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THE DEVELOPMENT OF THE CEREBELLAR CORTEX IN THE OPOSSUM.

I. THE FORMATION AND GROWTH OF THE CORTICAL LAYERS.

II. THE MATURATION OF THE PURKINJE CELL.

DISSertation

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By
Leah Carol Laxson, B.S.

* * * * *

1982

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Another example of a Purkinje cell during the perisomatic dendrite stage. A recurrent collateral (open block arrow labelled RC) originates from the Purkinje cell axon (open block arrow). Camera lucida drawing. Scale, 10μm. X1000

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THE DEVELOPMENT OF THE CEREBELLAR CORTEX OF THE OPOSSUM.

I. THE FORMATION AND GROWTH OF THE CORTICAL LAYERS.

INTRODUCTION

The distinctive laminar pattern of the adult mammalian cerebellum results from complex changes in the location and morphology of neuronal and glial elements which occur during development. In the opossum almost all of the development of the cerebellum occurs postnatally (Ulinski, '71). The opossum is born after 12 days gestation and matures in an external marsupium or pouch for approximately 77 days. The immaturity of the brain of the newborn opossum facilitates the analysis of cerebellar developmental processes that occur prenatally in other species. In the present account the formation and growth of the laminar pattern of the opossum cerebellar cortex will be described from birth to postnatal day (PN) 77. The processes involved in cortical growth were analyzed qualitatively and quantitatively at the light microscopic level.

The cerebellar cortex of the opossum was studied for three reasons: 1) to provide developmental data for comparison with other species regarding the time period and sequence of growth; 2) to provide
a background for the Golgi and electron microscopic analysis of the maturation of the Purkinje cell; 3) to contribute to ongoing studies on the temporal relationships between cerebellar growth and the development of the afferent systems to the cerebellum from the spinal cord and precerebellar nuclei (Martin et al., '80; Maley and King, '80a and b).

The early development of the cerebellum has also been analyzed prenatally in the human (Rakic and Sidman, '70), the whale (Korneliussen, '65) and the rat (Korneliussen, '68; Altman and Bayer, '78a). Additionally, the changes in the cortical layers which can be observed postnatally have been the focus of quantitative studies in several species (human, Raaf and Kernohan, '66; mouse, Haddera and Nooreddin, '66; rat, Altman, '69, Heinson, '77; hamster, Oster-Granite and Herndon, '76).
MATERIALS AND METHODS

The cerebella of 36 pouch young opossums were examined at different ages ranging from birth to approximately PN 77. The postnatal age of the pouch young opossums was determined by using a growth chart comparing snout-rump length to postnatal age (Cutts et al., '78); all ages in the present study were assigned based on this chart. Three series of littermates were used with each litter consisting of at least 6 animals. Young were removed initially from each litter when they were approximately 20mm in snout-rump length and at 20mm intervals thereafter. Additional specimens of various ages were also used from other litters. All of these animals were anesthetized with Metofane and perfused with 10% phosphate buffered formalin. Following perfusion the whole brain was removed and immersed in 10% buffered formalin; the tissue was then dehydrated, embedded in paraffin and serially sectioned in the transverse or sagittal plane at 14μm. The sections were mounted on slides and stained with 0.05% cresyl violet.

For quantitative analysis photographs at a magnification of 35X were made of a sagittal section through the vermis and two parasagittal sections on each side of midline at 140μm intervals resulting in a total sample of five sections from each case. In younger animals the
sagittal plane was defined by the opening of the fourth ventricle. In later ages the two sections containing the most medial aspect of the deep nuclei were identified and a section midway between these two sections was chosen as the midline. The perimeter and the total sectional area of each of these sections was determined using a morphometric computer (Zeiss Videoplan). Measurements of the perimeter were made by following all contours of the pial surface from lobule I to lobule X. In addition the sectional area occupied by the individual layers of the cerebellum was determined using the stereological method of Weibel ('79). A grid of points spaced 1 cm apart was placed over the photograph of the cerebellar section and the area of each layer was then calculated from the number of points over the layer. When the total sectional area obtained using the videoplan versus the stereological method was compared, a maximum difference of 5% was obtained.

The cases were separated into 6 groups using snout-rump length as the criterion; each group consisted of all cases within a 20mm interval beginning with 20mm and continuing to 150mm. A mean value (+ standard error of the mean) for the sectional area of each of the layers and for the total sectional area of a vermal section was obtained for each group. The demarcation between the layers was determined by visual inspection.

In order to determine the width of the layers of the cerebellar cortex, measurements of the same sections referred to above were made at 250X using an ocular micrometer. At least five measurements of the width of each layer in lobules IV and V were made for each case.
Prior to the development of the lobular pattern, the measurements were made anterior to the primary fissure.

In addition to the quantitative analysis of sagittal sections, both transverse and sagittal sections were examined from birth to PN 21 (40mm) to obtain qualitative data on cerebellar development.
RESULTS

A. Qualitative Observations on Early Development

Postnatal Day 1 (12mm)

The cerebellar anlage in a newborn pouch young opossum consists of a thick cellular band (Fig. 1). In lateral areas two layers are evident; a thick ventricular zone (VZ) and an intermediate zone (IZ) (Fig. 1), but only the ventricular zone is present in the midline (Fig. 1, arrow). The ventricular zone is densely packed with ovoid cells oriented with their long axis perpendicular to the ventricular surface. The cells of the intermediate zone are spherical and less densely packed. There is no external granular layer or marginal zone at this time.

Postnatal Day 3-5 (17mm-21mm)

By PN 3 the cerebellar anlage has increased in size and is composed of five lamina (Fig. 2). The first lamina is the ventricular zone (Fig. 2, 1); it is relatively thinner than in the newborn and occupies less than one-half of the total cerebellar width. Above the ventricular zone is a second less densely packed cellular layer which
will be termed the ventral intermediate zone (Fig. 2, 2). The third layer which is dorsal to these two zones is a narrow acellular band (Fig. 2, 3). The fourth layer has numerous round cells and is referred to as the dorsal intermediate zone (Fig. 2, 4). The fifth layer is a very thin marginal layer which now covers the dorsal surface of the presumptive cerebellum (Fig. 2, 5).

At PN 5 the external granular layer (EGL) is beginning to appear in the caudal and lateral regions of the cerebellar plate (Fig. 4), but is not present in more medial areas (Fig. 3). The ventricular zone is much thicker in medial areas than in more lateral areas (compare Figs. 3 and 4).

Postnatal Day 7-14 (24mm-34mm)

A laminar pattern is evident during the period from PN 7 to PN 11 (Figs. 5, 6 and 7). The ventricular zone is thinner than at younger ages and is 5-6 cells in thickness. The marked difference between the width of the ventricular zone medially and laterally which was observed at younger ages is no longer seen. Dorsal to the ventricular zone a wide cellular layer is present throughout the cerebellum. The dorsal and ventral intermediate zones and the acellular zone are no longer clearly seen. The Purkinje cell layer is beginning to condense beneath a narrow presumptive molecular layer (Fig. 5). The EGL covers the caudal and lateral areas of the cerebellum by PN 9 and is 2-4 cells in width; by PN 14 the EGL completely covers the cerebellar surface (Figs. 5, 6 and 7).
Postnatal Day 14-19 (34mm-40mm)

From PN 14-17 the lamination of the vermal cerebellum is comparable to that described from PN 7-14 (Figs. 8, 9 and 10). However, in the lateral areas of the cerebellum the presumptive Purkinje cell layer is clearly present and is 6-9 cells thick (compare Figs. 7 and 9). The Purkinje cell layer has expanded and now can be observed throughout the entire extent of the cerebellum. The presumptive molecular layer located dorsal to the Purkinje cell zone contains small cells which may be migrating granule cells (Figs. 9 and 10). The EGL is 3-4 cells in width. Foliation is observed laterally before it is seen medially; also in lateral areas the Purkinje cell layer is more clearly demarcated and the EGL is thicker (compare Figs. 9 and 10; also Fig. 8). After PN 19 (40mm) the laminar pattern remains the same until PN 33 (60mm) when the internal granular layer (IGL) can first be differentiated from the white matter (WM) (Figs. 18 and 33).

B. Quantitative Analysis of Cerebellar Growth

I. The Growth of the Vermal Perimeter (Fig. 22)

The perimeter of vermal sections remains relatively constant from PN 7 (25mm) until PN 19 (40mm). From PN 19 (40mm) until PN 33 (60mm) the perimeter triples in extent; after PN 33 (60mm) a more rapid rate of increase is observed until PN 75 (128mm) at which time the rate of growth of the perimeter decreases (compare Figs. 11-15). Foliation begins around PN 25 (50mm) and the time period of rapid increase in perimeter reflects the maturation of the folial pattern (compare Figs. 12 and 13).
II. The Growth of the Total Cerebellar Area (Fig. 23)

From birth (Fig. 1) until PN 33 (60mm) (Fig. 13), the cerebellar area measured from vermal sections increases by a factor of six from .02$\text{mm}^2$ to 1.3$\text{mm}^2$ (standard error of the mean [S.E.M.] $\pm .18$) (Fig. 23). However, from PN 1 to PN 33 is a period of relatively slow growth in comparison to the time from PN 33 (60mm) to PN 77 (150mm) (Fig. 23) when the area increases rapidly from 1.3$\text{mm}^2$ ($\pm .18$) to 19.2$\text{mm}^2$ ($\pm 1.2$). In the adult opossum the area of the cerebellar vermis has increased by 7$\text{mm}^2$ to 27$\text{mm}^2$ indicating that some growth occurs after PN 77 (150mm) (Fig. 17).

III. The Growth of the Cortical Layers (Fig. 23)

Quantification of the area of the individual layers of the opossum cerebellum was started with specimens from PN 17 (38mm) when the external granular layer, the molecular layer and the Purkinje cell layer could first be distinguished clearly and was continued until PN 77 (150mm). This endpoint was selected because at this time the Purkinje cell has its mature morphological form and synaptic relationships.

A. The External Granular Layer

The external granular layer (EGL) is first seen at PN 10 (25mm) and is present until after PN 105 (220mm). The sectional area of the EGL increases from its first appearance (Fig. 5) until PN 75 (130mm) (Fig. 21) when it reaches its maximal extent (Fig. 23). Following this peak the area decreases rapidly until the disappearance
of the EGL after PN 105. The maximal width of 32μm (± 2.16) of the EGL occurs about PN 35 (60-65mm) (Figs. 18 and 25). At this time the depth as measured by the number of cells (10-12) is also maximal.

The EGL occupies about one-fourth of the cortical area from PN 10 (25mm) until PN 47 (80mm) when a precipitous decrease in the percentage occurs (Fig. 24). The decrease occurs concurrently with a relatively large increase in the fraction of the total area covered by the molecular area.

B. The Molecular Area

The molecular layer can first be demarcated around PN 14 (34mm) (Figs. 5, 6 and 7); at this age it occupies an area of 0.2mm². By PN 33 (60mm) the molecular layer measures 0.15mm² (± .03) which is a seven-fold increase in size (Fig. 18); however, this is a minor increase in comparison to the rapid growth occurring from PN 33 (60mm) to PN 77 (150mm) (Fig. 21) when the molecular layer is 7.9mm² (± .57) (Fig. 23).

The width of the molecular layer shows a similar pattern: a slow rate of growth until 33 days (60mm) followed by a more rapid rate of growth until PN 77 (150mm) (compare Figs. 16-21; Fig. 25).

The fraction of the total sectional area of the vermis taken up by the molecular layer is about 15% from PN 16 (35mm) to PN 47 (80mm). After this the molecular layer increases to occupy 43% of the sectional area of the cerebellum by PN 77. In terms of change in the fraction of the sectional area occupied by one layer, this is the greatest change observed in the maturing opossum cerebellum (Fig. 24).
C. The Internal Granular Layer

It is difficult to delineate the boundary between the internal granular layer (IGL) and the white matter (WM) prior to PN 33 (60mm). After PN 33 a boundary between these two layers can be distinguished (compare Figs. 12 and 13); however, the white matter still has many more cells than it does in the adult. The sectional area of the IGL increases rapidly from $0.5\text{mm}^2 (\pm 0.08)$ at PN 33 to $8.0\text{mm}^2 (\pm 0.46)$ at PN 77 (150mm) when the growth curve for this layer becomes almost level (Fig. 23). This layer shows the most rapid growth rate of any of the cerebellar layers. However, the percentage of the total area of a vermal section occupied by the IGL remains almost constant at 45% throughout cerebellar development (Fig. 24).

D. The Medullary Layer

The changes in the medullary layer are less dramatic: from PN 33 to PN 77 the white matter (WM) slowly expands from $0.3\text{mm}^2$ to $1.2\text{mm}^2 (\pm 0.17)$. The fraction of the sectional area of the vermis composed of the medullary layer decreases steadily from 12% at PN 33 to 3% at PN 77 (Fig. 24). Prior to PN 33 the IGL and the WM were included in one measurement. The percentage of these two layers combined increases from 38% at PN 21 (40mm) to 57% by PN 33 (60mm) but from PN 33 to PN 77 this value declines to 45%. This loss in percentage is at least partially accounted for by the decrease in the fraction of the cerebellum composed of white matter.
E. The Purkinje Layer

From PN 17 (38mm) the band of Purkinje cells slowly and steadily enlarges until PN 75 (128mm) when its area becomes level at $1.5\text{mm}^2 \ (\pm .16)$ (Fig. 23). The percentage of sectional area occupied by the Purkinje cell layer decreases rapidly from 20% to 8% during the period from PN 16 (35mm) to PN 40 (70mm) (Fig. 24). During this period (PN 16-40) the Purkinje cells are forming a monolayer (compare Figs. 16, 17, 18, 19). After PN 40 the percentage of the cerebellum occupied by the Purkinje cells does not change appreciably (Fig. 24).
DISCUSSION

Early Development

In the opossum the cerebellar anlage is present at birth (12 mm) in a very immature state. Its histological appearance corresponds to that of the rat cerebellar anlage at embryonic (post-conception) day 13 which is the first age at which it has been described in other species (Korneliussen, '68; Altman and Bayer, '78a). At this age in the rat only the ventricular zone was seen in midline sagittal sections; however, in more lateral sections the ventricular zone and a superficial zone with fewer cells were present (Korneliussen, '68). These observations are comparable to those made in the opossum at birth. No marginal zone is seen during this period of cerebellar development in either the rat or the opossum. In the cerebellum of the human fetus, the earliest description is from 7 week old embryos (Rakic and Sidman, '70). At this age in the human three layers are described: ventricular, intermediate and marginal zones. Thus, the opossum cerebellum at birth may be comparable to human embryos less than seven weeks of age.

From PN 1 to PN 19 the maturation of the opossum cerebellum follows a pattern of laminar growth similar to that described in the
rat (Korneliussen, '68; Altman and Bayer, '78a). However, some differences exist in the sequential appearance of individual layers in the human and the opossum. The Purkinje cell layer has been first identified in the human at 9 weeks after conception, but the external granular layer did not appear until 10-12 weeks (Rakic and Sigman, '70). This order of events is reversed in the opossum; the external granular layer can be distinguished on the cerebellar surface by PN 7-9 but the Purkinje cell layer cannot be clearly delineated until PN 14-17. These observations suggest that the temporal sequence of histogenetic events may be slightly different in different species.

Quantitative Studies

The major growth of the cerebellum in the opossum occurs postnatally and is a lengthy process occupying about 75 days. During this time the area of the vermis increases dramatically and the layers of the cerebellum undergo growth at varying rates resulting in distinct patterns of stratification at different stages of maturation.

The external granular layer (EGL) first appears at PN 7 (25mm) in the opossum and persists until after PN 105 (220mm). In the rat the EGL covers the cerebellum prenatally at embryonic day 20 and disappears by PN 21 except for sparse patches (Altman and Bayer, '78a; Palay and Chan-Palay, '74). Although in the opossum the area of the EGL increases continuously and gradually until PN 75 after which it decreases rapidly until its disappearance, it has achieved its maximal width of 10-12 cells at PN 33 (60mm). This decrease in width prior to a decrease in area of the EGL has been noted previously in the rat
The decrease in width of the EGL coincides with a rapid increase in the cerebellar surface due to the onset of foliation. As observed in the mouse (Mares and Lodin, '70), the EGL in the opossum is also thickest in the concavities of the fissures and thinnest in the convexities of the lobules. The percentage of cerebellar area occupied by the EGL in the opossum continuously declines after PN 10 (30mm). This can be accounted for by the accelerated growth of the other layers at this time.

The EGL persists for at least 30 days after PN 77 and therefore is still present during a time period when the Purkinje cell is mature in appearance (Laxson and King, '82b). This persistence of the EGL after the Purkinje cell is in the mature stage is also observed in the human (Rakic and Sidman, '70). For several months after birth in this species the EGL forms a thin layer over the cerebellar surface. The exact significance of the continued migration of neurons from the EGL and extended formation of synaptic contacts by the interneurons derived from the EGL is unknown. In the rat, the Purkinje cell dendritic tree has been observed to increase the area it occupies for several weeks after the Purkinje cell has attained adult synaptic relationships (Pysh and Weiss, '78; Altman, '72b). In the opossum an increase is also observed in the size of the area filled by the dendritic tree between the weanling and adult opossum (Laxson, unpublished observations).

Two zones can be distinguished within the immature EGL (Cajal, '60); these zones have been termed the proliferative and premigratory areas based on the orientation of the cells (Altman, '69). In the opossum both zones are present from PN 21 to PN 47, but mitotic
figures were not observed in the EGL after PN 47 (80mm) suggesting that the generation of granule, stellate and basket cells has ceased by this time. However, migration of these interneurons proceeds for about 50 more days in the opossum, to PN 105.

The molecular layer can first be clearly demarcated about PN 14 (34mm); it should be noted that an acellular band can be seen along the surface of the cerebellum prior to this time and even prior to the appearance of the EGL. The fine structure of this layer has not yet been determined although it has been suggested that this "fibrous layer" may contain olivary axons (Altman and Bayer, '78a). Since few granule cells have migrated at PN 14, it is possible that most of the axons of the "molecular" layer are not parallel fibers as is the case during later development and in the adult. Data from immunohistochemical studies on the localization of serotonin in pouch young opossum brains reveals that serotonergic fibers are present in the intermediate zone on PN 1 (Ho, DiTirro and Martin, personal communication).

From PN 21 to PN 75 the molecular layer shows a rapid rate of growth comparable to that of the internal granular layer (IGL). As has been observed in our Golgi and electron microscopic material this rapid increase in area and thickness is the result of several factors: an increase in granule cell migration, the production of parallel fibers, the growth of the Purkinje cell dendritic tree especially after PN 40 (70mm), as well as increased numbers and size of basket, stellate cells and glial cells. These same contributory factors have been described in the human (Rakic and Sidman, '70) and rat (Altman, '69). Very little of the increase before PN 40 (70mm) can be
attributed to the growth of the Purkinje cell dendrites; based on ultrastructural observations the presumptive molecular layer prior to this time is composed almost completely of parallel fibers or other small axons. The percentage of the area of a vermal section occupied by the molecular layer remains constant until PN 40 (70mm); after this it almost triples by PN 77 (150mm). This later period coincides with the time of rapid dendritic growth of the Purkinje cell during the perisomatic spine stage and the main dendritic stage (Laxson and King, '82b).

The area of the Purkinje cell layer is relatively small in comparison to that of the molecular layer and IGL because it only includes the Purkinje cell bodies and not their dendritic trees which are part of the molecular layer. The area of this layer shows a slow and gradual enlargement. During early cerebellar development, before the Purkinje cells became aligned in a monolayer at PN 35 (65mm), the Purkinje cell layer occupies about one-quarter of the area of a vermal section. However, after formation of a monolayer this amount decreased steadily to 5% by PN 77.

The IGL and the medullary layer cannot be easily demarcated prior to PN 33 (60mm). Before this the cellular layer below the Purkinje cell layer contains deep nuclear neurons as well as granule and Golgi cells. Therefore, measurements preceding PN 33 do not truly indicate the growth of the internal granular layer. This has previously been observed in the rat (Altman, '69; Heinsen, '77) and in the human (Rakic and Sidman, '70). During later periods, the IGL is the most rapidly increasing cortical lamina in both area and width,
but the percentage of the sectional area of the vermis covered by this layer remains almost constant at 45%. This indicates that the growth of the IGL occurs at the same rate as that of the total cerebellar vermis.

In summary, the maturation of the cerebellum in the opossum is a long process lasting approximately 75 days in comparison to rodent cerebellar growth which requires about 25 days (Korneliussen, '68). The entire process of cortical lamination occurs after birth while the young opossums are in the marsupium. Therefore, the cerebellum of the opossum provides an ideal model for experimental manipulation at very early stages of development (Martin et al., '78). Comparable stages of cerebellar development occur in utero and during a shorter interval of time in other mammals. For example, PN 1 in the rat corresponds to approximately PN 19 in the opossum. Many of the processes of cerebellar growth, such as arrival of afferents and migration of neurons, occur between birth and PN 19 in the opossum.
ILLUSTRATIONS
PLATE I

Figure 1  A transverse section through the cerebellar anlage at postnatal day (PN) 1. Two zones can be distinguished in the lateral area: the intermediate zone (IZ) and the ventricular zone (VZ). In more medial regions (arrow) only the ventricular zone is present. All of the cerebellar sections illustrated in figures 1-21 are stained with cresyl violet.

Figure 2  At PN 3 the cerebellar anlage in transverse section is composed of five layers: 1) ventricular zone, 2) ventral intermediate zone, 3) acellular zone, 4) dorsal intermediate zone, and 5) marginal zone. The numbered striped vertical lines show the approximate planes for the sagittal sections shown in figures 3 and 4.

Figure 3  A parasagittal section through a medial area of the cerebellar anlage at PN 5. The rostral aspect is indicated by the arrow; this same symbol is used to indicate the rostral direction in all subsequent photomicrographs of sections in the sagittal plane. The ventricular zone (VZ) is prominent. The acellular zone and dorsal intermediate zone can also be seen.
Figure 4 A parasagittal section through the cerebellar anlage at PN 5. The ventricular zone is thinner laterally. The external granular layer (EGL) is only seen in lateral parasagittal sections at this age (compare with Fig. 3). The ventral intermediate zone can be clearly demarcated but the other lamina cannot be distinguished. This lack of lamination is a characteristic of the lateral areas of the cerebellar anlage at this age.
The cerebellum at PN 14 as observed in a transverse section. The ventricular zone (VZ) is thin compared to this zone in earlier ages (Figs. 1 and 2). The external granular layer (EGL) now covers the cerebellar surface. The Purkinje cell layer (PK) is clearly seen in some areas. The vertical striped lines with numbers correspond to the planes of parasagittal sections shown in figures 6 and 7.

A parasagittal section through the medial cerebellum at PN 14. The ventricular zone (VZ) and the external granular layer (EGL) are labelled.

The lateral area of the cerebellum shown in the parasagittal plane of section. A similar pattern of lamination to that observed medially (Fig. 6) is seen; this observation is in contrast to the marked differences observed at earlier ages between medial and lateral areas (compare with Figs. 3 and 4).
Figure 8  A section in the transverse plane through the cerebellum of a PN 19 pouch-young opossum. Foliation is beginning laterally. The external granular layer (EGL) and Purkinje cell layer (PK) are indicated by arrows. The vertical line numbered 9 indicates the approximate plane of section shown in figure 9.

Figure 9  The opossum cerebellum at PN 19 as observed in a parasagittal section through a lateral region. A diminished ventricular zone (VZ) is present. The Purkinje cell layer (PK) is clearly observed throughout this section. The primary fissure (arrowhead) indicates that the process of foliation is beginning.

Figure 10  A section in the parasagittal plane through a more medial area of the cerebellum at PN 19. The primary fissure is not present yet in more medial areas nor is the Purkinje cell layer as thick as it is more laterally (compare with Fig. 9).
Figures 11-15 A series of photomicrographs from the vermis which illustrate the changing laminar patterns, the growth of the perimeter and area, as well as the increase in foliation. The approximate age of the pouch young opossum used to obtain these specimens is indicated.
The development of the cortical layers can be observed in this series of sagittal sections. All of these photomicrographs were taken in lobules IV and V. The layers of the cerebellum are labelled: the external granular layer (EGL, Fig. 16), the molecular layer (MOL, Fig. 16), the Purkinje cell layer (PK, Fig. 16), the internal granular layer (IGL, Fig. 18) and the white matter (WM, Fig. 18). The approximate age is indicated.
The graph shows the increase in the perimeter of the cerebellum from birth to PN 77. On the abscissae are two scales: the superior scale is the snout-rump length in millimeters (mm) and the inferior scale is the age in postnatal days. On the ordinate is the perimeter measured in millimeters (mm). The filled circles represent means from at least three animals at each age except for the 130mm point which represents one animal. The vertical bars are the standard error of the mean.
Figure 23 Graphic representation of the growth of the cerebellar cortical layers from PN 1 to PN 77. The abbreviations used to identify the lines are: EGL, external granular layer; MOL, molecular layer; PK, Purkinje cell layer; IGL, internal granular layer; WM, white matter; and vermal area, sectional area of the vermis. On the abscissae are two scales: the superior scale is the snout-rump length in millimeters (mm) and the inferior scale is the age in postnatal days. On the ordinate is the area measured in millimeters squared (mm$^2$). The filled circles represent means from at least three animals at each age except for the 130mm point which represents one animal. The vertical bars are the standard error of the mean.
This graph shows the percentage of the total cerebellar area occupied by each individual cortical layer. The abbreviations used to identify the lines are: EGL, external granular layer; MOL, molecular layer; PK, Purkinje cell layer; IGL, internal granular layer; and WM, white matter. On the abscissae are two scales: the superior scale is the snout-rump length in millimeters (mm) and the inferior scale is the age in postnatal days. On the ordinate is the percentage of the sectional area of the vermis. The filled circles represent means from at least three animals at each age except for the 130mm point which represents one animal. The vertical bars are the standard error of the mean.
Figure 25  
Graph showing the width of the external granular layer (EGL), the molecular layer (MOL) and the Purkinje cell layer (PK). On the abscissae are two scales: the superior scale is the snout-rump length in millimeters (mm) and the inferior scale is the age in postnatal days. On the ordinate is the width of the cortical layer in microns (μm). The filled circles represent means from at least three animals at each age except for the 130mm point which represents one animal. The vertical bars are the standard error of the mean.
THE DEVELOPMENT OF THE CEREBELLAR CORTEX IN THE OPOSSUM.

II. THE MATURATION OF THE PURKINJE CELL.

INTRODUCTION

The morphogenesis of the primary neuron of the cerebellar cortex, the Purkinje (Pk) cell, has been extensively studied in several species using the Golgi method (Ramon y Cajal, '11 and '60; Addison, '11; Dadoune, '66; Hendelman, '80; Zecevic and Rakic, '76; Berry and Bradley, '76; Oster-Granite and Herndon, '76; Purpura et al., '64) and electron microscopy (Altman, '76b; Kornguth, '69 and '72; Larramendi, '69; Mugnaini, '69; Oster-Granite and Herndon, '76; Meller and Glees, '69; Zecevic and Rakic, '76). Our knowledge of its morphology, cytology and synaptic organization in the adult is unsurpassed (Palay and Chan-Palay, '74; Mugnaini, '72; Fox et al., '67). These data provide an invaluable endpoint for the interpretation of its growth and development.

The Pk cell undergoes extensive changes in morphology and synaptology during its maturation. Based on observations using Golgi and neurofibrillary methods, Cajal described three stages of Pk cell development: the fusiform stage, the stellate stage of disoriented dendrites and the stage of dendritic orientation and flattening.
(Ramon y Cajal, '11 and '60). Five stages of Pk cell development have been identified in Golgi preparations of mouse cerebellum; the immature stage, the perisomatic dendrite stage, the perisomatic spine stage, the stage of the main dendrite and the mature stage (Hendelman and Aggerwal, '80). Altman divided Pk cell maturation in the rat into five phases based on fine structural characteristics of the neuron and its maturing synapses (Altman, '72b). Other electron microscopic studies have utilized Cajal's classification and concentrated on the stellate and dendritic orientation stages (Larramendi, '69; Meller and Glees, '69; Mugnaini, '69).

The development of the opossum Purkinje cell will be analyzed based on correlations between data obtained from Golgi preparations and electron microscopy. This study was performed to determine the temporal relationship between the development of the Purkinje cell and one of its major sources of afferents, the inferior olivary complex. Much data has been gathered on the connectivity, synaptology and development of the inferior olivary complex (Martin et al., '80; King, '80; Maley and King, '80a and b; Bauer-Moffett and King, '81) in this species. Further normative and experimental work is planned to study the maturation of other precerebellar nuclei and of the deep nuclei of the cerebellum. The overall aim of these studies is to elucidate the sequence of connectivity and synaptogenesis within these neuronal systems and to examine basic mechanisms of neural development. Also, a comprehensive study of all stages of Pk cell growth has not been performed previously at the light and electron microscopic level simultaneously. The prolongation of Pk cell growth in the opossum allowed clarification
of the nature and order of occurrence of the somatic processes of
the Pk cell.

The opossum was chosen for study because of its unique
embryology: twelve days after conception the young of this marsupial
are born and crawl to an external pouch where they mature until they
are weaned about 77 days postnatally (Cutts et al., '78). Most brain
development occurs postnatally while the animals are in the pouch and
accessible for experimental manipulation (Ulinski, '71; Martin et al.,
'78). Another distinct advantage of this animal model when compared
to other mammalian species commonly used for developmental studies is
the protracted time of neural maturation. Notably the Pk cell of the
opossum passes from the fusiform or immature stage to the mature stage
in approximately 60 days between postnatal days 17 and 77 (40mm-150mm),
whereas in the rodent these changes occur within 14-15 days (mouse,
Hendelman, '80; rat, Addison, '11 and Altman, '72b).
MATERIALS AND METHODS

Pouch young opossums were obtained from females already pregnant when captured in the wild or bred while in captivity. The age of the animals was estimated based on the snout-rump length using the growth curve chart of Cutts et al. (’78). All ages given refer to postnatal (PN) days. Since the first age at which the Purkinje cell layer can be identified clearly is 17 days (38mm), this point was chosen to begin the study. Animals were anesthetized using ether or Metofane and sacrificed at 7 day (10mm) intervals from 17 days (38mm) to 77 days (150mm). The cerebella were either processed by the Golgi method or prepared for electron microscopy. The morphology of the Pk cell was also examined in Golgi preparations of adult opossum cerebella.

We are greatly indebted to Dr. Kent Morest for the use of his collection of Golgi preparations of pouch young opossums.

Golgi Impregnations

The entire brains of pouch young opossums were processed by one of two Golgi techniques. Most of the brains were prepared using the rapid Golgi technique on tissue fixed by transcardial perfusion with a solution of 3.5% potassium dichromate and 0.25% osmium tetroxide or on
tissue perfused with 2% glutaraldehyde-2%paraformaldehyde in 0.12M phosphate buffer (Morest, '69). Following perfusion with either fixative, the tissue was immersed in the potassium dichromate-osmium tetroxide solution. A modification of the Golgi-Kopsch method was employed on 12 brains after perfusion with 2% glutaraldehyde-2% paraformaldehyde in 0.12M phosphate buffer (Maley and King, '80b). When using the aldehyde mixture, the brains were left in fixative for at least 2 months. Both methods gave satisfactory and comparable results. All brains were embedded in celloidin and sectioned in the sagittal or transverse plane at a thickness of 120-180μm; the sections were then dehydrated, cleared with a-terpineol and xylene, mounted on glass slides and coverslipped. Drawings of selected Pk cells were made using a Leitz drawing tube and oil immersion lens which had a magnification of either 95X or 100X. With the exception of the youngest cases examined (19 days; 40mm) all Pk cells illustrated were taken from vermal sections cut in the sagittal plane. It should be noted that within a vermal section Pk cells exhibited variable degrees of maturation at each of the ages selected for study.

Electron Microscopy

After the pouch young opossums were anesthetized with Metofane, intracardiac perfusions were performed using a solution of 0.9% NaCl which also contained 1% xylocaine for approximately 30 seconds followed by 2% glutaraldehyde-2% paraformaldehyde in 0.12M phosphate buffer with 0.6% dextrose adjusted to an osmolarity of 1200M osm and a pH of 7.2 for 10-20 minutes. The rate of flow was adjusted by changing the gauge of
the needle used and the height of the perfusion apparatus depending on the size of the animal. Following perfusion, the calvarium was opened to expose the cerebellum and the specimen was left in the same fixative at 4°C for 12-24 hours. The cerebellum was then removed from the brainstem and cut in the sagittal plane into 0.5-1.0mm slices which were rinsed in phosphate buffer and postfixed in 2% osmium tetroxide. After dehydration in a graded series of acetone solutions, the tissue was embedded in Spurrs resin and oriented so that the sections would be in the sagittal plane. Using a Porter-Blue or LKB microtome, sections 0.5-2.0μ in thickness were cut with glass knives and stained with toluidine blue for light microscopic examination and subsequent orientation of thin sections. Thin sections (80-100nm) were cut with glass knives, stained with uranyl acetate and lead citrate, and examined with a Philips 300 electron microscope. The area sampled for electron microscopy was just anterior to the primary fissure in pouch young from 14-33 days (35-60mm) and was within lobules IV and/or V in animals older than 33 days (60mm). Samples from at least three animals were examined at each of the following intervals (40-45; 50-55; 60-70; 80-95; 100-125mm). The description of the mature stage of Pk cell development was derived from the cerebellar of two juvenile opossums.
OBSERVATIONS AND RESULTS

Early Immature Stage (Postnatal Days 19-24)

At 19 days postnatally (40mm) the cerebellum has a well-defined Purkinje (Pk) cell layer which is 5-7 neurons in width when viewed in lum plastic sections (Fig. 26). These cells are separated from the external granular layer by the incipient molecular layer and can be distinguished easily from migrating granule cells within the Purkinje cell layer. Migrating granule cells are smaller, have much darker cytoplasm and nuclei, and are more elongated than the immature Purkinje cells. The majority of the Pk cell bodies are fusiform or ovoid in shape with the nucleus located basally and the apical pole of the cell often tapering into one or two processes (Fig. 26). However, some immature Pk cells have a more spherical cell body and a club-shaped apical pole.

A few small dark cells, which are probably postmigratory granule cells, are located below the Pk cell layer. The location of the granule cells and the presence of small axons in electron micrographs of the molecular layer (Figs. 33 and 36) indicates that some parallel fibers are present at this age.

In Golgi preparations Pk cells have irregular ovoid perikarya, each with one or two long thick processes oriented toward the pial
surface (Figs. 31 and 77A). Such processes could not be seen to contact the pial surface because of the heavy metallic reaction product in the pial area. The apical processes have large dilations along their course; these are more frequently seen on the more proximal part of the processes (Fig. 31, block arrows). Many appendages radiate for short distances from the soma and lower portion of the apical process; these appendages terminate in bulbous expansions or delicate filopodia (Fig. 31, arrowheads). Rarely, a longer process extends from the soma into the more superficial molecular layer (Fig. 77A). The more distal parts of the apical processes have fewer appendages and exhibit fewer spines than the soma.

The axon (Fig. 31, open block arrow) was followed from the basal aspect of the soma for a short distance; its course suggested that the deep nuclei might be its target. In contrast to the mature Pk cell axon, the axon at this stage manifests numerous varicosities. No recurrent collaterals were observed.

In electron micrographs the Pk cell is readily identified because of its large nucleus and homogenously distributed euchromatin. A dense rim of heterochromatin is located adjacent to the nuclear envelope (Figs. 33, 36 and 37). The apical pole of the nucleus is convoluted and in some neurons it extends into the most proximal part of the apical dendrite (Figs. 33 and 37). The cytoplasm forms a thin border around the lateral and basal aspects of the nucleus and contains a few mitochondria, strands of rough endoplasmic reticulum and lysosomes. The apical portion of the soma, however, has a greater accumulation of cytoplasm and organelles. An extensive Golgi apparatus is
located in the cytoplasm of this region and is often continued into the elongate apical processes (Figs. 32 and 37, GA). Subsurface cisterns of smooth endoplasmic reticulum are observed very infrequently subja-
cent to the Pk cell membrane (Fig. 35, block arrow). There is no glial ensheathment of the Pk cell body and often the somas are in apposition to one another (Fig. 37) or to migrating granule cells.

The one or two apical processes originating from the pial pole of the Pk cell have many of the cytological characteristics of adult dendrites: numerous microtubules, longitudinally aligned mitochondria, rough endoplasmic reticulum, and smooth endoplasmic reticulum (Figs. 32 and 33). The rough endoplasmic reticulum is abundant at this stage and is concentrated along the plasma membrane of the dilated areas of the apical processes (Fig. 33, block arrows). The lateral appendages which arise from the soma and proximal part of the apical process have organelles similar to those of the apical process (Fig. 34). The axon arises from the base of the soma and has microtubules, free ribosomes and polysomes (Fig. 36, open block arrow).

Synaptic contacts on the Pk cell body are infrequent (Fig. 37, curved arrow) although synapses on flocculent profiles in the surrounding neuropil are evident (Figs. 32, 36, 38 and 39, arrowheads). The presynaptic profiles have a dark cytoplasmic matrix and contain round vesicles, approximately 0.5 to 1.0μm in diameter, and make synaptic contacts of the asymmetrical type. Large dense core vesicles are not seen at this age.
Late Immature Stage (Postnatal Days 25-32)

At 25 days postnatally (50mm) the Pk cell layer is 2-3 cells in width; the cell bodies are fusiform in shape (Fig. 27). Closer examination using Golgi preparations and electron micrographs of the Pk cell reveals several distinctive features at this age. In Golgi preparations the soma is fusiform. The single long apical process or processes characteristic of postnatal day 19 animals have been replaced by several processes which radiate mainly from the superior pole of the neuron (Fig. 40, arrowheads). In some instances one of these processes is thicker than the others and some of the processes branch from this thicker process rather than from the soma (Figs. 77B and 77D). The tips of the neuronal processes are often enlarged and irregular in shape (Fig. 40, asterisk). The base of the cell at this age is broader than at the preceding age and has processes emerging from it (Fig. 40, small arrows). These appendages are much shorter and finer than those arising from the apex of the cell (Figs. 40, 52D and 52E).

Although the appearance of the Pk cell in electron micrographs is similar in some respects to that described at the preceding age (19 days; 40mm), differences are evident. The nucleus has become more elliptical in shape and the apical convolutions have decreased in depth and number (Fig. 41). The cytoplasm in the basal area has increased in amount and appears less electron dense than the rest of the Pk cell cytoplasm due to a paucity of organelles (Figs. 41 and 42). The organelles present in this region include glycogen, ribosomes, poly­somes, infrequent mitochondria and an occasional strand of smooth endoplasmic reticulum. The processes originating from the soma have
organelles typically found in dendrites: microtubules, ribosomes and smooth endoplasmic reticulum (Fig. 38, arrowhead).

In comparison to the 19 day Pk cells, synaptic contacts are more apparent on the cell body and processes and in the surrounding neuropil (Figs. 43-48). The terminals synapsing on or adjacent to the Pk cells of 25 day (50mm) opossums are of four types. The most frequent type of presynaptic element is large (1-2μm) with a lucent matrix and clear spherical vesicles. This first type forms asymmetric junctions on the cell body and somatic processes (Figs. 44, 45 and 46, open block arrows-1) and similar terminals are also seen on flocculent profiles in the neuropil immediately adjacent to the Pk cell (Figs. 38 and 40, open block arrows-1). These postsynaptic profiles probably originate from Pk cells because of their similar cytoplasmic characteristics. The second type of bouton has a darker cytoplasmic matrix, abundant round vesicles, and junctional complexes of the asymmetric type (Fig. 46, open block arrow-2). These boutons also contact the soma and processes of Pk cells. The third class of terminals is composed of smaller boutons (0.5-1μm) with a lucent matrix and spherical clear vesicles clustered near the asymmetric contacts (Figs. 47 and 48, open block arrows-3). The latter boutons, whose morphology closely resembles that of mature parallel fibers, are commonly seen synapsing in the neuropil immediately adjacent to the processes arising from the apical area of Pk cells (Fig. 48, inset). A fourth variety of terminal is present in proximity of Pk cells, but these have not been observed forming synaptic contacts on the Pk cell bodies. The terminals of this fourth type are larger than the first type described and contain spherical vesicles,
dense core vesicles and glycogen and are presynaptic to flocculent profiles in the neuropil (Fig. 47, inset).

**The Perisomatic Dendrite Stage (Postnatal Days 33-44)**

Examination of the cerebellar cortex in 1μm plastic sections at 37 days after birth (63-67mm) reveals that the Pk cells are now arranged in an uneven monolayer (Fig. 28). The amount of neuropil between the Pk cells has increased considerably. The external granular, the molecular layer and the internal granular layer have all grown in thickness. Granule cells migrating across the molecular layer are prominent.

The size of the perikaryon has almost doubled primarily because of the large increase in the apical region of the cell body (Fig. 50). In electron micrographs this area contains the same organelles as it did in the immature stage, but the rough endoplasmic reticulum is more extensive and the mitochondria are more numerous. The nuclei are more rounded. Subsurface cisterns are seen occasionally (Fig. 50, inset, block arrow).

Golgi preparations disclose an astounding increase in the number of processes arising from the circumference of the soma (Figs. 49 and 53). These multitudinous appendages radiate in all directions, but those originating from the apical one-half of the soma tend to be slightly longer (Fig. 53). These appendages can be divided into two classes. The first kind is the most common and measures 10μ to 50μ in length; these often branch into two or more terminal extensions (Figs. 49, 50 and 51). Many small protrusions and spines occur along
the length of these appendages which terminate with an expansion or very fine filopodia. Processes of this first type are also evident in electron micrographs (Fig. 50). Because these elongations contain the organelles characteristic of adult dendrites such as microtubules, neurofilaments, mitochondria, ribosomes and smooth endoplasmic reticulum, they are interpreted to be presumptive dendrites (Figs. 50, 51, 52, 56 and 57). Spines filled with a flocculent matrix are seen originating from the shafts of these dendritic processes (Fig. 51, asterisk).

The second class of appendage is rarely seen at this stage in Golgi preparations or electron micrographs. In Golgi preparations these projections are short (5μm) and knobby (Fig. 49). These knobs do not display the cytological features of dendrites, but rather have a light flocculent matrix with an occasional strand of smooth endoplasmic reticulum (Fig. 54, labelled S). This type of process has only been observed at the base of the soma at this stage and corresponds to the perisomatic spines of the next stage.

In Golgi preparations the axons of some Pk cells could be followed for 100μm into the white matter; recurrent collaterals were only observed infrequently (Figs. 49 and 53). In electron micrographs the axon is identified ultrastructurally by its site of origin at the base of the cell, by its straight course and by its numerous longitudinally oriented microtubules (Fig. 55, block arrow). Small protrusions or spines which arise from the axons do not contain organelles (Fig. 55, stars). Subsurface cisterns are located adjacent to the axonal membrane (Fig. 55, block arrow).
Synaptic terminals contact the soma and both types of somatic processes (Figs. 51, 52, 53, 55, 56 and 57). The large boutons synapsing with the cell body measure up to 3μm and contain spherical vesicles throughout the entire profile (Fig. 54). These boutons form asymmetric junctions with the Pk cell soma and simultaneously with flocculent processes in the adjacent neuropil or with proximal areas of the perisomatic dendrites. Perisomatic dendrites are contacted by synaptic terminals that can be divided into three groups: 1) large boutons which have spherical vesicles and asymmetric contacts on the shafts of presumptive dendrites or their flocculent protrusions (Fig. 51); 2) elongated terminals which are packed with spherical vesicles and form asymmetric junctions on apical perisomatic dendrites (Figs. 56 and 57); 3) small boutons which contain round vesicles and form asymmetric junctions on the shafts of the presumptive dendrites (Fig. 52). The few perisomatic spines are the postsynaptic site for boutons containing spherical vesicles and making asymmetric synaptic junctions. These boutons synapsing on the spines closely resemble the axosomatic terminals described above.

Perisomatic Spine Stage (Postnatal Days 45-60)

The perisomatic spine stage lasts from postnatal day 43-60 (75-100mm) and is a period of transition distinguished by major changes in the morphology of the Pk cell. Although this is a distinct stage in Pk cell development as observed in Golgi preparations and electron micrographs, only minor changes appear in 1μm plastic sections (Fig. 29). By 83mm the Pk cells are arrayed in an even monolayer with slightly
more neuropil between their perikarya than at the preceding stage. The
Pk cell soma has enlarged but an apical cap of cytoplasm is still
present. By the end of this stage (100mm) the cytoplasm is evenly dis­
tributed around the nucleus and the apical cap is not a prominent
feature. The molecular layer is appreciably wider due to the increase
in parallel fibers and growth of the Pk cell dendritic tree.

In Golgi impregnations of Pk cells at 43-47 days (75-85mm) post­
natally, the ovoid soma is encrusted with spines which vary in form
from short (5-7μm), blunt extrusions to longer gnarled excrescences
(Fig. 58, arrows). Longer lateral processes reminiscent of those of
the perisomatic dendrite stage are found near the apex of Pk cells at
42 days (Fig. 58, arrowhead); these cells also exhibit immature den­
dritic development in comparison to slightly older (47 days) Pk cells
(Fig. 59). Initially during this stage two or three dendritic branches
arise either directly from the apex of the perikaryon or from a very
stout and short apical dendrite (Fig. 58). The more immature dendrites
branch infrequently, have long thin tortorous spines and terminal bulbs
with fine, thread-like appendages. Quite often the branching points
are thicker than the parent dendrite (Fig. 58). During the late peri­
somatic spine stage a single main dendrite is present and the degree
of secondary branching increases (Fig. 59). Although the dendritic
tree extends into the molecular layer, the dendritic branches do not
reach the external granular layer. The perisomatic spines of Pk cells
with more differentiated dendrites are much smaller and more uniform
(Fig. 53, arrows). By 57-60 days (95-100mm) the number of perisomatic
spines is greatly reduced and those remaining are mostly located on the
upper area of the soma. The main dendrite has spines or thorns which closely resemble the perisomatic spines at the end of this stage.

The ultrastructural features of the dendritic arborization are beginning to attain a mature appearance (Fig. 67). Many spines arise from the dendrites; these protrusions have a flocculent matrix and in some cases contain vesicular elements (Figs. 65 and 66). A glial sheath now covers much of the dendritic surface (Figs. 66 and 67, labelled G1).

Ultrastructural examination of the Pk cell reveals numerous perisomatic spines (Hendelman and Aggerwal, '80) or pseudopodia (Mugnaini, '69) covering the lateral and basal aspects of the cell body (Figs. 60, 61, 62 and 63, labelled S). These are identical to the second type of somatic process described at the perisomatic dendrite stage. The perisomatic spines have a light flocculent matrix and scattered strands of smooth endoplasmic reticulum (Figs. 61, 62 and 63, labelled S). The presumptive dendrites which were the prevalent type of somatic process during the perisomatic dendrite stage are observed infrequently.

Major changes occur in the synaptic relationships of the developing Pk cell between the perisomatic dendrite stage and the perisomatic spine stage. Most boutons which contact the perisomatic spines contain spherical vesicles, glycogen and an occasional small mitochondria and form asymmetric synaptic junctions (Figs. 61 and 62). Another type of terminal which is found rarely has pleomorphic vesicles and forms indistinct symmetric junctions directly on the surface of the cell body (Fig. 64, block arrow).
Terminals are present on the shafts and spines of the dendritic tree (Fig. 67). Closer examination shows that these terminals have round vesicles and asymmetric junctions (Fig. 66). These terminals are presynaptic to dendritic spines (Fig. 66, asterisks). Also synaptic configurations where the presynaptic element forms a ring around the postsynaptic spine are often seen (Fig. 65); these have not been observed in the mature cerebellar cortex of the opossum.

The Main Dendrite Stage (Postnatal Days 61-75)

In 1μm plastic sections, this phase of Pk cell development is similar to the previous stage. Both the molecular layer and the internal granular layer have increased in width; the external granular layer has decreased. The Pk cells are in an even monolayer and the nucleus is centrally located in the perikaryon (Fig. 30).

In Golgi preparations Pk cells exhibit a decrease in perisomatic spines, an increase in the length of the primary dendrite between the cell body and the first branch point, and an enlargement of the dendritic arborization (Fig. 68). The few spines observed on the soma are much smaller and simpler in form than those of the preceding stage (Fig. 68, arrows). The primary dendrite is much longer than in the perisomatic spine stage and has thorns arising from it. The secondary and tertiary dendrites have numerous spines some of which exhibit filopodia.

The axons could be followed for up to 70μm into the white matter (Fig. 68, open block arrow) and in some cases collaterals were seen along their length. However, the total extent of the
recurrent collateral plexi may not have been impregnated in our Golgi preparations.

When viewed in electron micrographs the soma of the Pk cell is now pear-shaped with occasional small spines (Figs. 69, 70, 72 and 73, labelled S); the nucleus is in the center of the cell and the nuclear membrane at the apical pole is slightly convoluted. The rough endoplasmic reticulum has formed the adult array of Nissl bodies. Subsurface cisterns of smooth endoplasmic reticulum are abundant and line the membrane of both the cell bodies and the dendrites (Figs. 69, 70, 72 and 74, arrowheads). A glial sheath insulates the Pk cell perikaryon and dendrites except where terminals are apposed to the plasma membrane (Figs. 69 and 74).

The soma is the postsynaptic site of at least two classes of synapses. The first includes large boutons characterized by scattered pleomorphic vesicles and punctate symmetric junctions which in some cases are rather indistinct (Figs. 69, 70 and 72). These terminals arise from unmyelinated axons as intermittent swellings (Fig. 69). These axons have a light matrix with a paucity of microtubules and loosely aggregated synaptic vesicles localized at the junctional complexes. The second class of somatic terminals also has symmetrical synaptic junctions and pleomorphic vesicles (Fig. 71). These terminals are distinguished from the first type by the numerous synaptic vesicles which fill the terminal and by longer more distinct synaptic junctions and by a darker cytoplasmic matrix.

The thorns of the primary dendrite and the somatic thorns on the most apical part of the neuron are the site of synapses with
spherical vesicles and asymmetric junctions (Figs. 73, 74, 75 and 76). The boutons forming these synapses are in some cases very large and densely packed with vesicles; in other examples the vesicles are less closely aggregated. Occasionally one of these boutons is seen to contact a series of three or four thorns (Figs. 75 and 76).

In the molecular layer small boutons with spherical vesicles clustered near the symmetrical synaptic junctions contact the spines of the Pk cell dendrites. This type of terminal is frequently observed.
DISCUSSION

The Stages of Pk Cell Development in the Opossum Compared to Other Species

The present results from Golgi preparations and electron micrographs support the division of Pk cell growth into five distinct stages and, therefore, the terminology of Hendelman and Aggerwal ('80) was employed. Each of the stages of Pk cell development are easily defined in the opossum. For example, the immature stage or fusiform stage of Cajal lends itself to descriptive analysis because of its postnatal occurrence and long duration. The second stage of Cajal, the stellate stage, corresponds to the perisomatic dendrite stage of Hendelman and Aggerwal. The third stage of Cajal, the stage of dendritic orientation and flattening, is equivalent to the remaining three stages described by Hendelman and Aggerwal ('80). These are the stage of perisomatic spines, the stage of the main dendrite and the mature stage.

The Immature Stage (Postnatal Days 19-32)

Golgi impregnations of the opossum Pk cell at 19-25 postnatal days (40-50mm) during the immature stage resemble those illustrated by Cajal ('60) in the mouse and chick and Hendelman ('80) in the mouse.
However, the most immature opossum Pk cells of this stage have an elongated apical process oriented with the long axis perpendicular to the pial surface which has not been described in other species. The cytological features of the elongated apical process as seen in the electron microscope are those of immature dendrites. However, this elongate process is a transient feature of Pk cell differentiation. During the perisomatic dendrite stage the apical processes are not present. Concurrently the outgrowth of numerous shorter processes from the soma occurs. It is not evident from either our Golgi impregnations or electron micrographs whether these elongate apical processes die or are reabsorbed into the perikaryon. Their function is likely related to the initial orientation of the Pk cell and will be discussed in more detail in the section on possible mechanisms of Pk cell migration.

The fine structure of the Pk cell during the early immature stage in the opossum has features similar to those described during this phase in the rat (Altman, '72b) and the human (Zecevic and Rakic, '76). Neither of these investigations described processes originating from the lateral aspects of the Pk cell soma during the immature stage; however, such appendages were illustrated by Cajal ('60). By 25 postnatal days (50mm) in the opossum lateral processes originating from the soma of the Pk cell are seen infrequently. The apical process is still present although reduced in length; in addition several other processes also originate from the apical pole of the neuron. Our description of this later period of the immature stage coincides with that reported for the neonatal rat (Altman, '72b) and fetal human (Zecevic and Rakic, '76). During the subsequent perisomatic dendrite
stage the number of somatic processes greatly increases. This variation in the number of somatic processes may be related to the initial arrival of afferents and the subsequent process of synaptogenesis.

Synapses on both the Pk cell soma and apical process during the early period of the immature stage are very infrequent, but a few boutons with asymmetric synaptic junctions and spherical vesicles are evident. Possibilities for the origin of these boutons include basket cell axons, parallel fibers, Pk cell recurrent collaterals, climbing fibers, mossy fibers or afferents from the locus coeruleus and raphe nuclei. Since autoradiographic experiments in the rat have demonstrated that basket cells are not generated until the perisomatic dendrite stage or phase 2 of Pk cell development (Altman, '69), it is unlikely that any of the boutons contacting the opossum Pk cell during the immature stage are from basket cell axons. This of course assumes that basket cells are generated in a similar sequence in the opossum. Also the morphology of these terminals is not comparable with that of synapses formed by immature basket cell axons during later stages of Pk cell development in the mouse; these terminals have symmetric junctional densities and contact the Pk cell soma during stages of development subsequent to the immature stage (Larramendi, '69). The terminals with spherical vesicles and asymmetric junctions are larger than parallel fiber synapses observed during the later developmental stages in the opossum. These cytological features are most comparable to those of boutons which are tentatively identified as climbing fibers during the perisomatic dendrite and spine stages in the opossum (postnatal days 33-60). However, the other sources suggested above cannot be excluded as possibilities.
Synaptic terminals associated with the Pk cell increase toward the end of the immature stage at postnatal day 25 and are of four types. Boutons like those described at postnatal day 20 with asymmetric synaptic junctions and spherical vesicles form synapses with the cell body and the somatic processes. The second variety of terminal also has spherical vesicles and an asymmetric synaptic density and contacts the soma and processes of the Pk cell. However, it is distinguished from the first type by its darker cytoplasmic matrix and more densely packed vesicles. These two terminal types may not represent two distinct classes of afferents, but rather may be developmental stages of a single afferent system. As suggested above for synapses present earlier in the immature stage, they may be developing climbing fibers. The third type of terminal is small, contains clear round vesicles and makes asymmetric contacts. These are tentatively identified as originating from parallel fibers because of their similarities to parallel fiber boutons observed in the adult rat cerebellum (Palay and Chan-Palay, '74) and in later developmental stages in the opossum. The morphology of the fourth class of terminals observed at the deep border of the Pk cells that did not synapse with these neurons could be comparable to immature mossy fibers seen in other species (Larramendi, '69; Meller and Glees, '69; Altman, '72c; Mugnaini, '69) or to terminals containing monoamines (Chan-Palay, '77).

The Perisomatic Dendrite Stage (Postnatal Days 33-44)

The Pk cells of postnatal opossums at day 35 correspond to those described and illustrated as characteristic of the stellate stage of
disoriented dendrites by Ramon y Cajal ('11) in the dog. The somatic processes of this second stage are longer than the lateral somatic processes of the preceding immature stage or those of the following stage of perisomatic spines. This distinctive stage of morphological development of the Pk cell has been described using Golgi preparations in monkey and human fetuses (Zecevic and Rakic, '76), in the rat (Altman, '72b; Dadoune, '66) and mouse (Hendelman, '80).

The elongated somatic appendages of the perisomatic dendrite stage have the morphological characteristics of dendrites at the fine structural level. These processes, however, have been referred to as presumptive dendrites in this account because it is not clear whether these immature dendrites actually contribute to the adult dendritic tree. Our results in the opossum are at variance with the electron microscopic observations of Altman during phase 2 of Pk cell development in the rat (Altman, '72b). Somatic processes in the rat do not possess the ultrastructural characteristics of dendrites during this phase and are thus denoted by Altman as perisomatic processes. These perisomatic processes described in the rat have the same cytology as the perisomatic spines of the next stage in the development of the opossum Pk cell. The lack of processes with dendritic characteristics early in Pk cell development in the rat may be a species difference; alternatively, the protracted time span of Pk cell maturation in the opossum as compared to the rat may allow a better definition of major changes in Pk cell maturation. In the cerebellum of the chick embryo (Mugnaini, '69) and of the fetal monkey (Kornguth, '72) processes similar to the presumptive dendrites of the perisomatic dendrite
stage in the opossum were also identified as dendrites based on cytological features seen with the electron microscope.

During the perisomatic dendrite stage synapses are observed more frequently on both the Pk cell body and the somatic processes. All synaptic boutons during this stage form asymmetric junctions and contain clear spherical vesicles. The axosomatic terminals contacting the presumptive dendrites and the sparse somatic spines are tentatively identified as climbing fiber boutons based on their cytological characteristics. The elongate terminals which synapse with the apical perisomatic dendrites have a dark cytoplasmic matrix and densely packed round vesicles, features which are associated with more mature climbing fiber terminals of the main dendrite stage. If these tentative identifications are correct this suggests that all climbing fibers do not mature at the same rate. The small boutons which make asymmetric synapses with the presumptive dendrites have round vesicles aggregated near the synapse and correspond to parallel fiber terminals of later stages. The presence of parallel fiber terminals and possibly climbing fiber terminals on the presumptive dendrites supports the hypothesis that the perisomatic dendrites become part of the mature dendritic tree (Hendelman and Aggerwal, '80). The type of terminal which was observed during the late immature stage and identified as either a mossy fiber or monoaminergic terminal is not seen adjacent to the Pk cells.

The Perisomatic Spine Stage (Postnatal Days 45-60)

In pouch young opossums this period was clearly delineated by analysis of neurons in both Golgi preparations and electron micrographs.
This stage has been described previously in Golgi material of the developing mouse cerebellum (Hendelman and Aggerwal, '80) and has been illustrated by others in drawings from Golgi preparations (Zecevic and Rakic, '76; Ramon y Cajal, '11 and '60). The perisomatic spines are much shorter than the perisomatic dendrites and are complex knobby protrusions arising from all areas of the soma. At the beginning of this stage most neurons have two or three short dendrites with many spines and filopodia, but dendritic development progresses until one primary dendrite is present.

In electron micrographs the perisomatic spines are quite different in cytoplasmic composition from the presumptive dendrites of the previous stage; the spines contain only a flocculent matrix and a few strands of smooth endoplasmic reticulum. Numerous terms have been used to describe these protrusions from the soma: pseudopodia (Mugnaini, '69), perisomatic processes (Altman, '72b), filopodia (Sotelo and Arsenio-Nunes, '76) and spines (Kornguth, '72).

Analysis of the synaptology also shows that the perisomatic spine stage is a distinct period during the maturation of the Pk cell. The spines are contacted by terminals which form asymmetric junctions and contain spherical vesicles; these have the cytological characteristics of climbing fibers. Comparable terminals have also been described synapsing on the perisomatic spines in other species and identified as climbing fibers (mouse, Larramendi, '69; Meller and Glees, '69; rat, Altman, '72b; monkey, Kornguth, '72). In the opossum however, all of the presumptive climbing fiber terminals do not have the dense cytoplasmic matrix observed in the immature mouse (Larramendi, '69).
Comparison with later stages suggests that the matrix density increases as maturation progresses.

During this stage another type of terminal with pleomorphic vesicles and symmetric synaptic junctions infrequently contacts the upper area of the soma; these boutons closely resemble those of mature basket cell axons in the rat (Palay and Chan-Palay, '74). Terminals of a similar nature have been described in the developing mouse cerebellum (Larramendi, '69). In the molecular layer parallel fiber synapses with asymmetric junctions and spherical vesicles are easily recognized on Pk cell dendritic spines. The terminals of Pk cell recurrent collaterals could not be distinguished at this time, but were identified during the main dendrite stage.

The Stage of the Main Dendrite (Postnatal Days 61-75)

This stage occupies the period between postnatal day 61 to postnatal day 75 when the Pk cell has relatively few perisomatic spines compared to the preceding stage. During the early perisomatic spine stage two or three dendrites emerge from the apical pole of the Pk cell, but during the main dendrite stage the dendritic tree consists of a single apical dendrite with many secondary and tertiary branches covered with spines. Growth of the dendritic tree is still occurring during this stage as evidenced by filopodia, which are especially frequent at the tips of the dendritic branches. This association of filopodia with dendritic growth has been suggested in the cerebellum and many other areas of the central nervous system (Berry and Bradley, '76; Morest, '69).
At this stage the synaptology of the Pk cell is almost mature. Basket cell axons and recurrent collateral terminals synapse on the soma and their fine structure is very similar to that observed in the adult rat (Palay and Chan-Palay, '74), monkey (Fox, '62) and cat (Mugnaini, '72). The parallel fibers form numerous synapses with dendritic spines in the molecular layer; these also correspond to those previously described (listed above).

The climbing fibers are not completely mature yet; in the adult very few are present on the soma, but at this stage a few climbing fiber terminals still contact spines on the apex of the soma. The fine structure of the climbing fiber boutons is similar to that described in other species except that in some instances the terminals are still not as dark (Larramendi, '69). Climbing fiber boutons are also present on the thorns of the primary and secondary dendrites.

The Mature Stage (After Postnatal Day 76)

The maturation of the Pk cell in the opossum continues after the main dendrite stage for an undefined length of time until the adult size of the dendritic tree is attained. This phenomenon of an extended growth period is also evident in the rat (Pysh and Weiss, '78; Berry and Bradley, '76). Also, the perisomatic spines almost completely disappear. Filopodia are rarely observed on the dendrites of mature Pk cells. Examination of the Pk cell in the adult opossum reveals that its synaptology is comparable to that seen in the main dendrite stage except that climbing fibers are now located on the thorns of the dendrites.
Temporal Correlations Between Cerebellar and Inferior Olivary Development in the Opossum.

The inferior olivary complex first appears in the ventral medulla of pouch young opossums at postnatal day 3 as a ball of cells; by postnatal day 21 (40mm) the subdivisions of the nuclear complex are distinct and similar to those of the adult (Maley and King, '80a). The Pk cells at postnatal day 21 are in the immature stage and the Pk cell layer is 5-6 cells thick (Laxson and King, '82b). The axons of the inferior olivary neurons, the climbing fibers, are present in the cerebellum by postnatal day 14 (30mm) based on data obtained from the retrograde transport of horseradish peroxidase (HRP) from the immature cerebellum (Bishop, personal communication). At postnatal day 21 when synapses on the Pk cell are infrequent, HRP labelled neurons are numerous within the olive. This suggests that although olivary axons reach their target early, a period of time may elapse before synaptogenesis with their primary cerebellar target, the Pk cell, occurs. The phenomenon of a timed delay between afferent arrival and synapse formation has been observed in the cerebral cortex (Rakic, '76, '77; Wise and Jones, '76, '78; Wise et al., '77) in the optic tectum (Kahn, '74; Crossland et al., '74a) and in the inferior olive (Bauer-Moffett and King, '81).

Afferents to the inferior olivary complex also are present early in development: spinal afferents are located within the MAO by postnatal day 12, midbrain axons are present at postnatal day 17, cortical axons arrive later on postnatal day 30, and afferents from the deep cerebellar nuclei are observed at postnatal day 17 (Martin...
et al., '80). Taken together, these data suggest that spino-olivo-cerebellar circuits form prior to cortical and midbrain olivo-cerebellar circuits and cerebellar feedback projections to the inferior olive.

In the rat physiological studies demonstrate that climbing fibers are functional during the latter part of the immature stage (postnatal day 3) (Puro and Woodward, '77; Altman, '72b). Toward the end of the immature stage (postnatal day 25, 50mm) in the opossum an increased incidence of terminals with asymmetrical junctions and spherical vesicles on Pk cells is evident. Taken together this evidence suggests that climbing fibers may be forming functional synapses with immature Pk cells early in opossum cerebellar development. In the rat the physiological responses of Pk cells to climbing fiber stimulation are completely mature at postnatal day 21 (Puro and Woodward, '77). At 21 days in the rat the Pk cell has adult synaptic relationships (Altman, '72b). By the end of the main dendrite stage (postnatal day 60-75) in the opossum the Pk cell has its adult synaptic relationships and may be physiologically mature.

The Role of the Climbing Fiber in Purkinje Cell Development

The development of climbing fibers was described by Ramon y Cajal ('11) as occurring in synchrony with the maturation of the Pk cell dendritic tree. The climbing fiber passes through three phases during its development: 1) the nid stage, 2) the capuchon stage, and 3) the immature climbing fiber arborization stage. The nid stage coincides with the perisomatic spine stage of Pk cell development in the opossum and in rodents (O'Leary et al., '71; Ramon y Cajal, '11).
Several electron microscopic studies have reported that synaptic terminals on somatic spines of Pk cells during this stage are those of climbing fibers (Larramendi, '69; Altman, '72b). During the final two stages of climbing fiber maturation, the Pk cell is in the main dendrite stage. Thus, climbing fibers change their primary postsynaptic site from perisomatic spines to thorns on the primary and secondary dendrites during development (Larramendi, '69; Altman, '72b; Kornguth, '72).

At the beginning of the immature stage of Pk cell development numerous olivary axons are present in the opossum cerebellum (Bishop, personal communication; Martin, '80). Based on histological and autoradiographic observations, climbing fibers may also be present in the rat cerebellum at postnatal day 1; at this time the rat Purkinje cell is in the immature or fusiform stage (Altman, '78a). Because of technical difficulties, there is no physiological data regarding the innervation of Pk cells prior to postnatal day 3 in the rat (Crepal, '72; Puro and Woodward, '77; Mariani and Changeux, '81a and b); however, Mariani and Changeux ('81b) have recorded spontaneous activity in olivary neurons at postnatal day 1 in rat pups. Anatomical studies are needed to determine the initial time of arrival of climbing fibers in the cerebellum. Furthermore, the morphology of these axons has not been described prior to the perisomatic dendrite stage.

The climbing fibers have been hypothesized to induce the development of the Pk cell dendritic tree (Kornguth and Scott, '72). To test this hypothesis the effects of removal of olivary afferents during the immature stage of the Pk cell were investigated in the rat (Sotelo...
and Arsenio-Nunes, '76; Berry and Bradley, '76) and in the kitten
(Kawaguchi et al., '75). Following lesions of the inferior cerebellar
peduncle in neonatal rat pups the adult morphology of Pk cell dendrites
was examined. After this experimental manipulation spines on the
proximal dendrites increased in number and were contacted by parallel
fibers rather than climbing fibers. In contrast, the more distal spiny
branchlets maintained their normal synaptic relationships (Sotelo and
Arsenio-Nunes, '76). Although the dendritic branching patterns were
normal, areas occupied by the dendritic tree were reduced (Berry and
Bradley, '76; Kawaguchi et al., '75). Olivary axons are present in
the cerebellum prior to the immature stage of Pk cell development
(Bishop, unpublished observations). Thus the effect, if any, of pre­
venting climbing fibers from entering the cerebellum early in develop­
ment remains to be investigated.

The outgrowth of the presumptive dendrites which occurs during
the stage of periosomatic dendrites may be influenced by the arrival
of afferents and the subsequent formation of synapses with the Pk cell.
Each Pk cell is multiply innervated by several climbing fibers from
postnatal day 3 to postnatal day 11 (Crepel et al., '76; Mariani and
Changeux, '81). This multiple innervation is maximal at postnatal day
5 (Mariani and Changeux, '80) when the Pk cell is in the perisomatic
dendrite stage in the rat (Dadoune, '66). By postnatal day 11 most Pk
cells are contacted by only one climbing fiber. The morphological
correlates of this regression of the multiple innervation have not been
reported. Further information on the early relationship between the
Pk cells and climbing fibers would be of interest.
The olivocerebellar projection is organized in sagittal longitudinal zones at postnatal day 6 in the rat (Dupont, '81). However, there are no data available on the organization of the climbing fibers in the rat prior to this time. In other areas of the CNS such as the cerebral cortex (Rakic, '80) and striatum (Goldman-Rakic, '80) the afferent distribution is diffuse very early in development and lacks the distinctive columnar and patchy patterns which appear later. It is possible that the olivocerebellar projection undergoes a similar remodeling very early in cerebellar growth.

Possible Mechanisms of Purkinje Cell Migration

During development the Purkinje cells move from a location deep in the cerebellar anlage to form a layer several cells thick in a more superficial position and eventually rearrange to form a monolayer (Altman and Bayer, '78). The mechanisms involved in achieving the adult position of the Pk cell are not well understood. The Pk cells have been shown to migrate radially from the transitory zone located immediately dorsal to the ventricular or neuroepithelial zone to their location in the immature Pk cell layer (Altman and Bayer, '78a). This process occurs prenatally in the rat from embryonic day 15 to at least day 20 when a Pk cell layer can be clearly seen. Although the birth-date of Pk neurons in the opossum are not known, histological observations lead to similar conclusions regarding radial migration. A Pk cell layer several cells thick is first clearly identified from postnatal day 17 (38mm) to postnatal day 21 (41mm); the radial migration of Pk cells from the deeper zone likely occurs immediately prior to
and during this time. The morphology of the Pk cell at this early age in Golgi preparations is similar in some respects to that of immature neurons described during migration in the opossum forebrain (Morest, '70). The axon is present at the basal pole and emanating from the apical pole is a long process which is oriented toward the pial surface. The presence of such a process suggests that it may serve to orient the Pk cell during its movement across the transitory zone. Morest hypothesized that in neuroblasts of the developing forebrain the perikaryon migrates through a primitive process which extends into the marginal zone and that dendritic and axonal differentiation begins while this primitive process is still present. Another similarity is the loss of the apical process which occurs during the latter part of the immature stage of Pk cell development. The Pk cell axon and presumptive dendrites are beginning to differentiate while the apical process can still be distinguished. The sequence also occurs in the opossum forebrain (Morest, '70).

Our Golgi impregnations reveal the presence of radial glia at postnatal day 10 prior to the appearance of the Pk cell layer and also later during the immature stage. No consistent association between radial glia and Pk cells during the early immature stage were observed in our electron micrographs. The concept that neurons born during early stages of development migrate independently whereas those born later may use radial glia as guides is compatible with our observations (Jacobson, '78).

Beginning with the end of the immature stage and continuing through the remainder of Pk cell development in the opossum, Pk cells
are located progressively further from the pial surface. This phenomenon has also been observed in other species (human, Zecevic and Rakic, '76; rat, Altman, '72b; mouse, Hendelman and Aggerwal, '80; hamster, Oster-Granite, '76). Two possible explanations regarding this increase in distance between the cerebellar surface and the Pk cell soma have been proposed (Altman, '72b; Hendelman and Aggerwal, '80). Altman hypothesized that the Pk cell body is fixed in position and the molecular layer expands upward as new parallel fibers are added. Hendelman reviews the evidence for the proposal that the pia is the fixed point and the Pk cell soma undergoes perikaryal translocation. Our data does not provide conclusive evidence in favor of either proposal.

**Summary**

Olivary axons reach the opossum cerebellum prior to the formation of a Pk cell layer; this is earlier in development than has previously been demonstrated in other mammalian species. Our data indicate that there is a delay between the arrival of the climbing fibers and the formation of synaptic contacts by these axons with the Pk cell.

Pk cell development can be divided into five stages based on correlation of data derived from Golgi impregnations and electron micrographs. In particular, somatic processes of the Pk cell have differing cytological features during the perisomatic dendrite and perisomatic spine stages. These two stages can also be distinguished by changes in the synaptic relationships of the climbing fiber with these processes. Our data suggest that the climbing fibers form synapses on the shafts of presumptive dendrites and soma of the Pk cell during the
perisomatic dendrite stage. However, the somatic spines and dendrites of the Pk cell are the postsynaptic site of climbing fiber boutons during the perisomatic spine stage. By the mature stage the climbing fiber has achieved its adult synaptic relationships with the dendritic thorns of the Pk cell.
ILLUSTRATIONS
A series of 1μm thick plastic sections cut in the sagittal plane from the cerebella of pouch young opossums at various ages. The sections in figures 26 and 27 are from the region immediately anterior to the primary fissure. The areas shown in figures 28, 29 and 30 are from Lobule IV or V. All of these sections were stained with toluidine blue. The cortical layers are identified: external granular layer (EGL), molecular layer (MOL), Purkinje cell layer (PK) and internal granular layer (IGL). 1μm plastic sections, 100X.

Figure 26
The cerebellar cortex at PN 19. At this time in Golgi preparations and electron micrographs the Purkinje cells exhibit the features of the early immature stage.

Figure 27
The cerebellar cortex at PN 25. The Purkinje cells are in the late immature stage.

Figure 28
The cerebellar cortex at 33 days. The Purkinje cells are in the perisomatic dendrite stage.

Figure 29
The cerebellar cortex at PN 55. The Purkinje cells are in the perisomatic spine stage.

Figure 30
The cerebellar cortex at PN 73. The Purkinje cells are in the main dendrite stage.
Figures 31-33: The Early Immature Stage (Postnatal Days 19-24)

Figure 31  This Purkinje cell is in the immature stage of its development. An elongated apical process extends toward the pial surface. This process exhibits dilations (block arrows) along its length. Shorter processes (arrowheads) arise from the proximal part of the apical process and the soma. These processes have terminal enlargements and filopodia. The axon (open block arrow) originates from the base of the neuron. Camera lucida drawing. Scale, 10μm. X1000.

Figure 32  The proximal region of an apical process (PK) contains mitochondria, rough and smooth endoplasmic reticulum, lysosomes and a large Golgi apparatus (GA). A terminal forming an asymmetric synaptic contact (arrowhead) can be observed in the adjacent neuropil. Electron micrograph. X15,500.

Figure 33  A Purkinje cell in a PN 21 pouch young opossum. The nucleus is convoluted and extends into the lower area of the apical process. The apical process has numerous mitochondria, microtubules and rough and smooth endoplasmic reticulum. The edges of the dilations (block arrows) of the apical process contain
Figure 33 (cont.) aggregations of rough endoplasmic reticulum. A shorter process (asterisk) arises from the apical process; this corresponds to those observed in Golgi impregnations. Electron micrograph. X9,750.

Figure 34 An enlargement of the process indicated by the asterisk in figure 33. Electron micrograph. X38,750.

Figure 35 A subsurface cistern (block arrow) adjacent to the cell membrane of a Purkinje cell. Electron micrograph. X38,750.
PLATE VIII

Figure 36  
A Purkinje cell in a PN 21 opossum. The axon (open block arrow) originates from the base of the soma. A terminal with round vesicles forms an asymmetric junction (arrowhead) with a flocculent profile in the neuropil next to the Purkinje cell. Electron micrograph. X15,500.  
Inset: The synapse labelled with an arrowhead in figure 36 is shown at a higher magnification. Electron micrograph. X49,500.

Figure 37  
A bouton (curved arrow) with spherical vesicles and an asymmetric synaptic junction contacts an immature Purkinje cell. The nucleus is indented and the Golgi apparatus (GA) is present in the apical cytoplasm. Electron micrograph. X23,750.

Figure 38  
A flocculent process which arises from a Purkinje cell (PK) is contacted by a terminal filled with round vesicles. Electron micrograph. X49,500.

Figure 39  
A small bouton with round vesicles forms an asymmetric synaptic contact on a profile near the perikaryon of a Purkinje cell (PK). Electron micrograph. X49,500.
Figures 40-48: The Late Immature Stage (Postnatal Days 25-32)

Figure 40

An immature Purkinje cell with several processes (arrowheads) arising from the apical area of the fusiform cell body. In some cases these processes end with expansions (asterisks). Shorter processes (small arrows) originate from the base of the neuron. The axon is indicated by an open block arrow. Camera lucida drawing. Scale, 10μm. X1000.

Figure 41

The apical region of this Purkinje cell contains an extensive Golgi apparatus (GA). The soma is fusiform in shape. Less extracellular space appears in the surrounding neuropil than in the early immature stage (compare to Fig. 33). Electron micrograph. X9,750.

Figure 42

The basal area of an immature Purkinje cell exhibits a paucity of organelles. This was frequently observed and can also be seen in figure 41. Electron micrograph. X23,750.

Figure 43

A process (outlined with arrowheads) which originates from the lateral aspect of the Purkinje cell body (PK) contains microtubules, mitochondria and smooth endoplasmic reticulum. In the adjacent neuropil, terminals containing round vesicles form asymmetric synapses.
PLATE IX (CONTINUED)

Figure 43 (cont.) with flocculent profiles (open block arrows – 1).

Electron micrograph. X23, 750.
A bouton (block arrow - 1) with round vesicles and an asymmetric synaptic junction synapsing on a somatic process (SP) of a Purkinje cell (PK). Electron micrograph. X38,750.

Several terminals (block arrows - 1) with similar features to terminals in figures 43 and 44. They synapse on either a Purkinje cell process (SP) or on profiles in the surrounding neuropil. Electron micrograph. X19,000.

A terminal of the first type (block arrow - 1) synapses with a somatic process (SP) from a Purkinje cell (PK). The bouton indicated by the block arrow numbered 2 also has round vesicles and forms an asymmetric synapse, but the vesicles are much more densely packed. Electron micrograph. X23,750.

An area of neuropil adjacent to the Purkinje cell (PK) soma. Small boutons (block arrow - 3) contain round vesicles and are presynaptic to flocculent profiles. The other type of terminal illustrated (block arrow numbered 4) is larger than those previously described and is populated with both clear round vesicles and large dense core vesicles. Electron micrograph. X38,750.
Figure 47 (cont.) **Inset:** This type of terminal infrequently forms punctate synapses (block arrow). Electron micrograph. X38,750.

Figure 48 Neuropil near the apical region of a Purkinje cell soma with numerous boutons having a similar morphology to the terminal indicated by the block arrow - 3 in figure 47. Electron micrograph. X49,500.

**Inset:** An example of a terminal of the third type. Junctions are made with a process from the apex (asterisk) of a Purkinje cell and a flocculent profile. Electron micrograph. X23,750.
Figures 49-57: The Perisomatic Dendrite Stage (Postnatal Days 33-42)

Figure 49
A Purkinje cell during the perisomatic dendrite stage has numerous long processes arising from its soma. The axon (open block arrow) originates from the base of the cell. Camera lucida drawing. Scale, 10μm. X1000.

Figure 50
The long processes arising from the Purkinje cell body contain microtubules, mitochondria, smooth and rough endoplasmic reticulum and are therefore identified as presumptive dendrites (PD). A large region of cytoplasm packed with organelles is seen above the nucleus. Bundles of parallel fibers (stars) surround the soma. Electron micrograph. X9,750.

Inset: A subsurface cistern (block arrow) is present next to the Purkinje cell membrane. Electron micrograph. X49,500.

Figure 51
A higher magnification of a presumptive dendrite (PD) reveals that it is the postsynaptic site of terminals (arrowheads) with round vesicles and asymmetric junctions. These terminals are present both on the shaft and on spines (asterisk) arising from the dendrite. Electron micrograph. X38,750.
PLATE XI (CONTINUED)

Figure 52  A small bouton making synaptic contact with a presumptive dendrite (PD) which extends into the molecular layer. Electron micrograph. X23,750.
PLATE XII

Figure 53  Another example of a Purkinje cell during the perisomatic dendrite stage. A recurrent collateral (open block arrow labelled RC) originates from the Purkinje cell axon (open block arrow). Camera lucida drawing. Scale, 10μm. X1000.

Figure 54  An axosomatic terminal with round vesicles also forms synapses (arrowheads) with flocculent profiles (S) which are possibly somatic spines of the Purkinje cell (NUC). Electron micrograph. X38,750.

Figure 55  An axon (Ax) from a Purkinje cell which contains numerous microtubules, smooth endoplasmic reticulum, occasional ribosomes and a subsurface cistern (block arrow). Small spines (stars) are present; similar protrusions can be observed in Golgi impregnations at this age (Fig. 53). Electron micrograph. X23,750.

Figures 56, 57  Presumptive dendrites (PD) from the apical area of Purkinje cells are postsynaptic to elongate terminals which are filled with round vesicles and form asymmetric synaptic junctions (arrowheads). Electron micrographs. X38,750.
Figures 58-60: The Perisomatic Spine Stage (Postnatal Days 43-60)

Figure 58  A Purkinje cell at the beginning of the perisomatic spine stage. Numerous somatic protrusions (arrows) are present. A process (arrowhead) reminiscent of the presumptive dendrites of the previous stage arises from the apical area of the soma. The axon is indicated by an open block arrow. Camera lucida drawing. Scale, 10µm. X1000.

Figure 59  A Purkinje cell toward the end of the perisomatic spine stage which is characterized by sessile spines (arrows). The dendritic tree has increased in size and complexity (compare to Fig. 58). Camera lucida drawing. Scale, 10µm. X1000.

Figure 60  Numerous somatic spines (S) originate from all areas of the Purkinje cell body. They are characterized by a flocculent matrix and occasional profiles of smooth endoplasmic reticulum. Electron micrograph. X14,500. 
Inset: Climbing fibers during the nid stage surround the Purkinje cell soma. At this time the Purkinje cells are in the perisomatic spine stage. Camera lucida drawing. X900.
Figures 61-63  Higher magnifications of selected areas from figure 60 illustrating the cytological features of the perisomatic spines (S) and the morphology of the presynaptic terminals. The terminals (Figs. 61 and 62) contain round vesicles and form asymmetric synaptic junctions. Electron micrographs. X30,250.

Synaptic configuration which is frequently observed in the molecular layer during this stage. The postsynaptic element (asterisk) in some instances can be identified as a Purkinje cell dendritic spine. Electron micrograph. X49,500.

The plane of section is parallel to the surface of a Purkinje cell dendrite. Glial processes (GI) approximate the dendrite at this stage. The dendritic spines (asterisks) are postsynaptic to boutons filled with clear round vesicles. One of these boutons (outlined by block arrows) is large and is penetrated by a spine (asterisk). Electron micrograph. X49,500.

An area of the molecular layer immediately above the Purkinje cell layer. The main dendrite (PK D) of a Purkinje cell branches and is contacted by synaptic boutons (arrowheads). Glial processes (GI) partially cover the surface of the dendrites. Electron micrograph. X14,500.
Figures 68-71: The Main Dendrite Stage (Postnatal Days 60-75)

Figure 68  A Purkinje cell during the main dendrite stage of development. The primary dendrite is longer and the dendritic arborization has increased in comparison to the previous stage. Some somatic spines (arrows) are still present. The axon (open block arrow) arises at the base of the soma. Camera lucida drawing. Scale, 10μm. X1000.

Figure 69  A bouton of a basket cell axon is closely apposed to a Purkinje cell (PK) soma. A somatic spine (S) is partially surrounded by glial processes. Subsurface cisterns (arrowheads) line the cell membrane. Electron micrograph. X49,500.

Figure 70  A basket cell axon terminal (B) contains pleomorphic vesicles and forms a symmetric synaptic junction with the soma and the neck of a somatic spine (S). Electron micrograph. X49,500.

Figure 71  A terminal tentatively identified as originating from a Purkinje cell recurrent collateral. This synaptic terminal has a darker cytoplasmic matrix and is more densely packed with vesicles than an adjacent basket cell axon terminal (B). Electron micrograph. X49,500.
Figure 72 A region of a Purkinje cell body with three somatic spines (S); in comparison to those of the preceding stage these spines are small and are usually not postsynaptic elements. Subsurface cisterns are indicated by arrowheads. Electron micrograph. X23,750.
Figure 73  A terminal containing round vesicles forms a long asymmetric synaptic junction with a somatic spine (S) on the apical part of the cell body. Electron micrograph. X49,500.

Figure 74  A climbing fiber travels in apposition to the Purkinje cell main dendrite and makes a synaptic contact on a dendritic thorn (asterisk). Subsurface cisterns (arrowheads) also line the dendritic membranes. Electron micrograph. X15,500.

Figure 75  An extensive climbing fiber bouton which is characterized by a dark cytoplasmic matrix forms synaptic contacts on dendritic thorns (asterisks). This terminal is adjacent to the most proximal portion of the main dendrite (PK D). Electron micrograph. X23,750.

Figure 76  A higher magnification of an area in figure 75. The climbing fiber bouton can be seen to contain spherical vesicles and numerous microtubules. Electron micrograph. X49,500.
Figure 77

A series of drawings of Golgi-impregnated Purkinje cells from the immature stage (upper left corner) to the main dendrite stage (lower right corner). Transitional forms between stages are shown. The bar represents 10 microns.
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