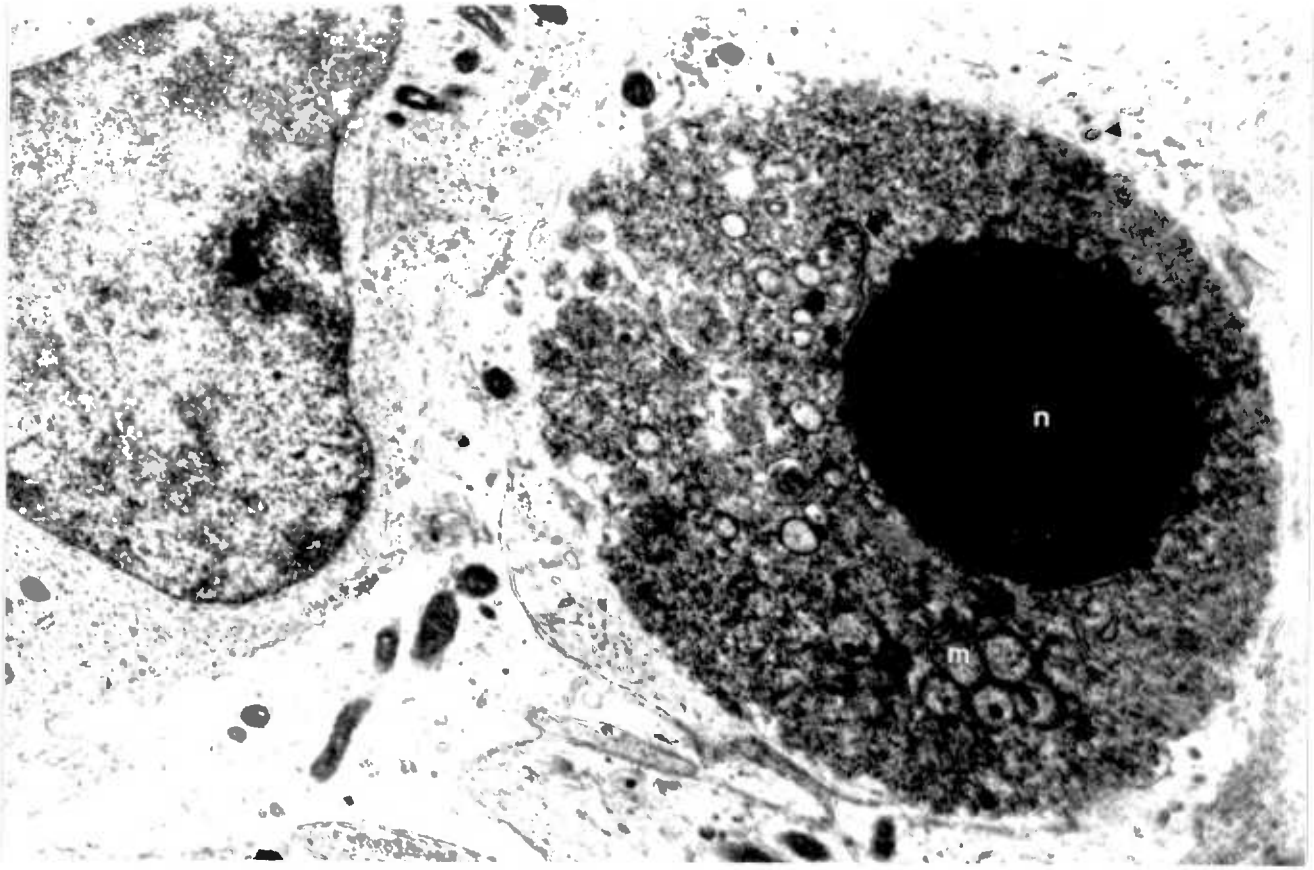


Fig. 105 Ultrastructure of cell death in the external granular layer of the 9 day old rat 6 hours after the injection of 2.0 mg/g hydroxyurea

(for legend please see overleaf)

Legend to Fig. 105

The apoptotic body has a condensed nucleus (n) with a "wandering nuclear" membrane (arrowed). The mitochondria appear normal, cristae are still detectable (m). A primary lysosome seems to be in the process of being released into the (intraphagosomal) apoptotic body (arrowhead). The adjacent EGL cell appears to be normal.

Uranyl acetate/lead citrate stained sagittal section (x 18,900)

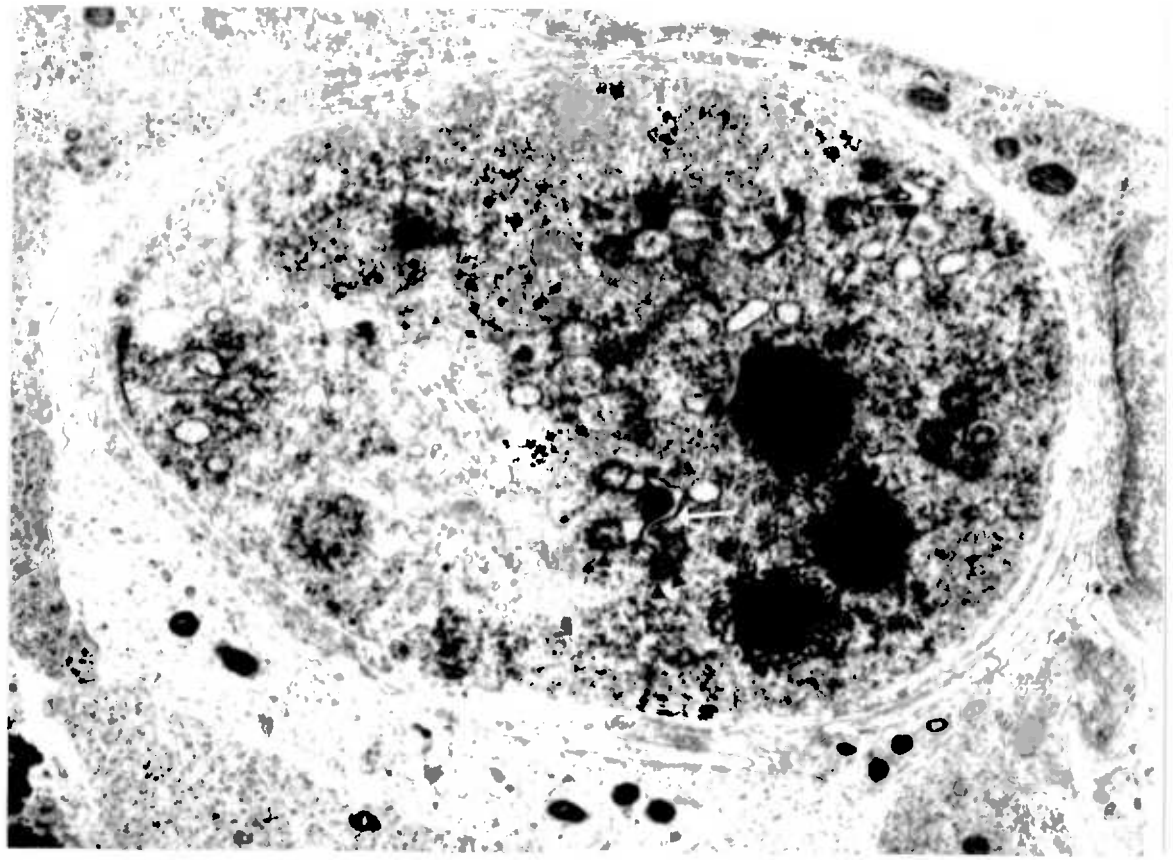


Fig. 106 Ultrastructure of cell death in the external granular layer of the 9 day old rat 6 hours after the injection of 2.0 mg/g hydroxyurea. (for legend please see overleaf)

Legend to Fig. 106

This is a typical example of an apoptopic body. The nucleus is very condensed and has fragmented into three pieces. There is possible evidence of nuclear membrane remnants in the cytoplasm (arrow); the presence of apparent pore structures cut en face (arrowheads) within the dark chromatin mass is suggestive.

Uranyl acetate/lead citrate stained sagittal section (x 16,000)

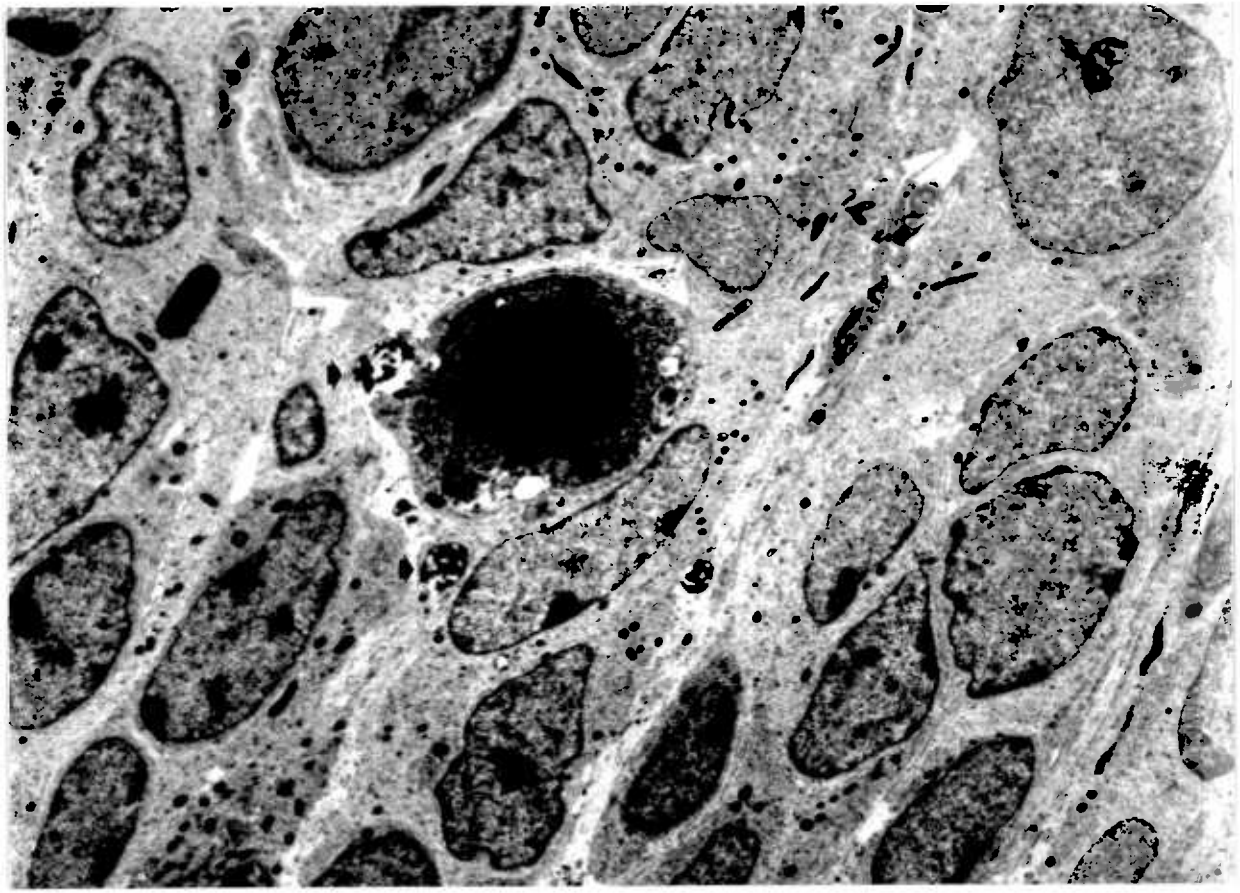


Fig. 107 Ultrastructure of cell death in the external granular layer of the 9 day old rat 6 hours after the injection of 2 mg/g hydroxyurea.

(for legend please turn over)

Legend to Fig. 107

Although this apoptotic body appears to be extracellular, the close proximity of lysosomal residual bodies (arrowed), suggests that it is at least partially surrounded by a phagocytic cell. It is possible that it is intraphagosomal itself.

Uranyl acetate/lead citrate stained sagittal section (x 5,600)

iii. Comments

Nuclear pyknosis and cell degeneration was found in all regions of the vermal EGL at all ages studied. The animals were perfusion fixed with a mixture of formaldehyde and glutaraldehyde (see materials and methods), and imperfectly perfused animals were discarded. It follows that the consistent detection of degenerating nuclei must represent a naturally occurring level of cell death in the EGL, rather than an artefact.

The level of cell death was generally higher in lobules I and I than in lobules IX and X, in keeping with the fact that the neocortex continues its development longer than the earlier-formed regions. However, lobule VI possessed the highest pyknotic index of the three regions studied. Mareš and Lodin (1970) showed that differential rates of cell proliferation might be responsible, in part, for the process of foliation in the rat cerebellum. If cell acquisition can be considered as a factor in the production of folia, cell deletion must also be considered. Lobule VI is one of the most convoluted of the rat cerebellar folia, so the relatively high level of cell death might be a factor in causing the invaginations in its cortex.

The pyknotic nuclei in the EGL may be derived from post-mitotic cells (Lewis, 1975) and thus the percentage of newly-formed cells which die can be calculated, provided the mean duration of a pyknotic cell is known. From the experiments in which HU was used to cause a wave of cell death, the mean duration for a pyknosis was found to be 6.5 hours. This is of the same order of magnitude as some of the times calculated by other

workers. In the larval toad, ventral horn motor neurons were estimated to degenerate within 3.2 hours (Hughes, 1961) and for similar cells within the foetal mouse a time of 1.4 hours was estimated (Harris-Flanagan, 1969). However, the relevance of these two figures to this study is uncertain. Both values were obtained indirectly, by calculating the time required to account for the disappearance of a known number of cells in a known time. Furthermore, Hughes (1961) only took into account a part of the degenerative process. A possibly more accurate figure is the calculated half-time of 9 hours for pyknotic nuclei in the x-irradiated rat EGL (Altman and Nicholson, 1971) from which a mean duration of 13 hours can be obtained. A mean duration of pyknoses of 7 to 9 hours can be obtained from data on the untreated or x-irradiated tadpole eye and the tadpole brain (Spear and Glücksmann, 1938; Glücksmann, 1940). Deo et al (1979) calculated a mean duration of 10 hours in x-irradiated rat cerebellum; Lewis (1975) assumes a mean duration of 10 hours and derives the formula - proportion of newly acquired cells which are lost by cell death = pyknotic index/mean duration x 2 x mitotic index.

A mean mitotic index of 1.4 was calculated from data presented in Mareš et al (1970), Lewis (1975), Lewis et al (1976) (maximum 2.3, minimum 0.9). In this study, since a mean duration for a pyknosis of 6.5 hours was calculated, the percentage of newly acquired cells lost by cell death was pyknotic index/18.1 x 100. Thus, the maximum loss of newly acquired cells from lobule VI was 9.5 per cent at 12 days after birth. At the same age lobules I and II were losing 7.4 per cent of their newly acquired cells.

At other ages the mean loss was less than 3.7 per cent. In order to achieve a steady state, the loss of newly formed cells would have to reach the 50 per cent level. In the case of proliferation influencing foliation the difference in proliferation rates of the EGL betwen the fissures and the convexities of the gyri was found to be 38 per cent in 2 day old mice and 13 per cent in 7 day old mice (Mareš and Lodin, 1970). Even the peak cell loss in lobule VI does not approach this level. It therefore seems unlikely that cell death is a major factor in controlling cell acquisition or foliation in the EGL. There is certainly no parallel here with the view held by Smart (1961) that in the subependymal layer cell formation is balanced by cell death, nor is there any support for the speculations of Hughes (1961) who calculated a loss of 8 or 9 cells for every neuron formed. The present findings are supported by a study using a [^{14}C] tracer technique to quantitate cell death in the developing rat cerebellum; this gave the result that cell death was not a significant factor; indeed cell death was at too low a level to be measurable in the first three postnatal weeks (Griffin et al, 1979).

The morphological events following the onset of cell death in the central nervous system remain to be clearly established. Systems in which cell death can be indeed experimentally have the advantage that the first stages of cell death can be detected, and a sequence of morphological changes following the insult can be described. Following the irradiation of 9 day old rat brains with 100 Rads of x-irradiation Altman and Nicholson (1971) distingushed four stages of imminent cell death. However, since changes in metabolic processes can be detected before changes in morphology (Pollak and Fallon, 1974) what Altman and Nicholson (1971) described

was probably actual (rather than imminent) cell death. The ultrastructural appearance of dying neurons in the spinal cord has been described in detail. The morphology of dying presumptive visceromotor neurons at the cervical level was found to be the same whether naturally occurring or induced by peripheral deprivation (O'Connor and Wyttenbach, 1974). This was not found to be so in the chick ciliary ganglion. Here the ultrastructure of normal cell death (designated type II cell death) was found to be different from peripheral deprivation-induced cell death Type I) (Pilar and Landmesser, 1976). Amongst the lateral motorneurons of the lumbar spinal cord of the chick embryo two morphologically distinct types of cell death were described, and termed type I and type II. Type I and II degenerations were also found when cell death was induced by peripheral deprivation, which increased the speed and number of cells undergoing degeneration (Chu-Wang and Oppenheim, 1978). In the trochlear nucleus of the duck embryo both induced and normal cell death showed the same sequence of degeneration, and this resembled the type II degeneration of Chu-Wang and Oppenheim (1978), (Sohal and Weidman, (1978)). The somewhat contradictory nature of these findings cannot be easily explained; they do not represent species-specific differences because the chick was the experimental animal in all the studies except that of Sohal and Weidman (1978). In no instance did the descriptions of cell death exactly match the description of apoptosis, especially since dilatation and/or vacuolation of mitochondria and rough endoplasmic reticulum was a common feature. The type II degenerations of Pilar and Landmesser (1976) and Chu-Wang

and Oppenheim (1978) and the sequence described by Sohal and Weidman (1978) were similar to apoptosis. In particular, all involved a compacting of nucleus and cytoplasm. Although "classical" apoptosis has not been linked with cell death in these studies of the normal central nervous system, its occurrence in the normal rat EGL seems to be demonstrated in the present study. The purpose and mechanisms of cell death in the EGL can as yet only be speculated on. This study has shown that it did not make a major contribution to either cell acquisition or foliation. This does not exclude the possibility that the cell death in the EGL plays some part in these processes. It is possible that the cells that die are those that fail to make contact with their targets as in other parts of the nervous system in which cell death has been described, notably the isthmo-optic nucleus of the chick (Clarke et al, 1976). However, the EGL of the Staggerer mutant mouse has not been described as showing a high level of cell death; rather it is the post-migratory granule cells that die when they fail to make contact with their targets, the Purkinje cell spines, which are defective (Sotelo and Changeux, 1974). Another explanation for the cell death in the EGL is that cells which are unable to make contact with the Bergmann glial fibres die. In the Weaver mutant mouse, there is defective migration from an elevated level of cell death in the EGL. This is secondary to the presence of abnormal Bergmann glia (Rakic and Sidman, 1973). It may be that the proliferating cells have a limited lifespan and those that fail to exit and establish synaptic contacts before this is reached die. However, this opinion does not account for the frequent observation of ectopic granule

cells (see page 27) which appear to be indistinguishable from the microneurons of the IGL in all but location. A further possibility is that the cells that die in the EGL represent the occurrence of mitotic death. In cultures of Ehrlich ascites tumour cells a small percentage of the cells that die have been thought to be mitotic, and it was suggested that mitotic death represents a means of eliminating cells with unstable chromosome constitutions (Lala, 1972).

The signal that initiates cell death in the EGL is also a matter of speculation. Lockshin and Beaulaton (1974) reviewed the hypothesis that during development cells were capable of effecting their own destruction by releasing autolytic enzymes from lysosomes. However, in the case of degenerating neuroblasts in chick embryo ganglia, the involvement of lysosomes was rejected (Pannese et al, 1976). Further, Pollak and Fallon (1974) in a study of cell death in the chick limb bud reported that a decrease in protein synthesis preceded any cytological evidence of ensuing necrosis; this would include the synthesis of lytic enzymes.

In summary, in the EGL of the rat there is a small amount of cell death, at too low a level to be considered as a rate limiting factor in cell acquisition. The regional differences suggest that the cell death may be a morphogenetic event, but this must remain a matter of speculation. The morphology of the cell death is similar to the type II degenerations found in the spinal cord (Pilar and Landmesser, 1976; Chu-Wang and Oppenheim, 1978) and bears a striking resemblance to the type of cell

death described as apoptosis (Kerr et al, 1972).

10. DISCUSSION

In this present work, the EGL of the postnatal rat cerebellum has been examined by a range of techniques in order to cast new light on some of the unresolved questions regarding the nature of its consituent cells.

The widely held view (see Table I) that the EGL may be a source of glial cells as well as neurons dates from Schaper's (1894) theory that the cells comprising this layer were bipotential. It cannot be denied that at some early stage in ontogenesis there are populations of neural cells which ultimately give rise to both neurons and glia. However, the postnatal proliferation of the EGL is an exceptionally late event in the development of the nervous system. Furthermore, the cells populating this layer have already undergone a migration which is an event that in the rest of the central nervous system is followed by differentiation (Sidman and Rakic, 1973).

The evidence on which the concept of EGL bipotentiality is based has been derived from light microscopic examination (Schaper, 1894) and autoradiography (Uzman, 1960a, b; Fujita, et al, 1966; Fujita, 1967; Meller and Glees, 1969). Support for this view has also been derived from the study of medulloblastoma (Jones, 1932; Globus and Kuhlenbeck, 1944). Light microscopic techniques have inherent weaknesses which can lead to misinterpretation. Examination of paraffin-wax sections in particular can easily yield ambiguous results. Detection of glial cells within or leaving the EGL could be the result of the imprecision of identification from nuclear morphology. This point has been made by Swarz and del Cerro

(1977), who pointed out that endothelial cells, pericytes and ectopic granule cells could easily be confused with astroglia and oligodendroglia. They also suggested that microglia might be confused with oligodendroglia.

The present study was also unable to define any cells morphologically identifiable as astrocytes or oligodendrocytes within the EGL. The examination of the molecular layer of the mouse cerebellum 25 days postnatally showed a complete abscence of astrocytes and oligodendrocytes (Swarz and del Cerro, 1977). Thus, on the evidence of more sensitive morphological examination (1 μm resin sections for light microscopy and electron microscopy) the EGL cannot be considered a source of glial cells.

With the introduction of autoradiography as a tool for tracking migrating cells it was hoped to improve on the classic histological methods for identifiying cells produced by the EGL. However, autoradiographic studies were prone to the same type of error when identifying cell types as standard, unlabelled paraffin wax sections. That is, endothelial cells, pericytes and ectopic granule cells all have dark nuclei that may be confused with oligodendroglia in the molecular layer (Swarz and del Cerro, 1977), and all may be labelled. This may account for the reported production of oligodendroglia by the EGL (Privat, 1973). Furthermore, autoradiographic examination as a technique for detecting the source of a particular cell is complicated by the fact that there are other germinative zones in the postnatal rat cerebellum, e.g. the residual subependymal layer which, if homologous with the forebrain subependymal layer, generates astrocytes, microglia and possibly oligodendrocytes

(Lewis, 1968; Lewis and Lai, 1974), and the IGL which is a source of proliferating astrocytes (Lewis et al, 1977; Moskovkin et al, 1978). Thus, even if the identification of labelled cells as glia is correct, the identification of their source may be in doubt. Astrocytes are generated in the IGL and presumably by the subependymal layer, which also produces oligodendrocytes; to assume that a labelled glial cell must have its origin in the EGL ignores these other possibilities.

In this study various immunocytochemical markers were used to examine the EGL for the possible production of glia, but the results were entirely negative. Antisera to astrocyte-specific proteins were found in processes spanning the EGL. However, electron microscopy confirmed that these were exclusively Bergmann glial processes. It has been speculated that the EGL is the source of the Bergmann glia (Ishida, 1973; Das, 1976) which would account for the presence of their processes and endfeet in the layer. The data are questionable however, and a more probable source of the Bergmann glia is the IGL (Basco et al, 1977). The positive staining of the Bergmann glial fibres and endfeet, together with the positive staining and morphological identification of astrocytes in the IGL confirmed the specificity of the two astrocytic markers used. The EGL was equally devoid of material staining positively for oligodendrocytic markers, whereas in the older animals, cells in the white matter stained intensely. It may be argued that the EGL cells are not sufficiently mature to exhibit specific glial characteristics, and that these only become demonstrable when they have reached their final destinations. However, immunocytochemical techniques have been used successfully in animals at much earlier stages.

Astrocytes have been detected in human foetal cerebrum at 10 - 18 weeks of gestation (Antanitus et al, 1976) and in the human foetal cerebellum as early as 7 - 10 weeks of gestation (Choi and Lapham, 1978). There is no direct evidence suggesting that myelin basic protein may be synthesised by immature oligodendrocytes. However, in the anterior commissure of the rat, oligodendrocytes have been detected with this marker, prior to myelin sheath formation from the fifth postnatal day (Stemberger et al, 1978b). Thus, the results of the immunocytochemical studies contribute further to the proposal of Athias (1897) and Cajal (1911) that the EGL does not generate neuroglia.

If the EGL cells is not bipotential than the concept of the medulloblast (Bailey and Cushing, 1925, 1926; Kershmann, 1938) must be reappraised. It has been asserted that the astrocytic elements sometimes found in medulloblastomas are neoplastic rather than invasive (Rubinstein et al, 1974). Though this must remain a matter for speculation an answer would not explain the presence of the oligodendroglial elements which are commonly found in these tumours. If the generation of medulloblastomas cannot be explained by an uncontrolled proliferation of a cluster of bipotential EGL cells an alternative hypothesis is necessary. The most straightforward explanation is that the parent cells of the tumour are dedifferentiated cells, whether EGL cells, ectopic granule cells or glial cells. That is, the tumour development is preceded by a focal dedifferentiation to primitive neuroepithelial cells.

The present investigations conclude that the EGL is not composed of bipotential cells, but rather of a uniform population of dividing neural

cells. Since Cajal (1911) they have been conceived of as being systematically arranged into two zones representing one subpopulation of proliferating primitive cells in the upper zone and another of postmitotic cells in the lower zone (Altman, 1972a). The present study does not lend support such a clear cut organisation of the layer.

The ultrastructural studies presented here confirm to a great extent previous observations of the morphology of the EGL (Mugnaini and Forströnen, 1967, del Cerro and Snider, 1972a). However, the detection of the processes extended by cells at all levels of the EGL, seen in scanning electron micrographs only with coronal fractures, is a significant deviation from the schema presented by Altman (1972a) derived from his work and that of Cajal (1911). In this, the process of the extension of a single process from each pole is strictly related to the depth of the cell within the EGL. This does not always appear to be the case. The pattern of proliferation, measured as distribution of mitoses or labelled nuclei, emphasises the homogeneous nature of the EGL. Rather than being divided into two separate zones with the inner zone (seen as consisting of elongated cells in coronal section) being postmitotic (Mareš et al, 1970, Altman, 1972a) there is a gradient of proliferative activity across the whole layer. Investigation of the vascular supply revealed that the intrinsic vasculature in the outer regions of the cerebellum remained poorly supplied throughout the existence of the EGL. Only after its disappearance was there angiogenesis in the region which it had once occupied. Since there was an absence of any intrinsic capillary network, it is necessary to assume that the EGL is supplied by the pial membrane capillary bed. It has been proposed, though

without experimental support, that the position of this source of nutrients is the reason for the EGL being at the surface of the cerebellum, i.e. the cells have migrated to this locus attracted by the rich vascular supply (Klosovskii, 1963).

However, in an experimental tumour system, a gradient of cell proliferation has been described with higher rates of proliferation near blood vessels, and decreased rates at a distance from them (Tannock, 1968, 1970). This may be due to a gradient of oxygen concentration (Brosemer and Rutter, 1961) or nutrients (Tannock, 1968). It is therefore possible that with a rich blood supply adjacent to the pial surface, proliferation is most intense in the outermost EGL, and diminishes proportionally to the distance from the source of oxygen, nutrients or blood-bourne proliferation-regulating factors such as thyroid hormone (Patel et al, 1979). It is interesting to note that, while he recognised two morphologically distinct zones, Cajal (1911) did not consider the deeper layer to be exclusively postmitotic since he observed and illustrated the occasional mitotic figure among the elongated cells (Fig. 5).

Together with the opposition to the theory that the EGL cells are bipotential and the questioning of the division of the EGL into two functionally distinct zones, this thesis presents evidence to suggest the possible identity of the EGL cells. Stereological analysis of morphological parameters of differentiation has its limitations (Jacobson, 1978) but as yet alternative techniques remain inadequate. Successful use of a neuron specific marker might have yielded more conclusive information on the degree of neuronal maturity exhibited by the EGL cells. Whether the ambiguous results

obtained in this work by immunocytochemical staining for neuron specific enolase and D2 protein were due to technical factors or the immaturity of the cells is not known. More fruitful results might be obtained by staining for such neuron specific markers as Thy-1 antigen and tetanus toxin receptors.

The results of the stereological analyses were clear; the round cells and the elongated cells of the EGL (in coronal section) were at the same stage of maturation at any given age of animal. That is, in terms of the proportion of nucleus, mitochondria, rough endoplasmic reticulum and Golgi apparatus to cytoplasm it could not be said that for any age the round cells were any less mature than the elongated cells. The same analyses showed that the EGL cells were similar to the IGL neurons, and both populations of cells were maturing at a similar rate for at least the first two weeks of postnatal life. This strongly suggests that the EGL cells are not primitive or undifferentiated cells but are non-functional neurons. When grown in culture EGL cells from the cerebellum of rats as young as 4 days cease to proliferate and begin to form synapses (Balázs, personal communication). This would seem to indicate very clearly that the EGL cells are fully committed neurons which do not express their definitive neuronal characteristics until they have completed their second migration into the IGL. However the presence of normal contacts between ectopic granule cells and, for example mossy fibres (Lemky-Johnson & Larramendi 1968a; Mugnani, 1972) suggests that position is not as important as the age of the animal for the expression of full neuronal characteristics by granule cells.

The proliferative nature of the EGL cells has been cited as evidence of their primitive nature. A conventional view is that DNA-synthesising and dividing cells cannot be neurons. However, the EGL is not a proliferative zone in every animal. In the frog the EGL only appears after the premetamorphic period (when the tadpole is about three years old)

and its constituent cells never proliferate (Gona, 1972, 1976). Since the EGL cells proliferate in more primitive animals than the frog, for example teleosts (Schaper, 1894; Pouwels, 1978 a-e), the lack of proliferative activity in the frog EGL cannot be interpreted as a characteristic of this layer in lower vertebrate phyla. Cell division in the EGL is thus probably an optional activity necessary to generate the large number of neurons destined for the IGL, and possibly to assist in the gyrification of the cerebellum (Mareš and Lodin, 1970). Precedents have been established for the reappraisal of established ideas regarding the proliferative activity of neurons. For example the concept that the adult mammalian brain is largely static appears to be no longer tenable. By the application of sensitive techniques it has been clearly demonstrated that growth and plasticity, including neurogenesis and synaptogenesis, can occur in the mature, unoperated, mammalian brain (Kaplan and Hinds, 1977; Graziadei and Monti Graziadei, 1978). The old embryological dictum that "differentiated cells do not divide" or, put another way, "cell division is incompatible with differentiation" must now also come under scrutiny. Though allegations of mitotic activity of neurons in the spinal cord have been made (Kjellgren, 1944), observations stemming from light microscopy alone must be subject to some doubt. However, there have been recent reports of division among differentiating neurons on the basis of autoradiographic detection of proliferating cells, and electron microscopy to confirm the neuronal nature of these. In the rat superior cervical and stellate ganglia, neurons were detected in S phase up to the fourth postnatal day (Hendry, 1977). In the chick, cells which were clearly identified as

differentiating adrenergic neuroblasts on the basis of their ability to synthesise and store catecholamine were demonstrated to be still capable of mitosis (Cohen, 1974). This author suggested that previous failure to detect proliferation amongst these cells was possibly due to the low probability of observing mitotic figures. Since S phase is many times longer than mitosis, in a population in which the level of mitotic activity is low, the chances of locating proliferating cells is considerably enhanced by the use of $[^3H]$ thymidine.

In summary, this study suggests a modified schema for the EGL.

1) The EGL cells are a cloned population and are therefore homogeneous.

2) Although they are proliferating, their strong morphological resemblance to the microneurons of the IGL suggests that they are nerve cells despite the lack of evidence of functional differentiation.

3) The gradient of proliferation across the EGL is a reflection of the poor intrinsic vasculature and the major blood supply deriving from the pial capillaries, rather than any gradient of differentiation.

4) There is no evidence of gliogenesis in the EGL, either on the basis of the ultrastructure of the constituent cells or from immunocytochemical localisation of glial specific proteins. If the EGL is the source of medulloblastoma then the cells must undergo dedifferentiation to a primitive stage to explain the occurrence of mixed tumours.

5) Cell death occurs in all regions of the EGL. Although there are regional differences in the vermis, nowhere does the rate of cell death appear large enough to be a major regulatory factor in the

acquisition of cells by the layer, though it may assist in the formation of the folia.

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