Postnatal Development of the Cerebellar Cortex in the Rat

II. PHASES IN THE MATURATION OF PURKINJE CELLS AND OF THE MOLECULAR LAYER

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ABSTRACT The growth and synaptic maturation of Purkinje cells and of the molecular layer were studied in the cerebellar cortex of rats aged 0, 3, 5, 7, 10, 12, 15, 21 and 30 days with histological (including Golgi), histochemical, autoradiographic and electron microscopic techniques. Five phases were distinguished in the maturation of Purkinje cells. During the first phase, Purkinje cells become dispersed and aligned in a monolayer but as yet few or no synapses are formed. Next, two transient structures appear: a hypertrophied apical cone composed of "reticular" cytoplasm, and lateral perisomatic processes which establish conspicuous asymmetrical synapses with climbing fibers. During the third phase the perisomatic processes disappear; the "reticular" cytoplasm streams upward into the growing dendrites; the soma is invaded by permanent inconspicuous, symmetrical synapses of basket cells; and, finally, it is surrounded by glial processes, which marks the end of the synaptic maturation of the soma. During the fourth phase parallel fibers form synapses with dendritic spines in the lower half of the molecular layer. During the fifth phase, which occurs after the disappearance of the external germinal layer, parallel fibers establish synapses with dendritic spines in the upper molecular layer. The "march" of synaptogenesis in the molecular layer from the bottom upward is characterized by three successive events: an initial gradient in the appearance and disappearance of coated vesicles, heralding synaptogenesis; a similar subsequent trend in the formation of synapses; and finally, the interposition in the same sequence of glial processes between Purkinje cell dendrites and parallel fibers, marking the cessation of synaptogenesis.

The postnatal acquisition and differentiation of the precursors of granule, basket and stellate cells in the cerebellar cortex of the rat proceed concurrently with the maturation of the prenatally-formed Purkinje cells. In the preceding paper (Altman, '72a) we described the first steps in the formation and maturation of cerebellar microneurons. This paper deals with some of the distinguishable phases in the differentiation of Purinje cells, in terms of cytological and ultrastructural changes occurring within the cells and the synapses they establish with other maturing elements of the cerebellar cortex. Aspects of the maturation of the granular layer will be described in the succeeding paper (Altman, '72b). The normal growth of Purkinje cells is dependent on the synchronous maturation of cerebellar microneurons. In a separate series of studies we have been examining the effects of selective elimination of microneurons by low-level x-irradiation on the development of Purkinje cells and the cortex as a whole. The evidence indicates (Altman and Anderson, '72) considerable autonomy in the growth of Purkinje cells but also drastic alterations in their mode of differentiation, most of which can be attributed

to the changed morphogenetic environment produced by elimination of the microneurons.

MATERIALS AND METHODS

The material utilized for this study was described in detail in the preceding paper (Altman, '72a). Cerebella from several hundred Long-Evans rats were prepared for histological, histochemical, autoradiographic and electron microscopic examination. Over 100 cerebella, ranging in age at daily intervals, from 0–21 days, and also some 30-day material, were stained with cresyl violet and hematoxylin and eosin. Autoradiograms of cerebella were obtained from 64 rats that received at different ages a single dose of thymidine-H^3, from 20 rats that received two successive daily doses between 0–19 days, and from ten rats that received four successive daily doses during the same period. Sections from the cerebella of 53 rats aged 0, 3, 5, 7, 10, 15, 21 and 30 days were prepared for the following histochemical reactions: AChE, SDH, LDH, CYO, NAD, and NADP. Cerebella from 28 rats of the preceding age groups were prepared with the Golgi-Cox technique. Finally, the pyramis of 49 cerebella of the preceding ages (and 12 days of age) were examined with electron microscopy. (Summarized in tables 1–7 in the preceding paper.)

RESULTS

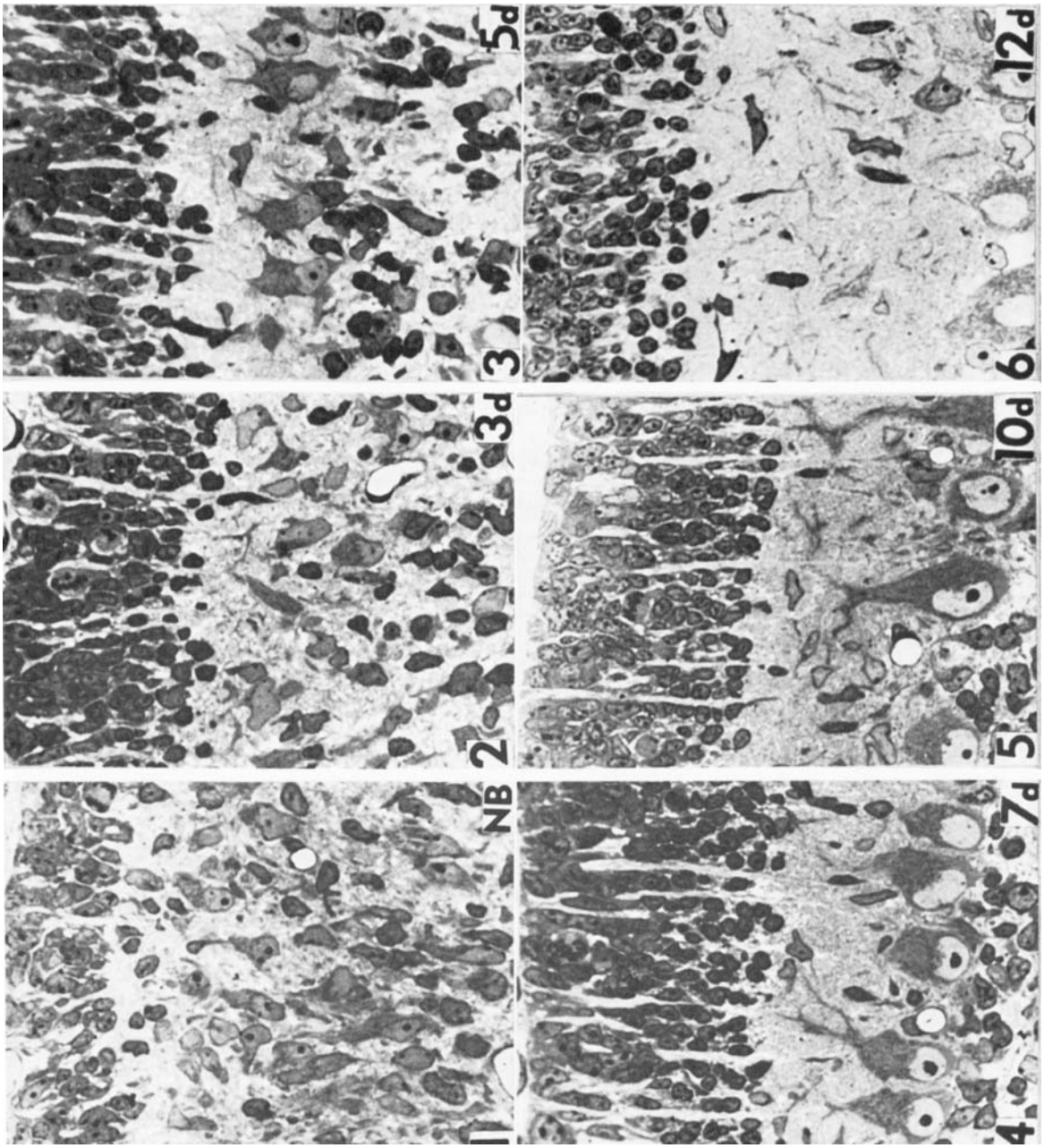
1. *Migration and dispersion of Purkinje cells: the presynaptogenic phase*

Light microscopic observations. The Purkinje cells are the most numerous cellular elements of the cerebellar cortex of the newborn rat and they are distributed haphazardly about 6–12 cells deep between the thin molecular layer and the white matter (fig. 1). The cytoplasm of the Purkinje cells is sparse, the nuclei are polymorphous, and many of the Purkinje cells are spindle-shaped. In general, the nuclei of the more superficially situated Purkinje cells are larger and paler than those situated deeply, and the latter are intermingled with and not clearly distinguishable from cells of the medullary layer. At three to four days, the majority, though not all, of the Purkinje cells have become dispersed over the cortex in a monolayer (fig. 2) and an occasional Purkinje cell is seen in the pyramis which shows indications of the commencement of formation of an apical mitre or cone (many such cells are seen at this age in the earlier-maturing nodulus and ventral uvula).

The undifferentiated state of the cerebellar cortex in general at zero and three days was indicated by the sparseness or absence of histochemical staining for various enzymes. Among the oxidative enzymes, staining for succinate dehydrogenase at birth indicated very mild reaction in the undifferentiated cells of the external granular layer and some reaction in the upper half of the wide zone of Purkinje cells, mostly located among the cell nuclei. In the three-day old animals, there was some indication of preferential staining in the apical cytoplasm of the more superficial Purkinje cells. The staining for succinate dehydrogenase was somewhat more pronounced in the anterior than posterior vermis. Comparable results were obtained in the distribution of mild staining for tri- and diphosphopyridine nucleotide diaphorase. There was minimal or no reaction at these ages in the cerebellar cortex for cytochrome oxidase. More marked staining was seen for lactate dehydrogenase than the other enzymes examined. In the newborn animals, there was mild staining around the nuclei of external germinal cells; stronger staining in the cytoplasm of Purkinje cells outlining their unstained nuclei. In the 3-day old animals, there were signs of a preferential staining of the apical cytoplasm of Purkinje cells.

Electron microscopic observations. In newborn animals, two types of Purkinje cells are seen. There is a high concentration of cells with darkly-staining lobulated polymorphous nuclei which give the impression of being ameboid, migratory cells. More superficially situated are lighter staining cells with less irregularly shaped nuclei. The cells with polymorphous nuclei often have the lobulated face of their nuclei oriented toward the surface of the

Figs. 1–6 Low-power photomicrographs of "thick" Epon sections of the cerebellar cortex in infant rats of different ages in the region of the pyramis (0, 3 days) or the pyramis itself. Stained with azure B. × 640.



Figures 1–6

cortex (fig. 14). In these cells, the cytoplasm is usually sparse; but cells with bizarre-shaped perikarya are also seen, such as extremely elongated spindle-shaped cells, cells with large club endings, and the like (fig. 15). The cytoplasm of the migratory cells has few organelles, except for a rich accumulation of clusters of free ribosomes. Even the axons have this structure, although some microtubules could be identified. Occasionally a microsome-studded, large amorphous inclusion is present in the cytoplasm of these cells. In the more regularly-shaped and lightly-staining Purkinje cells, the large apical portion of the cytoplasm tends to have a "reticular" organization, with a high concentration of Golgi apparatus, agranular cisterns, an occasional multivesicular body and coated vesicle, and more rarely, basal bodies or a cilium. This structural organization is indicative of intensive growth.

Some of the Purkinje cells that are seen in the three-day old animals are indistinguishable from the embryonic cells seen in the newborn animals, but the proportion of the polymorphous cells is decreased. Most of the Purkinje cells, which are now partially strung out in a monolayer, have lightly staining, more regularly shaped nuclei and the apical cytoplasm has become enlarged with a peripherally placed, ribosome-rich portion and a variably sized central core that has a "reticular" organization (fig. 16). The apical portion of the cytoplasm typically has several multivesicular bodies, in the interior of which there are many, few or no vesicles. Fused subsurface cisterns, usually short but sometimes running for some length, are quite common in a lateral or apical position (fig. 17); open coated vesicles in this position are less frequently seen (see below). The axon, in the few instances in which it could be identified, typically had a high concentration of parallel microtubules (fig. 16). In general, synapses are absent on the Purkinje cell soma in the newborn animals (this conclusion is based on the observation of several hundred cells) and they are extremely rare in the three-day old animals. Very rarely, an asymmetrical synapse is seen with round large vesicles and long boutons in an apical position. More often, desmosome-like attachment membranes are visible between the perikarya of Purkinje cells and unidentified processes with a tubular organization, and sometimes even between adjoining Purkinje cells.

In summary, during this early phase of development, covering about four days after birth, the Purkinje cells move into their final position in the cortex and become strung out in a monolayer. The nuclei are being transformed from a shape suggesting migration to one indicative of a stationary state; the cytoplasm is showing early signs of the commencement of growth in an apical direction, but as yet few synaptic contacts are established with the soma.

2. *Growth of the transient apical cone and the perisomatic processes of Purkinje cells*

Light microscopic observations. In the molecular layer of five-day old rats, a slight increase is seen in depth to which contribution is made by the growing apical cones of the Purkinje cells (fig. 3). These apical cones may be rectangular or pyramidal in shape and are often quite bizarre in appearance. The apical cones tend to be formed where the Purkinje cell soma is contiguous with the molecular layer, that is, toward the surface of the cortex. In counterstained Nissl sections, the nucleus of the Purkinje cells which have become round and large, tend to stain very lightly, whereas in the apical cone, two well-stained zones may be distinguished: a basophilic rim (demarcating the location of the region rich in ribosomes) and in the center of the apical cone a more extensive acidophilic region (see below). With the growth of the extensive apical cones into the molecular layer, the nonoverlapping Purkinje cells perforce become strung out and form strictly a monolayer. Associated with this is an enlargement of the surface area of the cortex through the accelerated formation of gyri and sulci that give rise to the formation of sublobules.

Further increase is seen in the growth of the apical cones in the seven-day old rats at which age the development of this transient cytoplasmic enlargement reaches its apex (fig. 4). But regional differences are present. For instance, in

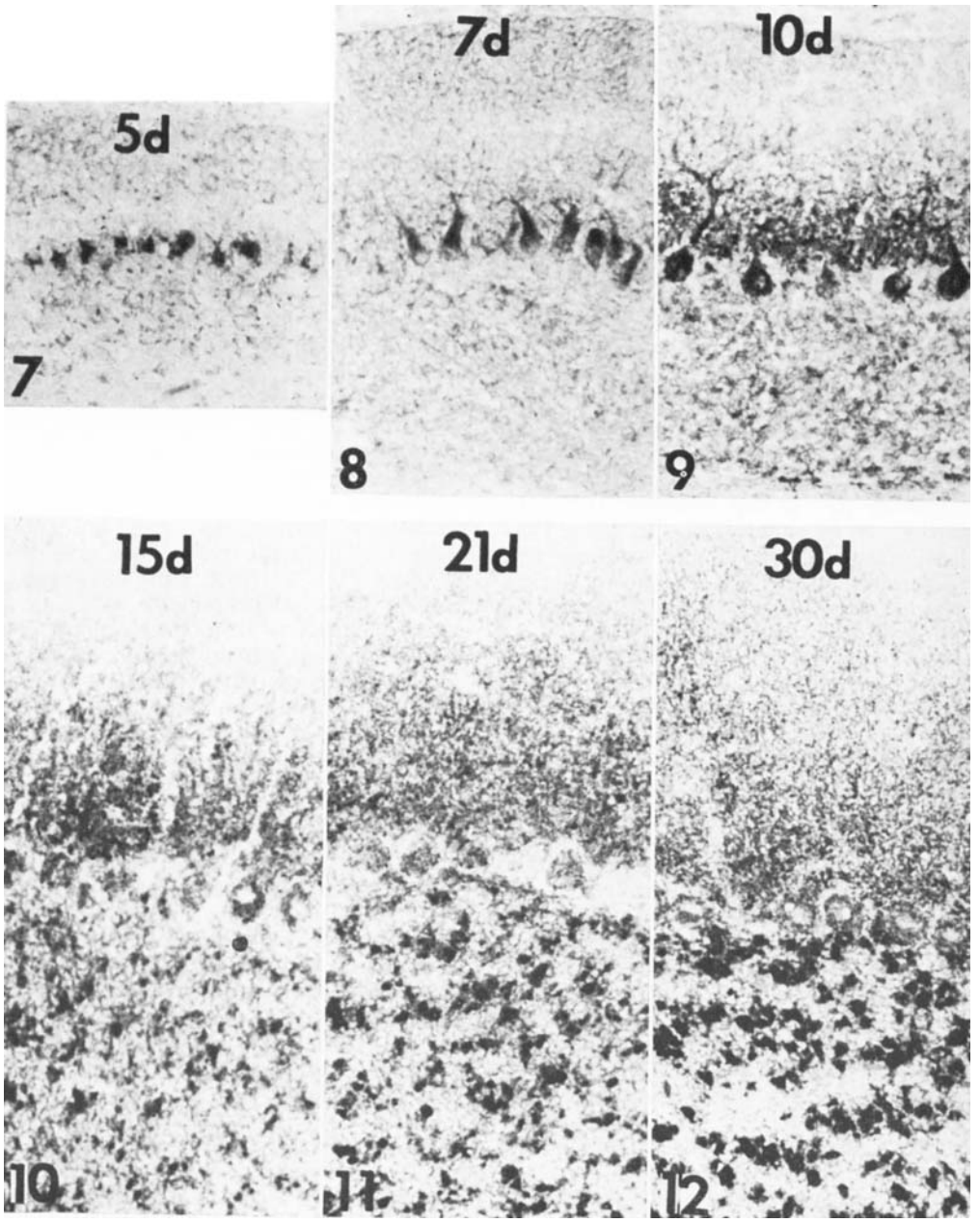
the posterior vermis, a ventrodorsol gradient is discernible in the maturation of the Purkinje cells and in the increasing depth of the molecular layer. Thus, at seven days in the nodulus, the Purkinje cells have developing primary dendrites, several such cells are seen in the ventral uvula, but only a few in the dorsal uvula. Within the pyramis, the Purkinje cells are more mature in the ventral than the dorsal half, and in the tuber, many Purkinje cells are seen that have not reached the apical cone phase at this age.

At five days of age the cerebellum is characterized by a marked staining of the cell bodies of the Purkinje cells for succinate dehydrogenase, against a background of light or no staining of the external germinal, molecular, granular, and medullary layers (fig. 7). In the Purkinje cells, the staining was pronounced in the apical cytoplasm; thus, this procedure outlines well the changing shape of this growing region. In the pyramis of five-day old animals (fig. 7) the apical cone shape predominated; in the seven-day old animals (fig. 8) several Purkinje cells have an outgrowing primary dendrite with an occasional larger branch. At this age Purkinje cells with well developed primary dendrites outlined by this reaction are more frequent in the nodulus and uvula; and along the central sulcus the earlier maturing Purkinje cells in the depth of the fissure are mostly in the stage of primary dendrite development, those situated more dorsally still in the apical cone stage. The staining reactions for the other oxidative enzymes (lactate dehydrogenase, cytochrome oxidase, triphosphopyridine nucleotide and diphosphopyridine nucleotide diaphorase) were similar to succinate dehydrogenase. All except one of these techniques (cytochrome oxidase) outlined the apical cytoplasm of the Purkinje cells in the different lobules according to the developmental stages they have reached. At these ages, staining is not seen with any of these oxidative enzyme reactions in the molecular layer itself.

In Golgi-impregnated sections in the pyramis, and also in the nodulus and uvula, the somata of Purkinje cells in the seven-day old rats was full of irregular, long and thick lateral processes. Occasionally these perisomatic processes had club-like endings. The axons of Purkinje cells tended to be thick and a few were seen with recurrent collaterals. Presumed climbing fiber terminals were seen in appreciable numbers that had a bushy ending resembling the shape of the Purkinje cell soma.

Electron microscopic observations. There is an appreciable increase in the size of the cell bodies of the Purkinje cells at five days. This is due to an enlargement of the nucleus which assumes a lightly-staining, round shape, and to the extensive growth of the apical cytoplasm commonly in the shape of a miter or a cone but sometimes in quite irregular forms. Occasionally an elongated primary dendrite is present at this age. But other Purkinje cells still have darkly-staining lobulated nuclei and poorly developed apical cytoplasms. The typical Purkinje cell of the pyramis has a thin rim of cytoplasm around the nucleus basally and laterally, containing a few mitochondria and being richly supplied with free and membrane-bound ribosomes ("ribosomal" structure). The enlarged apical cytoplasm has ribosomes concentrated in a perinuclear and peripheral position but the extensive core itself has a reticular structure being extremely rich in vesicles of the agranular reticulum and in mitochondria. Several coated vesicles, multivesicular bodies and an occasional microtubule are also present. Where an outgrowing dendrite is seen, the periphery is rich in ribosomes; the central core contains a few microtubules. Where axons could be identified, they always had a tubular organization. An occasional axon had a dense longitudinal band, composed of an aggregate of contiguous microtubules.

At this age, as in younger animals, short or long subsurface, fused cisterns are commonly seen in the lateral or apical part of the Purkinje cell soma (figs. 17–18). These fused cisterns are frequently situated opposite parallel fibers and inside the cell they are occasionally contiguous with mitochondria. The glial processes that surround the somata of Purkinje cells in older animals were not yet identifiable in these. Synapses are extremely rare in the molecular layer at this age, but they are



Figs. 7–12 Low-power photomicrographs of sections of the cerebellar cortex (pyramis) stained for succinate dehydrogenase. × 256.

seen in growing numbers on the somata of Purkinje cells. Usually these are Gray, type I synapses, with conspicuous, asymmetrical dense membranes (the post-synaptic membrane being thicker than the presynaptic), situated directly on the soma (fig. 19) or less frequently at this age, on small protrusions, or perisomatic proc-

esses (fig. 20). The concentration of vesicles varies; the dense membranes are usually present over most of the contact area between the bouton and Purkinje cell soma. It is assumed that these are transient climbing fiber synapses which, in somewhat older animals, are always seen on perisomatic processes (fig. 21).

In the pyramis of seven-day old rats, darkly-staining polymorphonuclear Purkinje cells are no longer seen. The nuclei are consistently round, large and lightly staining, and have a prominent nucleolus (fig. 22). An even more marked change is the appearance of many perisomatic processes on the basolateral and lateral aspect of the soma. These processes, which are quite rare in the adult, are seen in a variety of shapes: as light protrusions or long slender cylindrical structures, but most commonly as tortuous invaginated structures surrounding or being surrounded by equally complex boutons. Their cytoplasm is devoid of ribosomes but cisterns are common in them. These perisomatic processes generally have synapses on them, frequently very complex ones (fig. 21). The synapses are of the conspicuous, asymmetrical and polyvesicular kind, and typically there are many attachment sites present, of which one is sometimes directly on the soma. In several instances, instead of a perisomatic synapse, similar complex junctures are embedded entirely in the soma. These observations suggest that the formation of perisomatic processes at this age represents the extension of the surface of the soma for this transient synapse, presumably that of climbing fibers. In the material available, symmetrical, oligovesicular type of synapses which are common in adults, were only rarely seen at this age. They were preferentially located on the apical cone and were associated with elongated boutons.

Associated with these changes in the perinuclear region, there is a considerable increase in the size of the supranuclear cytoplasm of the Purkinje cells (fig. 22). This growth of the apical cone is attributable to a further enlargement of the transient reticular core which is extremely rich in the typical organelles of this growth region. In many Purkinje cells, a primary dendrite may also be seen, with some larger branches and occasionally with a few finer branchlets. These primary dendrites are extremely rich in mitochondria (often elongated in shape), in cisterns (some having an irregular tubular shape), and in microtubules. Frequently, a few multivesicular bodies and lysosomes are also seen.

In contrast to the soma, the transient apical cone has few developed synapses, but a few open coated vesicles or dense membrane junctions are seen in contiguity with parallel fibers. On the outgrowing primary dendrites, occasionally a mature synapse of the asymmerical type is seen, but much more frequent are coated vesicles opposite parallel fibers in various stages of presumed unfolding, and symmetrical dense membrane junctions, or "early synapses" (figs. 23, 24). Where open coated vesicles and "early synapses" are seen, they are often present in large numbers. The open coated vesicles of the Purkinje cell dendrites typically have the parallel fibers drawn toward them and occasionally a protruding portion of the parallel fiber is engulfed by the coated vesicle (see below). The coated vesicles are less frequently present in the parallel fibers and in rare instances, open coated vesicles in both the Purkinje cell dendrite and the associated parallel fiber are facing each other. Not infrequently the parallel fiber situated opposite a coated vesicle has a dense junctional membrane and sometimes the unfolded coated vesicle of the Purkinje cell dendrite is itself continuous with a dense membrane. Rarely, synaptic vesicles are seen in the parallel fibers which are facing coated vesicles or flat dense membranes.

At this age, the parallel fibers and other cells or cell processes are in most regions directly contiguous with the soma or dendrite of Purkinje cells. But in some regions of the soma, glial processes are interposed between the initial portion of the primary dendrite and parallel fibers. Where these glial barriers are present, there are usually no coated vesicles, but quite often the Purkinje cell cytoplasm has one or more fused subsurface cisterns in these positions.

Conclusion. During the second phase of the maturation of Purkinje cells, two

transient cytoplasmic formations appear: the apical cone composed of a large concentration of mitochondria and agranular vesicles (which is coupled with intense oxidative enzyme activity) and many perisomatic processes on the lateral aspect of the soma. The appearance of the latter is associated with the establishment of complex asymmetrical synapses, presumably those of climbing fibers. Another phenomenon may be the appearance of "early synapses" between parallel fibers and the outgrowing dendrite of Purkinje cells; these may be transient junctions in view of the fact that in adults parallel fibers do not form synapses with the smooth surface of the primary dendrite.

3. *Maturation of the synaptic domain of the Purkinje cell soma (and the Maturation of basket cells)*

Light microscopic observations. In the pyramis of eight-day old animals, the majority of Purkinje cells have well-developed apical cones. In the nine to ten day old animals, the apical cones are still prominent in the dorsal half of the pyramis (fig. 5), but in the earlier-maturing ventral half, the perikarya of the Purkinje cells begin to assume a disc or pear shape. (At this age, many of the Purkinje cells of the nodulus have prominently stained enlarged basal cytoplasms which represents another transitional stage of maturation, whereas in the folium and tuber, they still are in the early apical cone stage.) By the eleventh to twelfth day, the disc or pear shaped Purkinje cells are predominant in the ventral half of the pyramis (fig. 6) and are also numerous in its dorsal aspect. A few Purkinje cells have enlarged basal cytoplasms.

Histochemical staining for succinate dehydrogenase in the ten-day old rats outlines the large apical cones, the primary dendrites, and a few short secondary branches of the Purkinje cells (fig. 9). In early maturing regions of the vermis (nodulus and ventral aspect of uvula) the Purkinje cell soma assumes a doughnut shape due to the apparent retraction of staining reaction from the apical regions together with a more uniform spread of perikaryal staining around the unstained nucleus. Concomitantly diffuse staining appears in the lower one-third of the molecular layer which replaces the discrete staining of the Purkinje cell primary dendrites. In the pyramis this is seen at a later age (fig. 10). Similar changes are seen with LDH, CYO, NAD staining. Of these, LDH and CYO provide the best resolution and there were indications of preferential staining at this age of the soma and short processes of what were tentatively identified as basket cells. The transient reaction of the perikarya of Purkinje cells with AChE staining was described elsewhere (Altman and Das, '70). This staining is much more pronounced in some lobules (like the nodulus) than in others. Mild staining of Purkinje cell perikarya is seen at three days, it is moderate at five, becomes intense at seven, but declines by the tenth day (to disappear permanently a few days thereafter).

In Golgi-impregnated sections, two types of Purkinje cells are seen: some with a rich plexus of perisomatic processes but no dendrites and, more frequently, others with no perisomatic processes, but one or two vertically oriented smooth dendrites with tufts of fine branches at their tips. The most mature Purkinje cells seen have the branching configuration of adult Purkinje cells but with a limited spread of the arborizing dendrites. Purkinje cells whose axon was visible usually had one or several recurrent collaterals. In one instance, such a collateral was seen to form a contact with the soma of another Purkinje cell.

Electron microscopic observations. In ten-day old rats, Purkinje cells with a primary dendrite and a few secondary branches are frequent, but in almost all instances these issue from a maximally enlarged apical cone (fig. 25). This hypertrophy is associated with further growth of the reticular core of the apical perikaryon filled to capacity with mitochondria and agranular reticulum. The soma of the Purkinje cell is now surrounded by patches of glial covering, by the cytoplasm of other cells such as descending granule cells, and by a few boutons with synapses. Perisomatic processes on the soma which have asymmetrical synapses on them have become much less frequent

than in the seven-day old animals, but they are still encountered. The concentration of boutons with symmetrical synapses directly on the surface of the soma has increased. Generally, these boutons have few vesicles and the symmetrical membrane "thickening" is quite thin and inconspicuous. It is presumed that these are early basket cell terminals.

The primary dendrites of Purkinje cells are characterized at this age by an excessive concentration of mitochondria which in some instances appeared to occupy as much as three-fourths of the available space (fig. 26B). Typically, these mitochondria are elongated in shape and give the impression of streaming in the outgrowing dendrites. Among the mitochondria, scattered rosettes of microsomes, microtubules, and elongated vesicles, or macrotubules, are common, and often a multivesicular body or a closed coated vesicle is present. In most places, the Purkinje cell primary dendrite and the thinner or shorter secondary branches are directly contiguous with parallel fibers. In other places, particularly in the main dendrites close to the soma, thin glial processes are interposed between the parallel fibers and the dendrites. Where there is direct contiguity between parallel fibers and the primary dendrite or branches of the Purkinje cells, open coated vesicles in various stages of unfolding and apposed dense membranes were quite frequent. Occasionally, the parallel fiber was partially "sucked" into the cavity of the open coated vesicle and in other instances, continuity was evident between the "coat" of the coated vesicles and the dense membrane of the desmosome-like early juncture (these are illustrated in 12-day old rats; see below). Rarely a large bouton with symmetrical synapse and a high concentration of vesicles was seen nestled in the hollow of a bifurcating branch. Even less often an asymmetrical synapse of presumed parallel fibers was seen on the dendrite or branchlet of the Purkinje cell. The axons of Purkinje cells typically had in their core a system of fused microtubules.

In the pyramis of 12-day old animals, some of the Purkinje cells have large apical cones with extensive reticular cores, but more common are perikarya with regressed apical cones (fig. 26); a rounder soma with an accumulation of ribosomes that have now become predominantly membrane bound; a massive apical dendrite; and a variable number of short secondary and tertiary branches (fig. 30). Typically, these dendrites have a high concentration of elongated and apparently streaming mitochondria (which are lined up parallel to the long axis of the dendrites) and various other "growth" organelles such as multivesicular bodies, macrotubules, and closed coated vesicles (fig. 27).

On the somata of several Purkinje cells, complex perisomatic synapses are still present. The boutons, which are rich in vesicles and often also have a few dense core vesicles, are engulfed by the perisomatic processes and the synaptic dense membranes are of the conspicuous asymmetrical type. In several instances, in addition to the asymmetrical synapse, desmosome-like symmetrical junctions were also seen at the contact point between the bouton and the membrane of the soma itself. On the somata of other Purkinje cells, elongated or round boutons are seen with a sparser concentration of vesicles and these have inconspicuous symmetrical synapses with the soma itself (fig. 28). This type of synapse which is identified as that of basket cells has become quite numerous at this age.

On the primary dendrites of Purkinje cells, where there is direct continguity with parallel fibers, open coated vesicles opposite parallel fibers are often seen (fig. 29). Where the parallel fibers are separated from the primary dendrite by a glial sheath, these open coated vesicles are absent (fig. 31), although closed coated vesicles are occasionally seen in the interior of the dendrite (fig. 27). Open coated vesicles opposite parallel fibers are more common on the secondary and tertiary dendrites, and often several of them are seen along a single branch or branchlet (fig. 29). In some instances, a dense membrane is present in the parallel fiber opposite the coated vesicle. Not rarely, the parallel fiber appears to be drawn into the coated vesicle and a portion of it is partially or totally pinched off and incorporated (figs. 33–36). Elongated boutons with variable concentrations of loosely aggregated syn-

aptic vesicles form inconspicuous symmetrical junctions with the primary dendrites. These relatively rare synapses are of the kind that are identified on the soma as those of basket cells. They may be basket cell axon or early stellate cell synapses. In addition, a few asymmetrical synapses are seen on peridendritic processes or "thorns" of smooth dendrites (fig. 26A); these are presumably mature climbing fiber synapses (see below). The number of asymmetrical synapses, identified as those of parallel fibers with Purkinje cell dendritic spines, is still quite low at this age.

In the pyramis of 12-day old rats the glial processes which cover large portions of the surface of the somata of Purkinje cells were identified as processes of neighboring glia cells (fig. 32). Glial sheath is also present over much of the surface of the primary dendrites, separating the dendrites from parallel fibers and other cellular processes (fig. 27). In general, an upward and outward migration of glial covering is discernible over Purkinje cells with age, and at this age the secondary and tertiary dendrites are still devoid of glial covering.

Paralleling these changes in the soma and dendrites of Purkinje cells, changes are also seen in the molecular layer. The molecular layer is still relatively thin at ten days, but a rapid growth is evident from that age onward (see fig. 4, in Altman, '72a). In the ten-day old rats, the molecular layer is densely packed with mostly contiguous parallel fibers which are lacking in varicosities. Parallel fiber synapses, which are of the asymmetrical type, are quite rare at this age, but here and there are seen synapsing with unidentified dendritic processes. At 12 days, parallel fiber synapses with the spines of Purkinje cell dendrites are seen, but these are still not numerous in the molecular layer at this age.

With regard to the stationary cells of the molecular layer (which was described in detail in the previous paper of this series), at ten days, differentiating cells are seen one to two cells deep which either have open coated vesicles or symmetrical junctional densities, but rarely mature synapses, on their soma or processes in apposition to what are probably parallel fibers. These cells, identified as differentiating basket cells, often have extensive lateral dendrites. In the 12-day old rats, the stack of lightly staining differentiating cells has increased to a depth of three to four cells. These cells have parallel fiber synapses on their soma and dendrites with conspicuous asymmetrical dense membranes and usually a few synaptic vesicles. If these observations are compared with the autoradiographic results (described in the first paper, according to which the cells in the lower one-third of the molecular layer are formed by the end of the first week) it follows that three to five days may elapse between the formation of the basket cells and their differentiation both in terms of synapses formed with them and of the basket cell axons forming synapses with the somata of Purkinje cells.

Conclusion. The transient apical cone of Purkinje cells shows maximal enlargement by ten days, but it declines rapidly by the twelfth day. This is coupled with the outgrowth of the primary dendrite and a few smooth branchlets. These are filled with mitochondria and agranular vesicles that are apparently streaming upward, supplying the outgrowing branchlets. Concurrently, the soma shows several signs of synaptic maturation. Symmetrical basket cell synapses are seen in appreciable numbers at ten days and they further increase by the twelfth day. Asymmetrical synapses with perisomatic processes are still present but are definitely declining and are no longer seen in the older animals. As another sign of the maturation of the soma, glial processes spread around it, except where boutons are present. Basket cells have parallel fiber synapses on their soma and dendrites, but parallel fiber synapses with Purkinje cell dendrites are still rare.

4. *Maturation of the lower synaptic domain of Purkinje cell dendrites*

Light microscopic observations. In Nissl-stained sections, the Purkinje cell soma has an adult appearance by the beginning of the third week of life, except in the late-maturing folium and tuber. In Golgi-impregnated material, perisomatic processes are no longer seen; instead there

are clear signs of the growth of the dendritic system with many smooth branchlets and increasing numbers of spiny branchlets. That this is the period of the onset of maturation of the dendritic expanse of the Purkinje cells is suggested not only by the rapid increase during the third week in the width of the molecular layer (fig. 13), but also by the histochemical observation of the enzymatic maturation of the lower part of the molecular layer which gradually moves upward.

Staining with SDH shows that in 15-day old rats, the Purkinje cell soma has a doughnut-shaped silhouette due to the uniform staining of the round perikaryon (fig. 10). The discrete staining of the dendritic shaft seen in the younger animals which reflected the high concentration of streaming mitochondria in the smooth dendrites and the scarcity of mitochondria in other components of the molecular layer, is no longer seen. Instead, the lower half of the molecular layer is stained diffusely and uniformly, except for a light narrow band just above the perikarya of Purkinje cells (the site of the supraganglionic plexus). At this age, the upper half of the molecular layer is essentially unstained. Similar observations were made in the 15-day old rats with the other oxidative enzyme stains, indicating that the maturation reflected by the diffuse distribution of oxidative enzymes is restricted at this stage of development to the lower half of the molecular layer.

Electron microscopic observations. In the 15-day old rats, the perikarya of Purkinje cells typically has a mature appearance, being round or pear-shaped, is filled with membrane bound ribosomes, and is devoid of perisomatic processes (fig. 37). The perikaryon is covered by glial processes which in several instances are seen to belong to the somata of Bergmann glia cells that are located in the vicinity. Basket cell boutons with synapses on the somata of Purkinje cells are seen in variable numbers: few in some sections, more than a dozen in others (fig. 37). The shape of the boutons ranges from long (fig. 38) to small round or oval-shaped ones (fig. 39), presumably depending on the plane of sectioning. In sections where they appear elongated, they may remain contiguous over a long distance, as much as one-third of one side of the cell body with intermittent patches of *en passant* synapses; where they are round and small, often several of them are closely packed (fig. 39). (This suggests that they are arranged in strips lying alongside one another.) The synapses are without exception of the inconspicuous symmetrical type. The narrow synaptic cleft is often filled with dense material and sometimes a thin parallel central band is discernible. These boutons are typically surrounded by glial processes which extend on the two sides of the Purkinje cell soma. Where the axon of the basket cell terminal is visible, a few microtubules and numerous neurofilaments characterize it.

The primary dendrite of Purkinje cells at this stage is still filled with elongated, presumably streaming mitochondria and with elongated cisterns. Much of the surface of the initial portion of the primary dendrite is covered by glial processes, except where boutons with a high concentration of large, round vesicles form conspicuous, asymmetrical synapses with variably shaped protrusions or "thorns" of the primary dendrite. The synapses are often quite complex with adhesions seen also on the smooth portion of the dendrite. These, which were seen in the twelve-day old rats (fig. 26), are identified as climbing fiber synapses. As frequently, boutons of another type with a lower concentration of smaller vesicles, form inconspicuous, symmetrical synapses directly on the surface of the smooth dendrites. These are presumed to be synapses of the ascending branches of basket cell axons. Parallel fiber synapses are not seen on the primary dendrite. These are seen at this age in small numbers on the secondary and tertiary dendrites in the lower half of the molecular layer. Where these synapses occur, the dendrites and parallel fibers are usually separated by a glial sheath. Typically the bouton forms a ring around the spine; there is a small accumulation of round vesicles closely adhering to the conspicuous, asymmetrical adhesion sites, and there are one or two cisterns below the subsynaptic web of the spine. Quite often these spine synapses are partially or fully surrounded by glial covering. In the middle and upper

molecular layer, such parallel fiber synapses are rare at 15 days. Instead, open coated vesicles are present in variable numbers on the surface of smooth dendritic branchlets opposite parallel fibers or conspicuous, symmetrical junctions without vesicles are formed with parallel fibers. At these immature regions, the dendritic branchlets of Purkinje cells are still contiguous with parallel fibers, without a glial barrier being present.

As we have described it earlier, differentiated cells are seen in the 15-day old animals in the lower and middle molecular layer, which must include basket and stellate cells. In the upper molecular layer in these animals, which represents the lower-to-middle molecular layer of the adult (fig. 13), the horizontally oriented cells are just beginning to differentiate. These cells, which are presumed to be middle stellate cells, have coated vesicles, junctional dense membranes and occasional parallel fiber synapses on their soma and processes. Relevant in this context are the autoradiographic results described in the preceding paper which show that injection with thymidine-H^3 during the third week of life (whether with 2 or 4 successive daily doses) yields few intensely labeled cells in adult rats in the middle or upper molecular layer. Evidently, the majority of stellate cells come into existence before the end of the second week, but their differentiation is prolonged for several days afterwards.

In summary, in the 15-day old animals, the differentiation of the lower domain of Purkinje cells has begun with parallel fibers starting to form synapses with the outgrowing dendritic spines and connections being established with this portion of the Purkinje cell dendritic domain by way of the lower stellate cells.

5. *Maturation of the upper synaptic domain of Purkinje cell dendrites*

Light microscopic observations. In Nissl-stained sections, the Purkinje cells and the molecular layer with its differentiated neurons, appear mature at 21 days. The only signs of immaturity are the presence of scattered cells in the remaining external germinal layer and vertically oriented, spindle-shaped migratory cells in the molecular layer. An indication at the light microscopic level, that the molecular layer as a whole is not completely mature comes from examination of the histochemical material. In the sections stained for succinate dehydrogenase, lactate dehydrogenase, cytochrome oxidase and the other oxidative enzymes, the reaction product is still restricted at this age to the lower three-fourths of the molecular layer; the upper one-fourth remains essentially unstained (fig. 11). The complete spread of staining to the surface is seen in the 30-day old animals (fig. 12) and even in these, staining intensity is still somewhat lower near the surface than elsewhere. This observation suggests that the maturation of the upper domain of the molecular layer continues for some time after cessation of cerebellar neurogenesis.

Electron microscopic observations. The primary dendrites of Purkinje cells and the secondary smooth branchlets are characterized at 21 and 30 days (figs. 40, 41) by the large concentration of longitudinally oriented and regularly arranged microtubules, some macrotubules, rare patches of granular endoplasmic reticulum, a few multivesicular bodies, subsurface cisterns

Fig. 13 Diagrammatic illustration of some major events in the maturation of a Purkinje cell and of the molecular layer. The width of the molecular layer (left abcissa) as a function of the animal's age (5 discrete columns) is based on measurements (see fig. 4, in Altman, '72a). Considering the principle of the stacking of parallel fibers from the bottom upward, and disregarding the expansion produced by the invading dendritic processes during synaptogenesis, their location within the molecular layer indicates their "age" or time of origin (right abcissa). If the parallel fibers are traced through the columns from left to right it is seen that the fiber formed on day 7 has no synapses on that day but that it has synapses with basket cells on day 12, and increasing number of synapses from the fifteenth day onward with dendritic spines of Purkinje cells. Likewise, the parallel fiber formed on day 12 has no synapses at that time but it has synapses with a stellate cell on day 15 and with Purkinje cell dendritic spines on day 21. The parallel fiber formed on day 15 has synapses with Purkinje spines on day 21, but in the upper one-fourth of the molecular layer such synapses are still lacking at this age. The displacement and growth of a climbing fiber is indicated on the left side of the Purkinje cell. The upward "march" of glial sheathing of the Purkinje cell is also indicated. The cell-width of the two zones of the external germinal layer (see fig. 1, in Altman, '72a) is accurate but they are not drawn to scale.

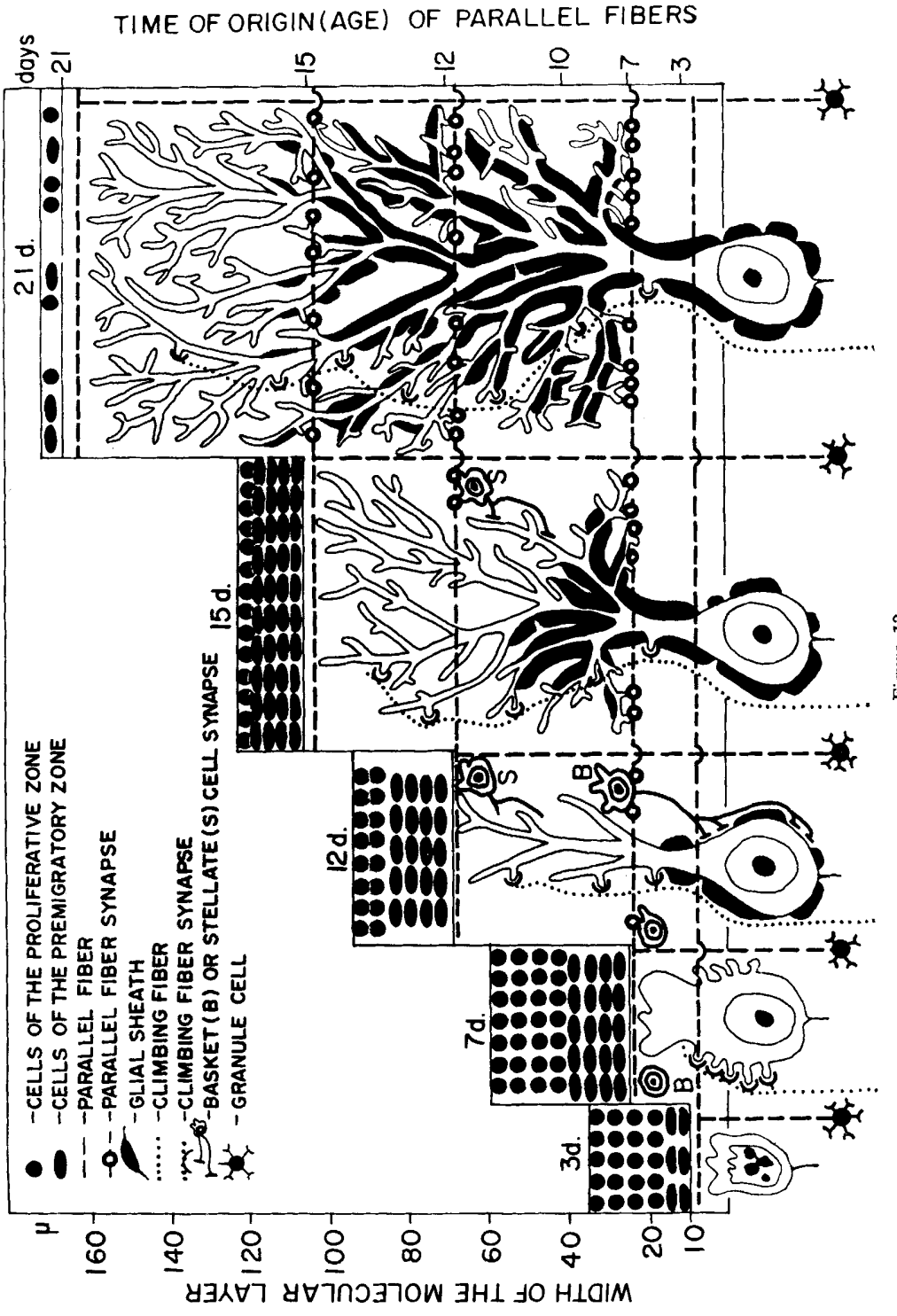

TIME OF ORIGIN (AGE) OF PARALLEL FIBERS
days
21
15
12
10
7
3
21 d.
15 d.
12d.
7d.
3d.
S
B
B
WIDTH OF THE MOLECULAR LAYER
μ
160
140
120
100
80
60
40
20
10
-CELLS OF THE PROLIFERATIVE ZONE
-CELLS OF THE PREMIGRATORY ZONE
-PARALLEL FIBER
-PARALLEL FIBER SYNAPSE
-GLIAL SHEATH
-CLIMBING FIBER
-CLIMBING FIBER SYNAPSE
-BASKET (B) OR STELLATE (S) CELL SYNAPSE
-GRANULE CELL

Figure 13

along the entire length of the dendrites, including occasional patches of stacked cisterns. The concentration of mitochondria which is located close to the outer membrane of the dendrite (fig. 41) is greatly reduced with respect to younger animals and only a few extremely long mitochondria are seen. The surface of these smooth dendrites is covered by glial processes which separate the dendrites from parallel fibers and other cell processes (fig. 40). The glial covering is interrupted at few points, where boutons form synaptic contacts either with the smooth surface of the primary and secondary dendrites (fig. 42) or with thorny outpouchings. The boutons of the former group tend to be of intermediate size and occur on the trunk of the primary dendrite, but more commonly near branching points of the secondary dendrites (fig. 42). These boutons are filled with a variable concentration of small, round or flat vesicles and the junctional densities are typically inconspicuous and symmetrical. The axon of these terminals has few microtubules and is rich in neurofilaments. These terminals are presumed to be those of basket or low stellate cells. The boutons of the second group, which terminate on outpouchings of the smooth dendrite form conspicuous, asymmetrical junctional densities. It is assumed that these are climbing fiber synapses (figs. 43, 44). The axons of these are rich in neurofilaments and microtubules.

In the lower and middle region of the molecular layer, the dendrites of the Purkinje cells are typically segregated from the parallel fibers and other cell processes, except where boutons form synapses on the smooth surface of dendrites or on their outpouchings. In the lower part of the molecular layer the parallel fiber synapses are numerous with identified or assumed Purkinje cell dendritic spines (fig. 47) and with the somata and dendrites of basket cells. The concentration of parallel fiber synapses on the perikarya and dendrites of basket cells is unusually high. In the middle portion of the molecular layer, there is a moderate concentration of parallel fiber enlargements and synapses with Purkinje cell dendritic spines (fig. 46). These synapses are typically surrounded by glial covering. In contrast, in the upper molecular layer, glial coverings on the smooth dendritic branches is either fragmentary or altogether absent at this age (fig. 48). The Purkinje cell dendrites which reach to the surface are filled with elongated mitochondria; macrotubules are abundant and the dendrites are contiguous with parallel fibers and other cell processes. Open coated vesicles are common in the Purkinje cell dendritic branchlets and these are typically situated opposite parallel fibers (fig. 48, inset). Developing dendritic spines are numerous and these may still be directly contiguous with parallel fibers. In general, a gradient is indicated in the concentration of parallel fiber enlargements and synapses from the bottom of the molecular layer, where they have become quite numerous at 21 days, to the top where they are yet to be formed (fig. 47).

The external germinal layer is no longer present at 30 days. Rarely an apparently undifferentiated cell is seen below the surface of the cortex with darkly-staining, coarse chromatin material, but such cells were also seen to have dendrites with synapses on them and are, therefore, presumed to be immature stellate cells. Beneath the subpial glial sheath, the parallel fibers are usually closely packed, often assuming a hexagonal pattern in cross section. The concentration of parallel fiber enlargements and synapses is still low near the surface in some regions, whereas in other regions, there is a fair accumulation of such enlargements with synapses on Purkinje cell dendritic spines and presumably other processes. In the upper aspect of the molecular layer, dendritic branchlets are in many places directly contiguous with parallel fibers, without interposed glial processes, and open coated vesicles are still present opposite parallel fibers. Junctional densities indicative of "early synapses" are also seen. In summary, these observations indicate that the maturation of the most superficial areas of the molecular layer, or of the uppermost dendritic domain of Purkinje cells, is still in progress nearly ten days after cessation of cerebellar neurogenesis.

DISCUSSION

The first phase of Purkinje cell maturation. The embryonic state of Purkinje cells in newborn mammals was pointed out by Ramon y Cajal ('60, p. 277). He distinguished three phases in the maturation of Purkinje cells: (a) the phase of fusiform cells; (b) the phase of stellate cells with disoriented dendrites, and (c) the phase with oriented and flattened dendritic "plumes" ('60, p. 304). The first phase was considered by Ramon y Cajal the neuroblast stage and he studied it in the cerebellum of mice before birth and in late maturing regions (flocculus) in newborn mice. The Purkinje cells are scattered at this stage in several rows and have dendrites that are budding from all sides. Ramon y Cajal pointed out that in the same region, the Purkinje cells closer to the surface appear to be more mature than the others. The scattering of Purkinje cells in several rows at birth was also described in rats by Addison ('11), although in contrast to our observations of a depth of 6–12 cells, he mentions a depth of two to three cells. Presumably he considered only the more mature cells in the upper rows of cells as Purkinje cells, i.e., those which began to have growing cytoplasms at their "ectal poles."

Ramon y Cajal's first phase is the same as the first phase described in this paper, except that the outgrowth of the lateral dendrites mentioned by Ramon y Cajal (obviously the perisomatic processes that appear later) are not seen during this early phase. There are not many references in the literature to this early phase, partly because of the great difficulty of staining the embryonic Purkinje cells with the Golgi technique, and the difficulty in fixing the cerebellum of newborn animals for electron microscopy. Our observations indicate that this early phase is characterized by the radial dispersion of Purkinje cells over the rapidly expanding (Altman, '69) and folding surface of the cortex. This process presumably begins before birth but continues for several days after birth. In the pyramis (which represents a lobe which is intermediate between early and late maturing regions of the vermis), the Purkinje cells located near the surface of the cortex are more mature in newborn rats than those located deeper. In the superficial cells, the beginnings are seen of the accumulation of "reticular" cytoplasm, which was described as the transient "growth" cytoplasm of the maturing Purkinje cell. In the three-day old rats, the majority of Purkinje cells have the latter characteristics. At this phase of development, the cytoplasm of the Purkinje cell is lacking in microtubules, except the axon which has a "tubular" organization. Synapses are not seen on the soma of Purkinje cells and synaptogenic activity (the presence of coated vesicles or complementary membrane thickenings) is minimal, confirming Ramon y Cajal's inference that the Purkinje cells are embryonic during this phase of development.

The second phase of Purkinje cell maturation. The transient apical enlargement of the somata of Purkinje cells, together with the concurrent outgrowth of temporary perisomatic processes, mark the second phase in the development of Purkinje cells. It begins with the permanent dispersion of Purkinje cells into a monolayer by about the fifth day after birth (Addison, '11) and in the pyramis it is evident for at least five additional days. This phase coincides with Ramon y Cajal's second phase which he referred to as the "phase of stellate cells with disoriented dendrites." He pointed out ('60, p. 308) that during this phase, the Purkinje cell "dendrites" have not yet become flattened in the sagittal plane.

The outgrowth of the apical cytoplasm of the growing Purkinje cell was noted in passing by several investigators and reference was made to the intense acidophilic staining of this region during early development (Addison, '11). Also the intense oxidative enzyme activity of the growing apical cytoplasm was noted or is detectable in the material presented by earlier investigators (Friede, '59; Robins and Lowe, '61; Das and Kreutzberg, '67; Woodward et al. '69; Ebels, '69). The large accumulation of mitochondria and of Golgi apparatus in this region was observed in electron microscopic investigations and contrasted with the scarcity of these organelles, and the greater accumulation of ribosomes, in the basal cytoplasm, in 75-day old monkey fetuses (Kornguth et al., '67) and one-week

old chick embryos (Meller and Glees, '69). Our present study shows clearly the association of temporary acidophilia and of intense oxidative enzyme activity of the apical region with the high accumulation of mitochondria, Golgi apparatus and a few other organelles in this "reticular" type of "growth" cytoplasm. The intense growth of this region for about a week leads to the formation of a greatly expanded supranuclear miter, or cone, which begins to regress when the dendritic system begins to mature and the mitochondria and other organelles stream into these growing regions. Evidently these organelles are produced in a perinuclear position and because of the rapid growth of the dendritic system, masses of these accumulate here before their upward displacement. The apparent streaming of these organelles, first through the primary and later the secondary and tertiary dendrites, and from the lower part of the molecular layer upward, and their great reduction in the maturing cerebellum in the former regions was described.

The transient lateral "dendrites" of the soma described by Ramon y Cajal do not have the properties of true dendrites, such as the presence of microtubules or other organelles, as noted already by Mugnaini ('69). But synapses are formed on their surface and this must be their temporary function. These perisomatic processes were described by many investigators (Athias, 1897; Addison, '11; Purpura et al., '64) and were recently identified with electron microscopy (Larramendi, '65; Larramendi and Victor, '67; Kornguth et al., '68; Mugnaini, '69). The inference of Ramon y Cajal ('11) that the presence of these processes is related to the "pericellular nest stage" in the development of climbing fibers is supported by the evidence of the presence of conspicuous, asymmetrical synapses on these processes (Larramendi and Victor, '67). In mice they were seen to appear first at six days (Larramendi, '69).

We were able to distinguish three phases in the growth of these temporary synapses. In five-day old rats conspicuous, asymmetrical synapses were occasionally seen either directly on the soma or on short protrusions. At seven days the same type of synapses commonly occurred on larger perisomatic processes, and these processes often had complex, invaginated joint surfaces and many synapses. Because conspicuous, asymmetrical synapses (Gray, type I) are never seen on the somata of Purkinje cells from the fifteenth day onward, it was postulated that the three variants of these synapses seen between 5–12 days represent stages in the maturation of temporary climbing fiber synapses with the Purkinje cell soma and its lateral outgrowths. In view of the temporary nature of this junction it is surprising that such intricately interdigitated synapses are formed. Either this interdigitation represents a necessary enlargement of the surface for adequate synaptic interaction (which are later dissolved) or else, the entire junction is translocated (Larramendi, '69) and the interdigitation is a mechanical device to prevent dissociation of the synapse during translocation from the soma to the dendrites.

In an earlier study (Altman and Das, '70) the possibility was raised that mossy fibers may also establish temporary synapses with Purkinje cells. We observed that Purkinje cells stain for cholinesterase at the time that they form perisomatic synapses, particularly in those lobes (e.g., nodulus) in which the mossy fibers are reactive (presumably because they are cholinergic). By the time the glomeruli stain, signalling the establishment of mossy fiber synapses with granule cells, the Purkinje cells cease to stain. Accordingly, an attempt was made to identify typical mossy fiber terminals with synapses on Purkinje cells at these early ages. However, typical mossy fibers could not be identified in five and seven day old rats (Altman, '72b). This suggests either that mossy fibers are not mature (and, also, that they do not form transient synapses with Purkinje cells) or else, that the immature mossy fiber terminal has other characteristics than the mature rosette and could not be identified with the criteria used. With the completion of these two phases the preparatory stage of Purkinje cell development comes to an end.

The third phase of Purkinje cell maturation. The synaptic maturation of the soma of the Purkinje cell was described as

the third major phase in its maturation. This event was indicated by the gradual disappearance of perisomatic processes and of temporary synapses with climbing fibers, and by the acquisition of permanent basket cell synapses.

At 10 days of age, Purkinje cell perisomatic synapses are still seen in the pyramis in appreciable numbers, but they are greatly reduced by the twelfth day. Likewise the apical enlargement is still prominent at ten days with maximal concentration of "reticular" cytoplasm, but by the twelfth day the supranuclear hypertrophy is receding and the "reticular" cytoplasm is concentrated in the outgrowing primary dendrite and its side branches. What are probably immature basket axon terminals are first seen to establish synapses on the perikarya of Purkinje cells at ten days. Evident synapses of this type, with inconspicuous, symmetrical junctional membranes (Gray, type II), are seen in appreciable numbers at 12 days. With the Golgi technique, Addison ('11) observed basket terminals on Purkinje cells in 11-day old rats. The maturation of synapses on the soma of basket cells was described in the previous paper (Altman, '72a). In 12-day old rats differentiated cells are seen in the molecular layer of the pyramis three to four cells deep, and these have mature parallel fiber synapses on their soma and dendrites. In mice, in which the maturation of the cerebellar cortex proceeds faster than in rats, basket cell axon synapses are first seen at nine to ten days but they increase in number up to 14 days (Larramendi, '69). Evidently the maturation of the dendritic (centripetal) and axonal (centrifugal) synapses of basket cells occurs concurrently.

A phenomenon that was noted in following the growth of Purkinje cells was a series of transformations in the structure of the cytoplasm that reflected stages in the maturation of the cell. These were tentatively referred to as "reticular," "ribosomal" and "tubular." The reticular type of cytoplasm, with excessive concentration of mitochondria and cisterns was seen first in a supranuclear position, producing the transient apical cone. It was also referred to as the "growth cytoplasm" because of its apparent streaming upward into the rapidly growing dendrites. When the dendrites matured, the concentration of mitochondria was greatly reduced and there was a great increase in microtubules, which is the characteristic of the tubular structure. Whereas the tubular stage was reached gradually in the dendrites, the axon had this organization almost from the beginning, suggesting its early maturation. The basal cytoplasm was throughout the entire period poor in mitochondria and rich in ribosomes. This ribosomal cytoplasm itself had two stages of development: initially the ribosomes formed predominantly free clusters; when the soma became mature, the ribosomes were generally bound to the membranes of the endoplasmic reticulum. (This transformation was studied in detail in spinal ganglia by Pannese ('68).) In the adult Purkinje cell the cytoplasm is essentially ribosomal, the dendrites tubular.

Because in 12-day old rats few parallel fiber synapses are seen with spines of Purkinje cells (which are yet to be formed in appreciable numbers) and because spiny branchlets of Purkinje cell dendrites are not especially numerous even in adults near the perikarya of Purkinje cells, it is suggested that the earliest maturing and deepest parallel fibers form synapses primarily with basket cells. The implication of this is that the maturation of the basket cell inhibitory input to Purkinje cells (Eccles et al., '67), which can be activated by the early-maturing parallel fiber synapses, antedates the maturation of excitatory input to the Purkinje cell by way of the mossy fiber-parallel fiber relay. The only excitatory channel to the Purkinje cells at this phase of development is by way of the climbing fibers. It is possible that, as in the adult (Scheibel and Scheibel, '54) climbing fibers form synapses also with basket cells at this age.

From the tenth day onward, conspicuous, asymmetrical synapses are seen on thorny outgrowths of the primary dendrite of Purkinje cells in small numbers, and at later stages on secondary and tertiary "smooth" branches. These were interpreted as climbing fiber synapses (Larramendi and Victor, '67). These boutons are typically larger than the varicosities of parallel fibers in longitudinal sections, but in cross

section they may be difficult to distinguish from larger parallel fiber terminals on Purkinje cell spines. The appearance of climbing fiber synapses on Purkinje cell dendrites represents the third and final stage in their maturation in Ramon y Cajal's ('11) scheme, from the pericellular through the capuchon to the dendritic stage. In contrast to Ramon y Cajal's belief that climbing fibers synapse with the smooth dendrites directly, the evidence is becoming compelling that they synapse with outgrowing spines. These, in order to distinguish them from spines on terminal branchlets, are referred to as "thorns" (Chan-Palay and Palay, '70). The development of climbing fibers examined in Golgi material was recently described in detail by O'Leary et al. ('71); experimental evidence that climbing fibers synapse with dendritic thorns will be presented in a forthcoming paper (Altman and Anderson, '72).

The fourth and fifth phases of Purkinje cell maturation. The fourth period covers roughly the third week of life after birth. At the beginning of this period the somata of Purkinje cells have an adult appearance in the pyramis, being disc or pear shaped (depending on the plane of sectioning) in Nissl stained sections. Growth is now shifted to the rapidly arborizing Purkinje cell dendrites, to the outgrowth of secondary and tertiary branches and terminal branchlets with spines. The appearance of the latter is coupled with the formation of parallel fiber synapses with them, which appears to be the most prominent event from the third week onward in the molecular layer. During this fourth phase of Purkinje cell maturation, parallel fiber synapses begin to be formed with dendritic spines in the lower half of the molecular layer, and at the end of the period, when the production of granule cell precursors comes to an end, the formation of these spines has spread upward to about the middle of the molecular layer. The progressive upward march of synaptogenesis was shown by the gradual increase in the proportion of parallel fiber varicosities, dendritic profiles and synaptic profiles over the thin strands of parallel fibers which predominate in immature regions. The absence of many synapses in the upper half of the molecular layer was suggested by the pattern of distribution of oxidative enzyme staining (a marker for the distribution of mitochondria) and demonstrated with electron microscopy. By the thirtieth day, numerous parallel fiber synapses with Purkinje cell spines have reached the surface of the cortex. Evidently the "march" of synaptic maturation from the bottom upward trails behind the morphogenic "stacking" of the parallel fibers.

In addition to the gradual upward "march" of parallel fiber-Purkinje spine synapses, two other phenomena were observed to display this trend, and both of these were interpreted as aspects of synaptogenesis. The first of these was the temporary appearance of coated vesicles opposite parallel fibers and of desmosoid junctional thickenings in the same position, and these were interpreted as events leading to the formation of permanent synapses (Altman, '71). The second was the gradual upward "march" of glial covering around the body of Purkinje cells and we postulate that this signals the upward "march" of the termination of synaptogenesis.

Signs of the onset of synaptogenesis: Coated vesicles. In infant rats, closed coated vesicles are seen in the cytoplasm of Purkinje cells in the vicinity of the Golgi apparatus, and in this position they are seen in variable numbers (usually small) at all the ages studied. Open coated vesicles, which are presumed to be derived from closed coated vesicles (Altman, '71), are occasionally seen, usually opposite parallel fibers, on the plasmalemma of the soma in infant rats, and later on the apical cone and primary dendrites. As the secondary and tertiary dendrites begin to grow, the open coated vesicles become numerous opposite parallel fibers and their upward march from the lower to the upper molecular layer is clearly seen between 12–30 days. Often these flask-shaped or flattened coated vesicles are continuous with desmosoid dense membranes or are intermingled with profiles of apposed desmosoid membranes of Purkinje cell processes and parallel fibers. Rarely, open coated vesicles in various phases of unfold-

ing are continuous with synaptic dense membranes.

The presence of these membrane specializations were interpreted as signals of synaptogenic activity, although their structural and functional significance remains to be determined. The interaction between these coated vesicles and parallel fibers is shown unmistakably when the parallel fiber or parts of it are drawn toward the cavity of the coated vesicle. But it is not clear whether this interaction is merely an event leading to synaptogenesis (e.g., the exchange of some material) or it is an actual step in the process (i.e., the production of material that becomes part of the synaptic dense membrane). That there is an association between the appearance of coated vesicles and subsequent synaptogenesis is indicated by the trailing upward march of parallel fiber-Purkinje cell spine synapses in the wake of these transient formations. However, the presence of these coated vesicles in infant rats opposite parallel fibers on the plasmalemma of the soma or primary dendrite is difficult to interpret, considering that permanent parallel fiber synapses are not formed at these sites. The other possibility is that these junctions are merely adhesion sites and synapses are formed after the adhesion has been sustained for some time, often in the face of various vicissitudes (such as translocation).

Signs of the end of synaptogenesis: Glial insulation. During the early days after birth, the only glial elements that could be identified in the cerebellar cortex were Bergmann glia cells. Their somata were seen below the zone of Purkinje cells, several of which showed mitotic activity, and their processes, which can be traced to the surface of the cortex, formed vertical columns through the cortex. During the first week the Bergmann glia processes do not surround the somata of Purkinje cells, which remain contiguous with parallel fibers and other cellular elements. During this period numerous short and long fused cisterns are seen below the plasma membrane of the Purkinje cell soma. This was of interest because in the somata of mature Purkinje cells, these subsurface cisterns are typically situated in apposition to glial processes (Altman, in preparation). Hence the presence of fused cisterns at certain points of the soma in infant rats may signal the reservation of these sites for neuronal/glial interaction.

Glial processes, usually those of Bergmann glia cells, are first seen to form a sheath around the perikarya of Purkinje cells on the seventh day, when small patches appear interposed between the soma and parallel fibers. Where these processes are present, coated vesicles are not seen on the soma, suggesting that the glial process presents a barrier and ends regional synaptogenic activity. By the tenth day, when permanent basket axon synapses are seen in increasing numbers on the Purkinje cell soma, the glial covering becomes more extensive and by the twelfth day essentially all the surfaces of the soma not covered by boutons are ensheathed in glia. By this time patches of glial covering are also seen on the thick, lower part of the primary dendrite, indicating the gradual upward "march" of glial sheathing, which by the 15th day covers the secondary and tertiary dendrites in the lower molecular layer and also surrounds here the parallel fiber synapses with Purkinje cell dendritic spines. In general, where permanent synapses are present on the processes of Purkinje cells, they are partially or completely ensheathed by glia, suggesting that at any region the establishment of a synapse is soon followed by glial insulation. This upward "march" of glial covering of the processes of Purkinje cells is a slow process and at 30 days there are still extensive regions where they are directly contiguous with parallel fibers without intervening glial barrier. Where this is seen, open coated vesicles are seen opposite parallel fibers, suggesting active synaptogenic activity.

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PLATES

PLATE 1

EXPLANATION OF FIGURE

14 Three Purkinje cells with polymorphous nuclei. It is assumed that these cells are in the process of migration to and dispersion over the surface of the cerebellar cortex. Posterior vermis, newborn rat. Inset: Purkinje cells from the same region. "Thick" Epon section, azure B, oil immersion, × 1600.

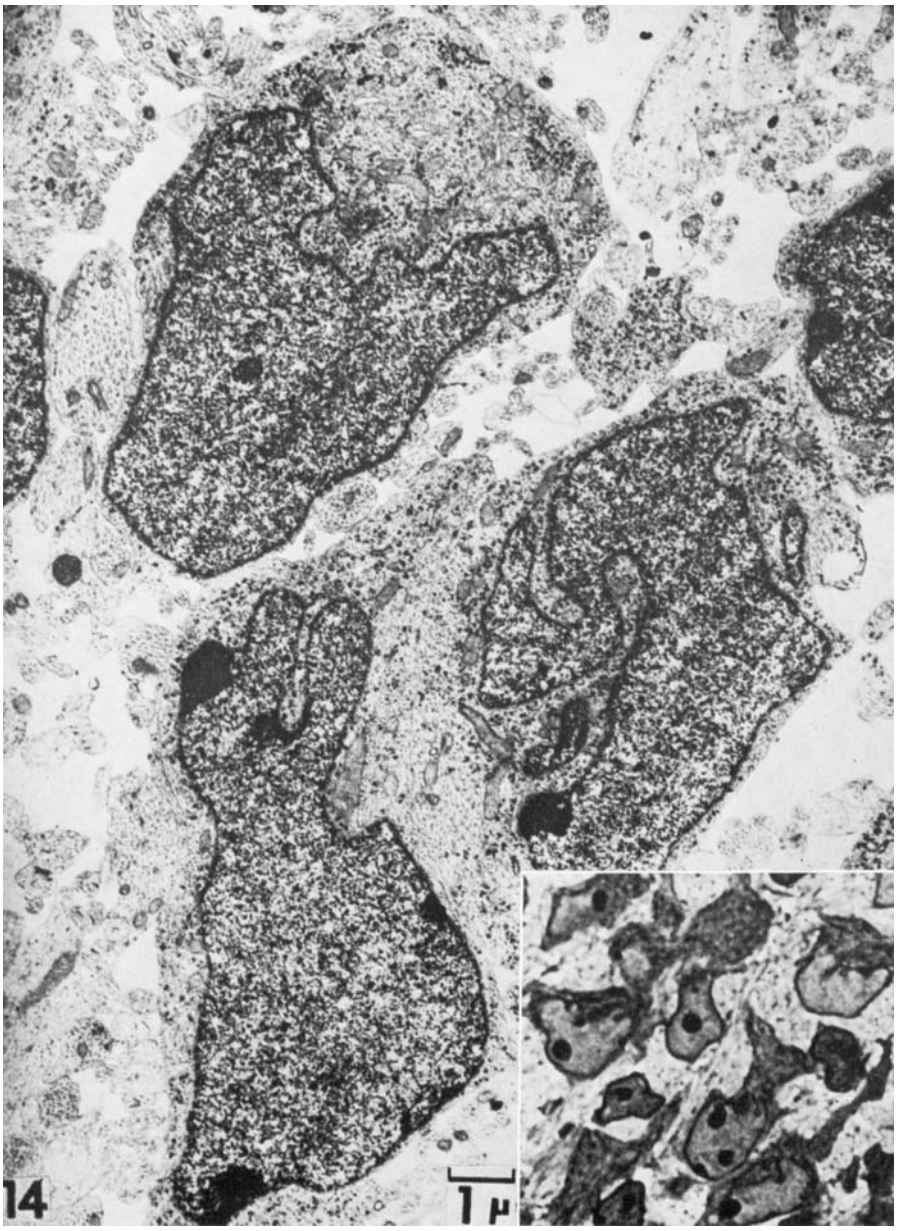

14
1 μ

PLATE 2

EXPLANATION OF FIGURE

15 An example of a bizarre looking immature Purkinje cell. As a rule, the lobulated surface of the nucleus is pointing upward toward the surface of the cortex. Less pronounced lobulation of this kind is also seen in maturing and, occasionally, mature Purkinje cells, and it may be related to intense metabolic activity in this region. Posterior vermis, newborn rat.

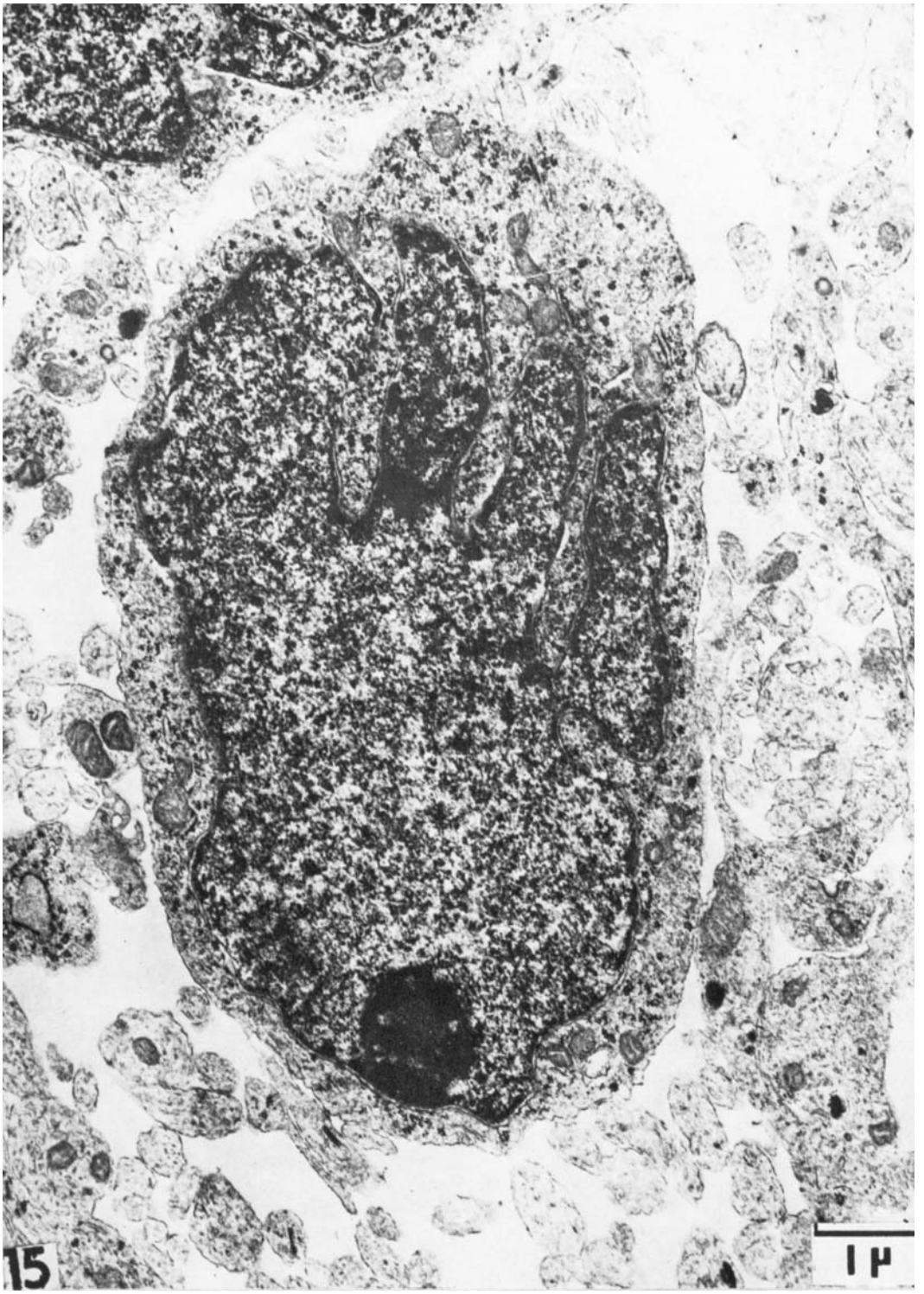

15
1 μ

PLATE 3

EXPLANATION OF FIGURE

16 Immature Purkinje cell with an apical cone that has a "reticular" structure characteristic of sites of intense growth. It consists of a large accumulation of mitochondria, Golgi apparatus, macrotubular cisterns, coated vesicles and multivesicular bodies (not seen in this picture). The basal cytoplasm has a "ribosomal" structure, that is, it is rich in free (later bound) ribosome clusters, but is lacking in other organelles. The axon has, at this age, a "tubular" structure, that is, it is poor in all the components described but is traversed by longitudinally oriented microtubules. Posterior vermis, three days. Inset: Purkinje cells from the same region. "Thick" Epon section, azure B, oil immersion, × 1600.

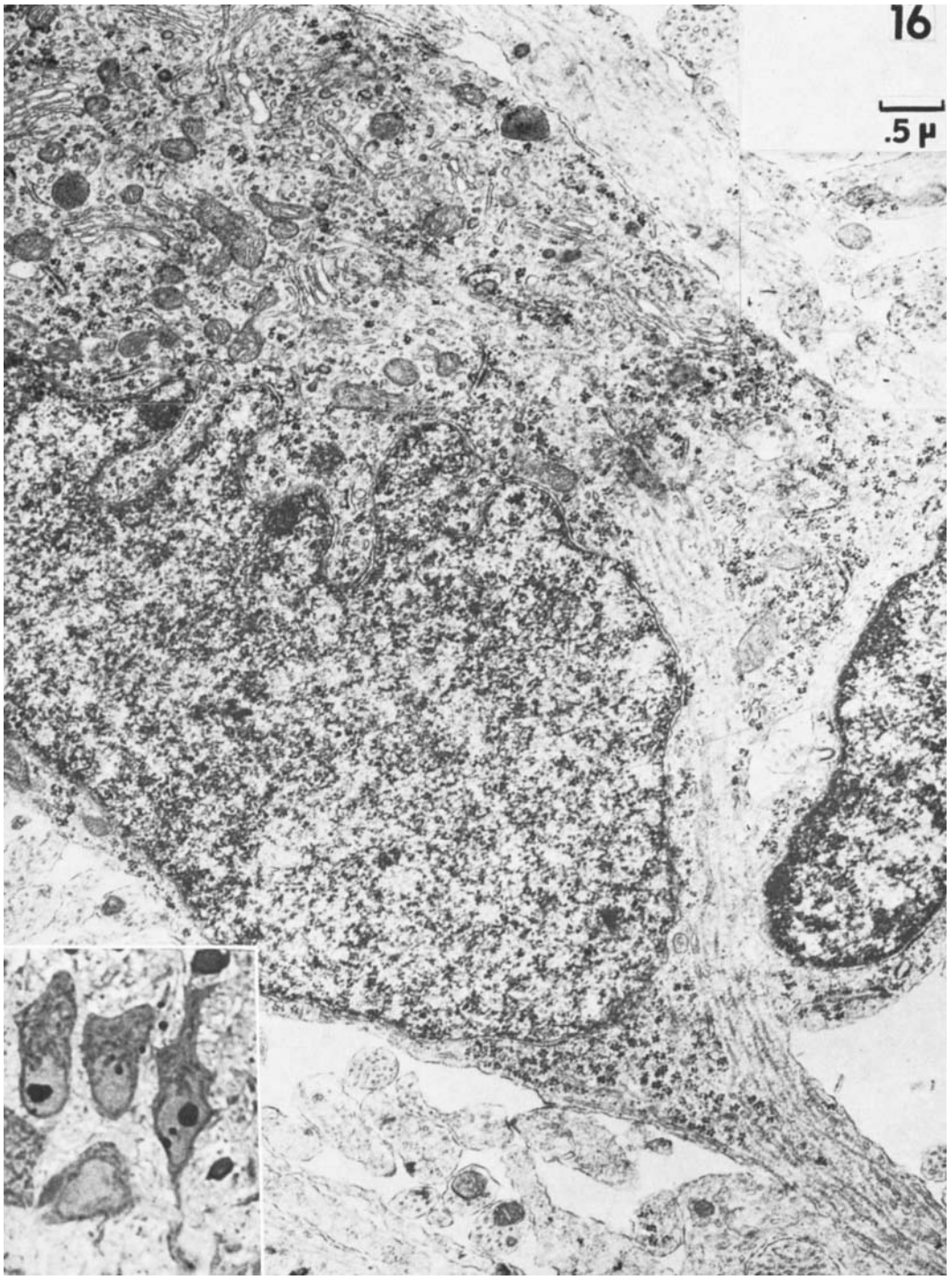

16
.5 μ

PLATE 4

EXPLANATION OF FIGURES

17 Purkinje cell whose apical cone is surrounded by parallel fibers. An open coated vesicle is seen opposite one of the parallel fibers and adjacent to it there is a subsurface, fused cistern (upper left quadrant). Symmetrical membrane densities, or early attachment membranes, may be present on the apical cone (upper right quadrant). Pyramis, five days.

18 Enlargement of the region with subsurface, fused cistern and open coated vesicle.

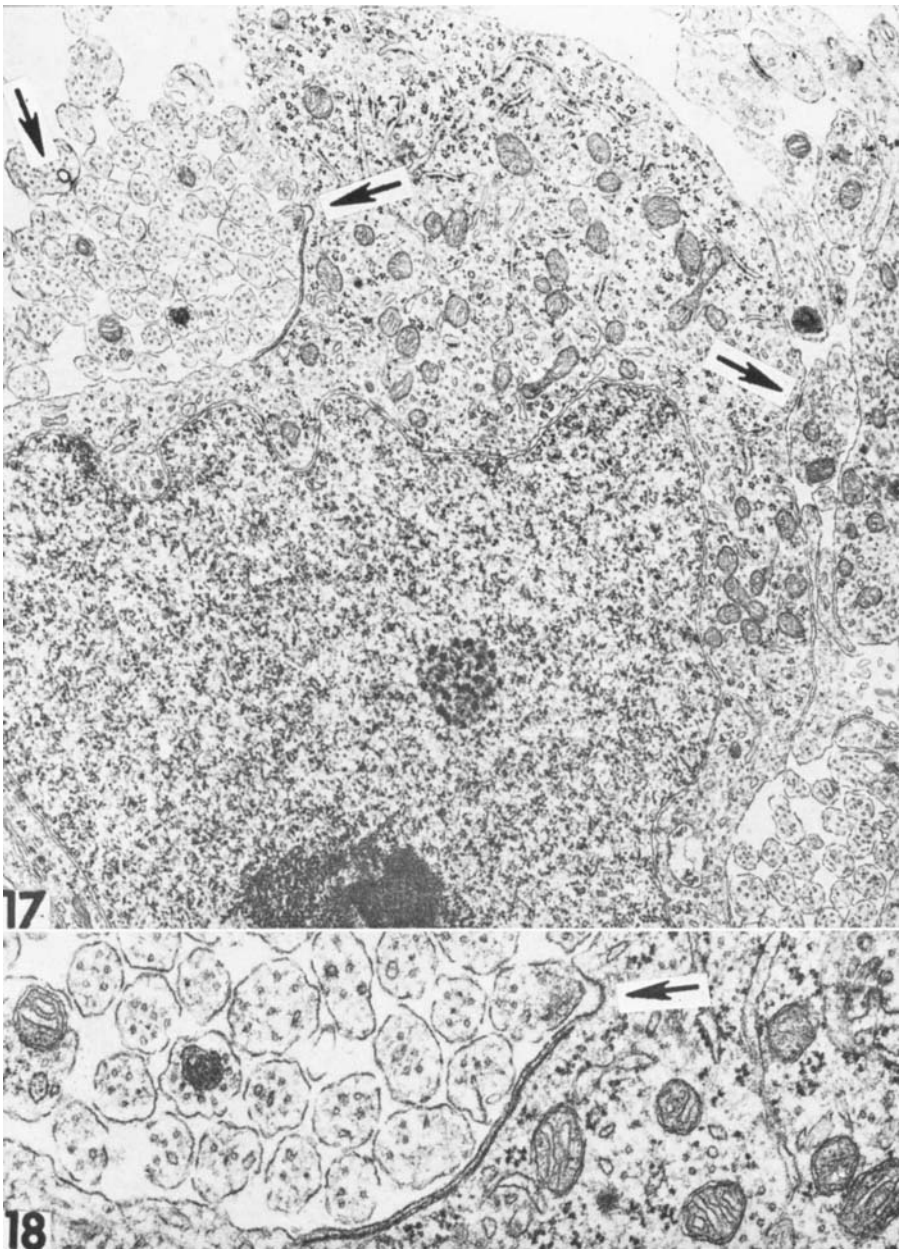

17
18

PLATE 5

EXPLANATION OF FIGURE

19 Two boutons with synapses on the basal aspect of a Purkinje cell. One of the synapses (lower left) is clearly the conspicuous, asymmetrical type (Gray, type I), and so is probably the other. In adults this type of synapse is never seen on the somata of Purkinje cells. They are seen directly on the soma only at this age, later (7–12 days) they are seen on transient perisomatic processes (figs. 20, 21). It is assumed that these are transient climbing fiber synapses. Pyramis, five days.

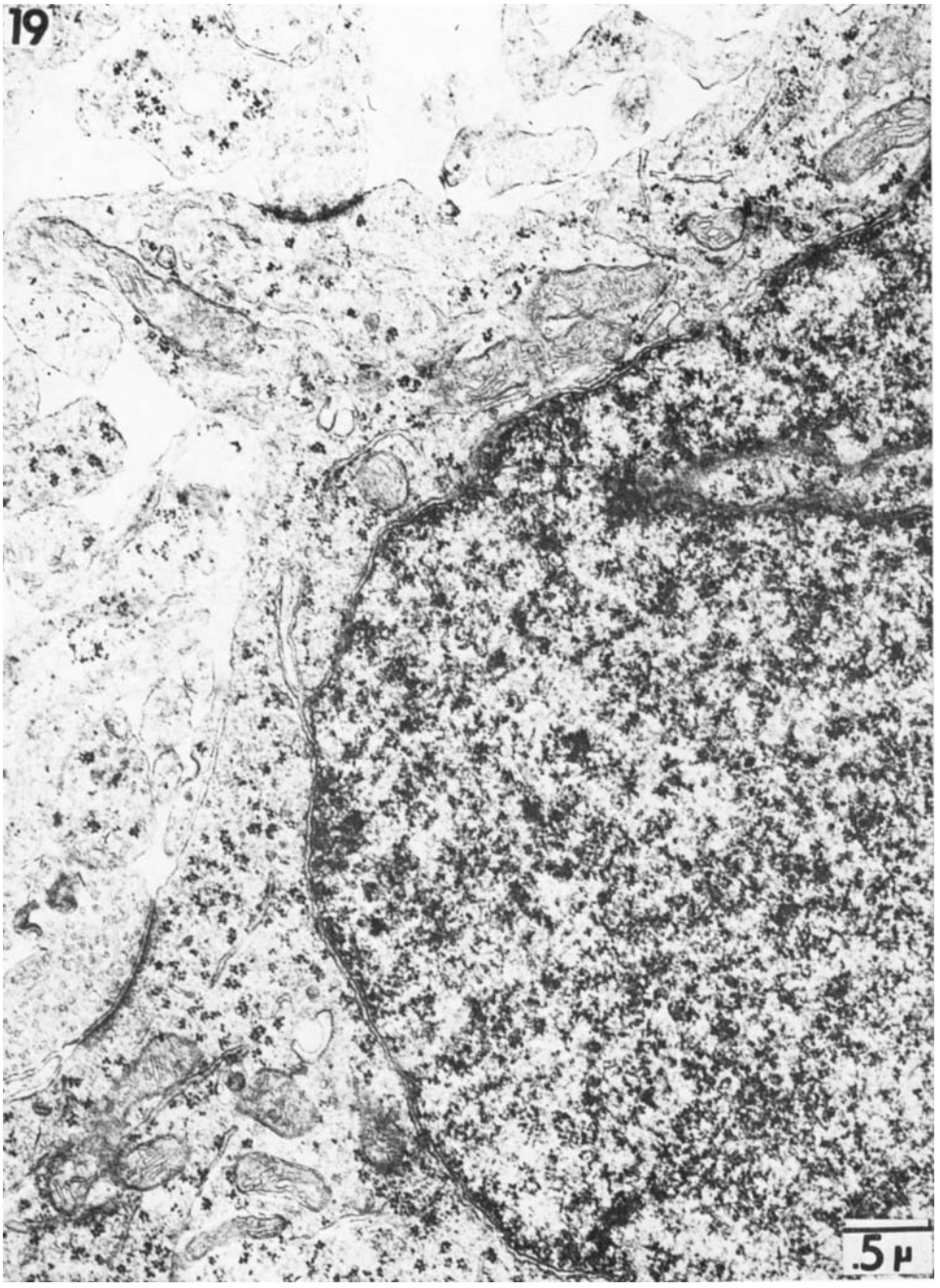

19
.5 μ

PLATE 6

EXPLANATION OF FIGURE

20 Two synapses, similar to those shown in figure 19, but these are clearly situated on rudimentary perisomatic processes. This represents the second stage in the growth of the transient climbing fiber synapse with the Purkinje cell perikaryon. A parallel fiber transient synapse may also be present (center, arrow). Pyramis, five days.

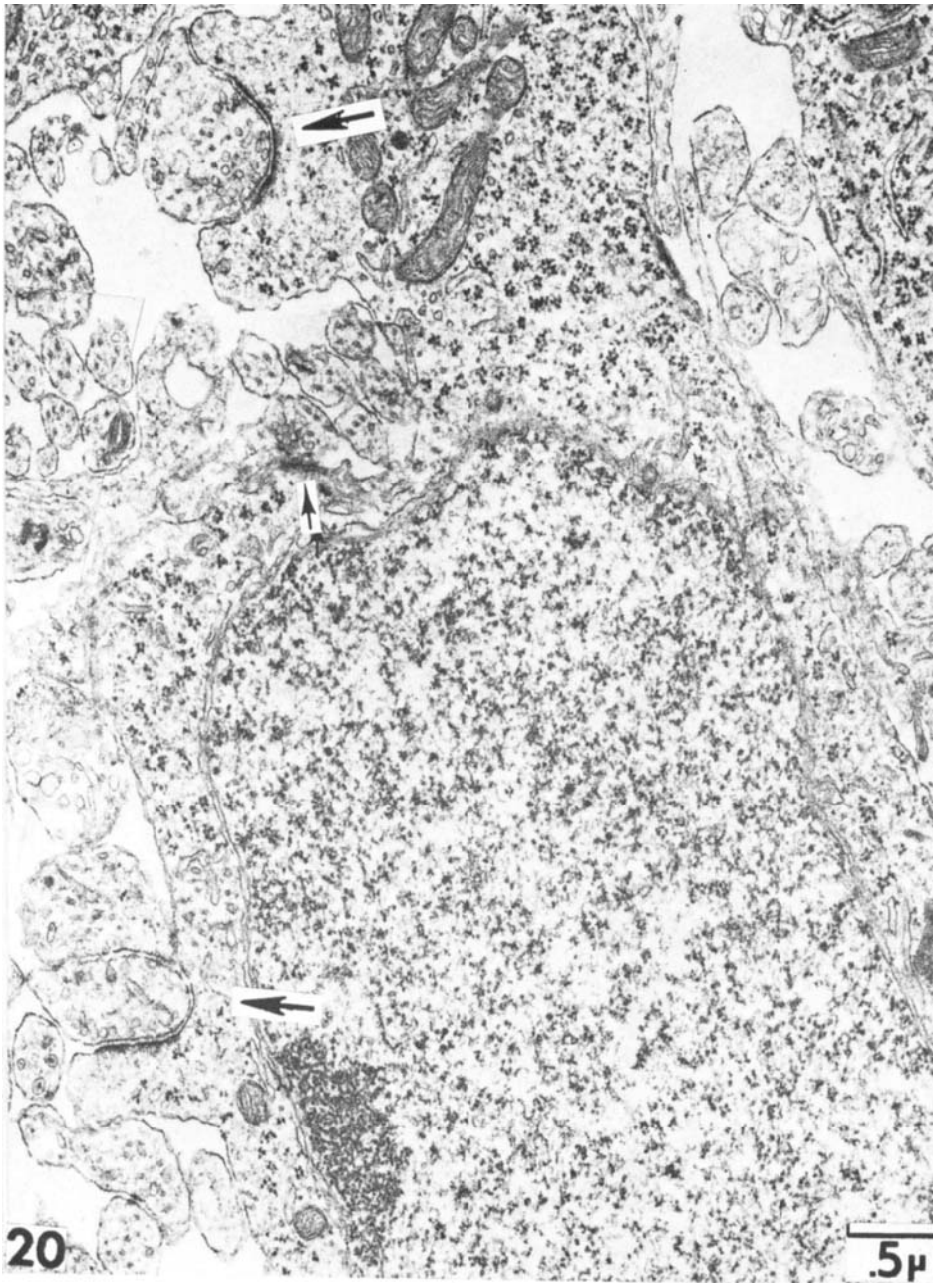

20
.5μ

PLATE 7

EXPLANATION OF FIGURE

21 Complex perisomatic synapse on the lateral aspect of a Purkinje cell soma and representing the last stage in the growth of perisomatic synapses. Note the scarcity of organelles in the perisomatic processes (arrows) and how they surround the terminal. In view of the transient nature of these synapses, this intimate relation (with serrated surfaces reminiscent of the synapses of granule cell dendrites with mossy fibers, see fig. 6 in Altman, '72b) can be explained as a way of enlarging the area of contact between the pre- and postsynaptic elements. Pyramis, seven days.

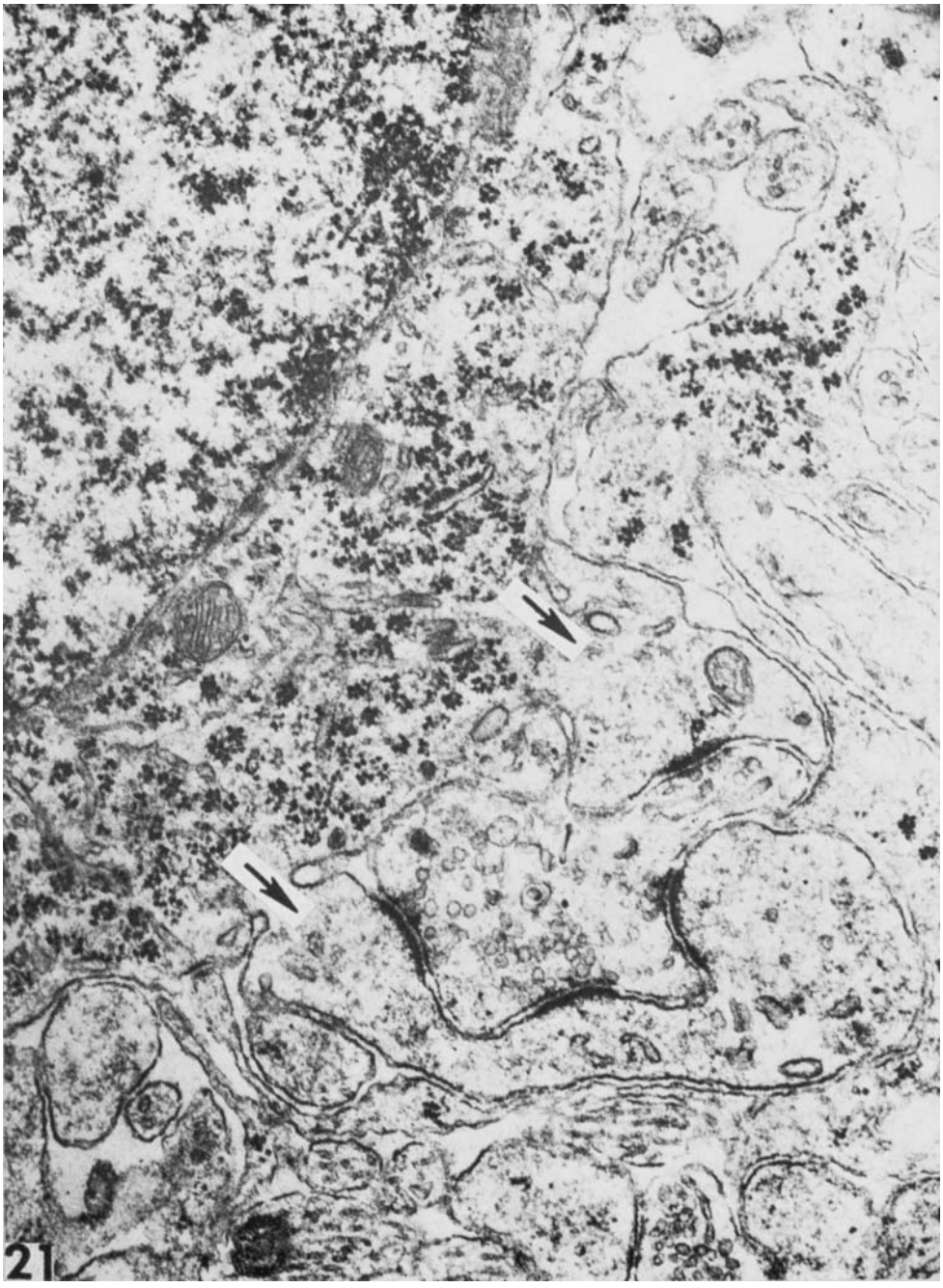

21

PLATE 8

EXPLANATION OF FIGURE

22 A Purkinje cell in the pyramis of a seven-day old rat. Note the enlarged and lightly staining nucleus and prominent nucleolus. The apical cone is very prominent and has a pronounced reticular organization. Fused cisterns (arrows) are common near the surface of the soma and its apical extension, and there are several perisomatic processes (PS). The dendritic branches are beginning to be formed.

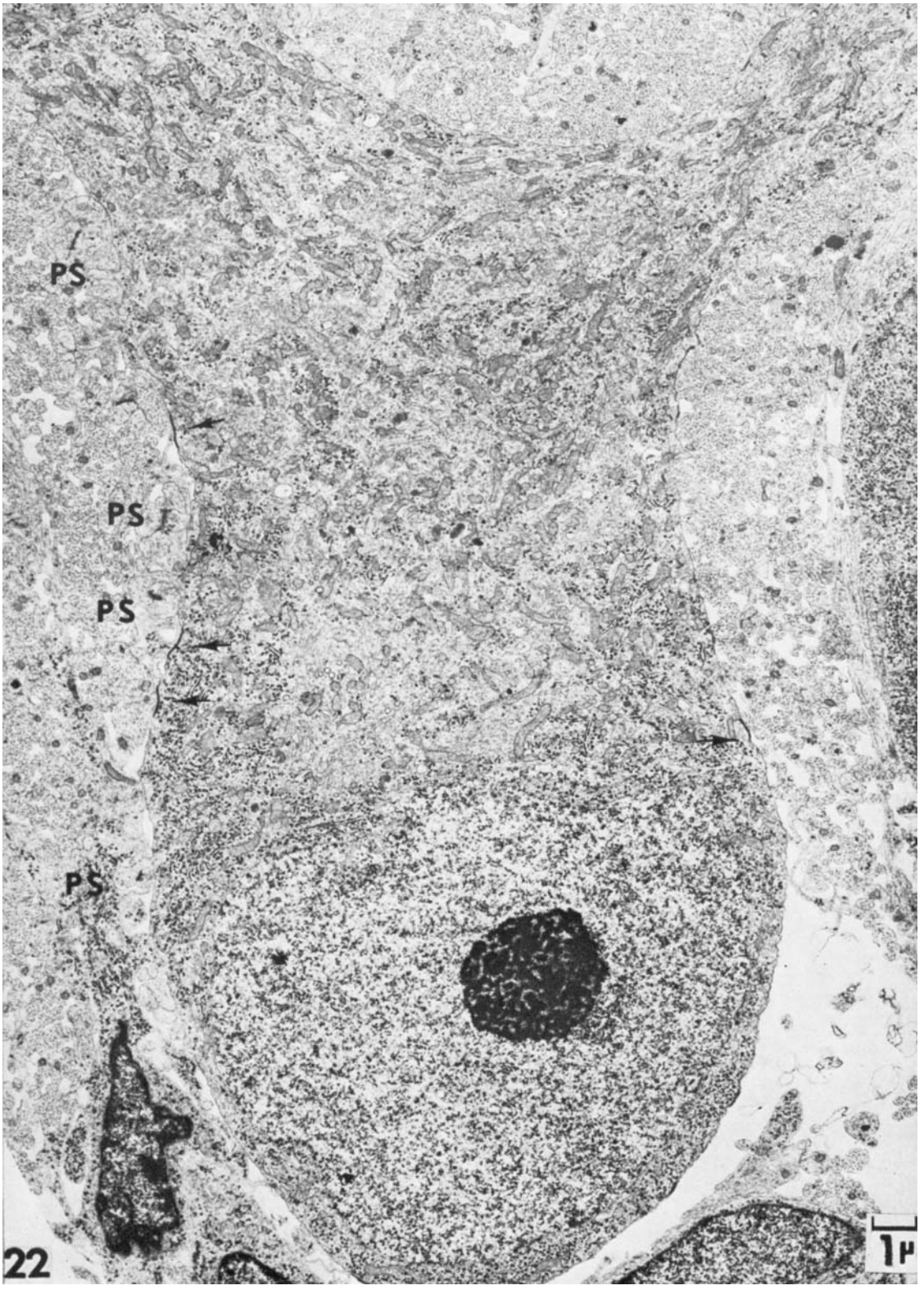

PS
PS
PS
PS
22
1μ

PLATE 9

EXPLANATION OF FIGURES

23–24 Closed coated vesicles (CV) in and open coated vesicles (OV) on the surface of Purkinje cell smooth dendrites opposite parallel fibers cut in cross section. Also visible are junctional, symmetrical membrane densities of the desmosoid type, reflecting attachment sites (AS), possibly early synapses, and mature synapses (MS) with synaptic vesicles and asymmetrical dense membranes. Note in upper left quadrant of figure 23 the presence of an opening (?) coated vesicle which is continuous with dense membrane junctions formed with a process that has synaptic vesicles. Pyramis, seven days.

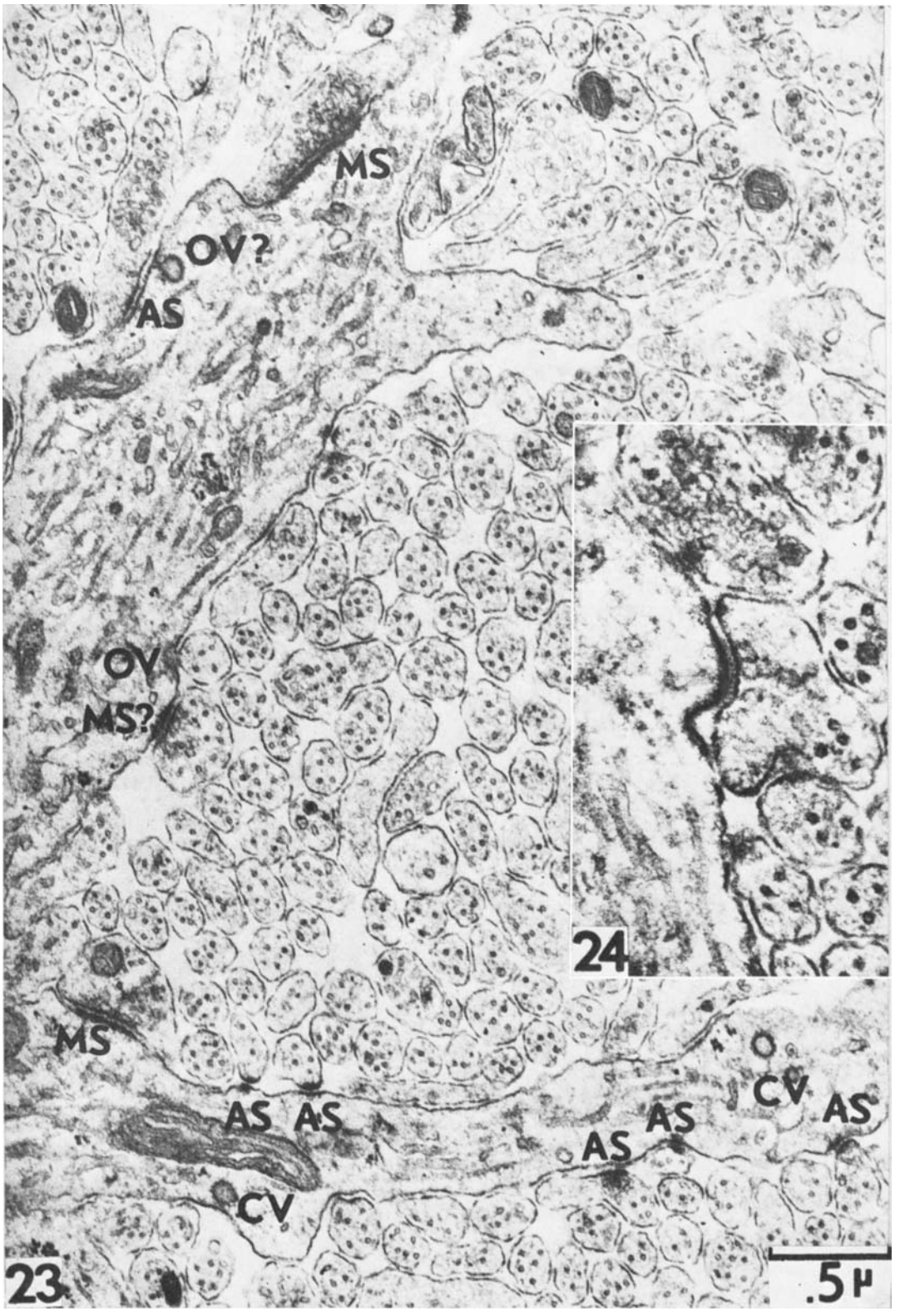

MS
OV?
AS
OV
MS?
24
MS
AS AS
CV
AS AS
CV AS
23
.5μ

PLATE 10

EXPLANATION OF FIGURE

25 Maximal enlargement of the apical cone with a reticular organization. Note the peripherally displaced ribosomal cytoplasm, still unbound, in the apical region and its predominance at the base. Note also the coexistence of newly forming inconspicuous, symmetrical synapses of basket cells (BS) with the perisomatic synapses (PS). Pyramis, ten days. Inset: a similar Purkinje cell from the same region; "thick" Epon section, azure B, × 640.

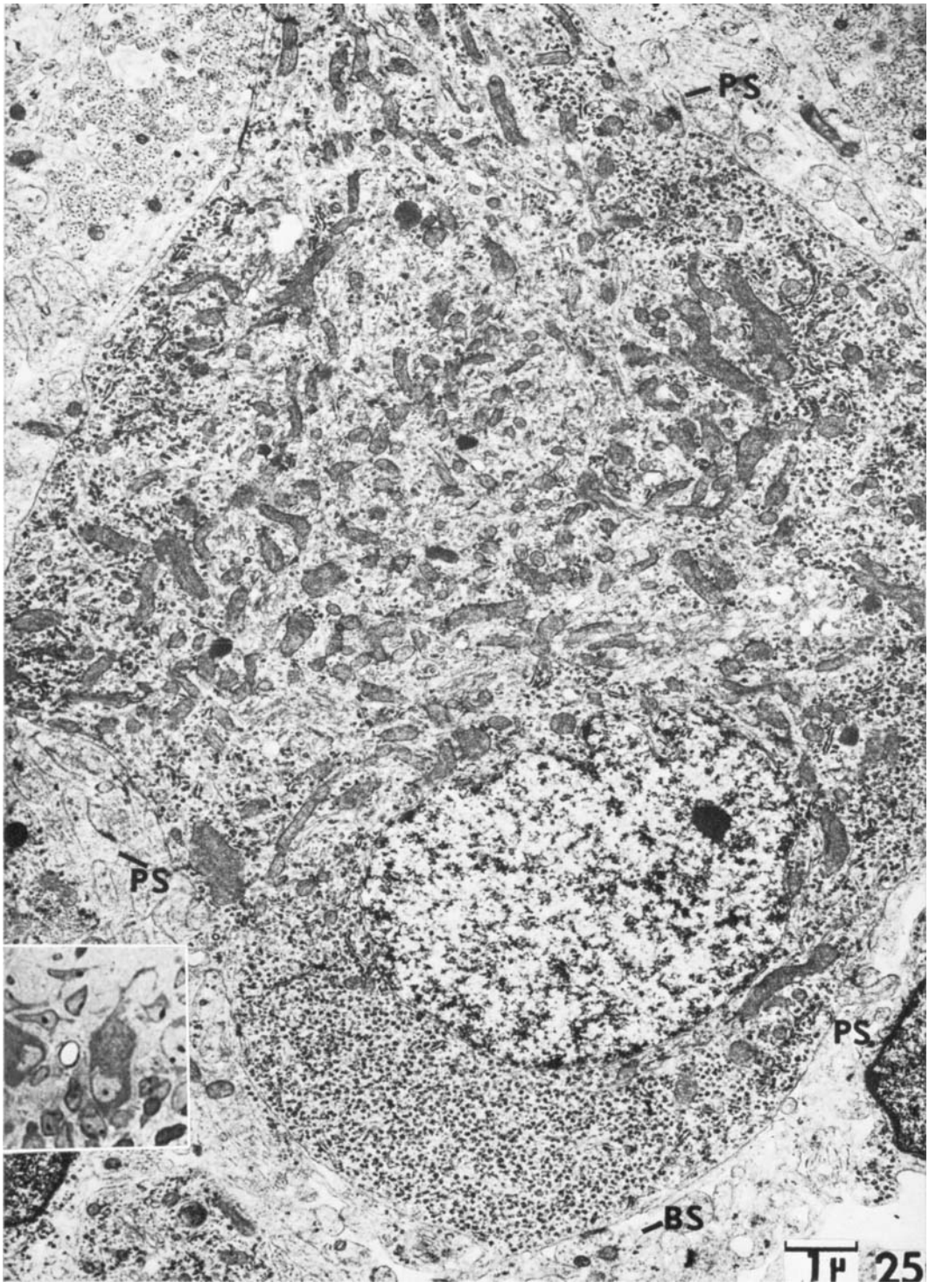

PS
PS
PS
BS
25

PLATE 11

EXPLANATION OF FIGURE

26 Purkinje cell with primary dendrite and smooth branchlets in the pyramis of a 12-day old rat. Note the regressed apical cone cytoplasm. At this stage of development the reticular cytoplasm predominates in the growing smooth dendrites (inset B). On the soma, perisomatic synapses (PS) are still present but synapses are becoming more common on outgrowing "thorns" (DS) of smooth dendrites (inset A). These synapses are similar to those on the perisomatic processes and are identified as the mature type of climbing fiber synapses. The beginning of the outgrowth of dendritic spines is seen in inset B, with synaptogenic interaction with a parallel fiber (arrow).

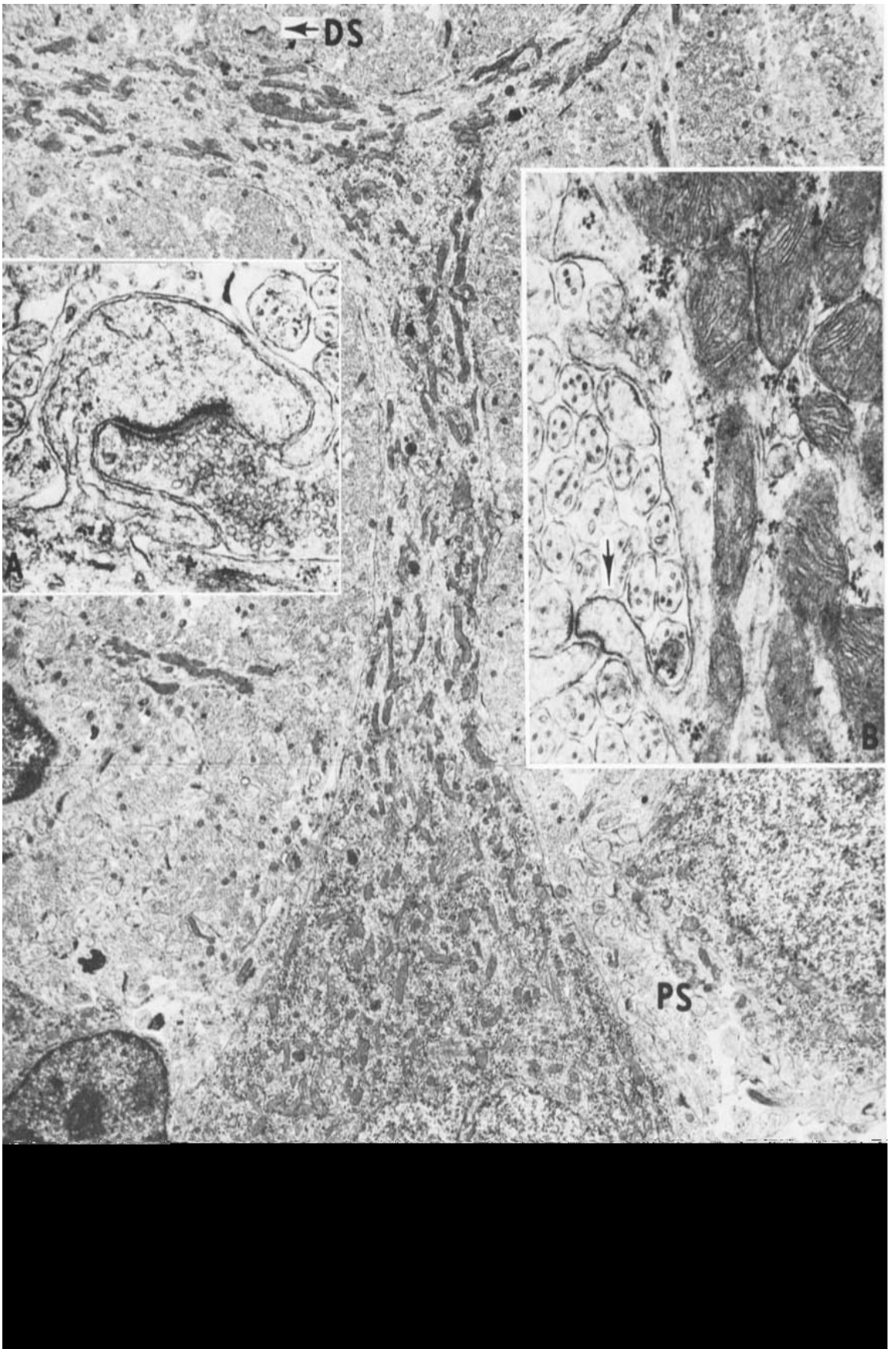

DS
A
B
PS

PLATE 12

EXPLANATION OF FIGURES

27 Closed coated vesicles (CV) in apparent upward streaming in the primary dendrite of a Purkinje cell. This dendrite is surrounded by glial covering. Pyramis, 12 days.

28 Basket cell bouton with inconspicuous symmetrical synapse on the soma of a Purkinje cell. Pyramis, 12 days.

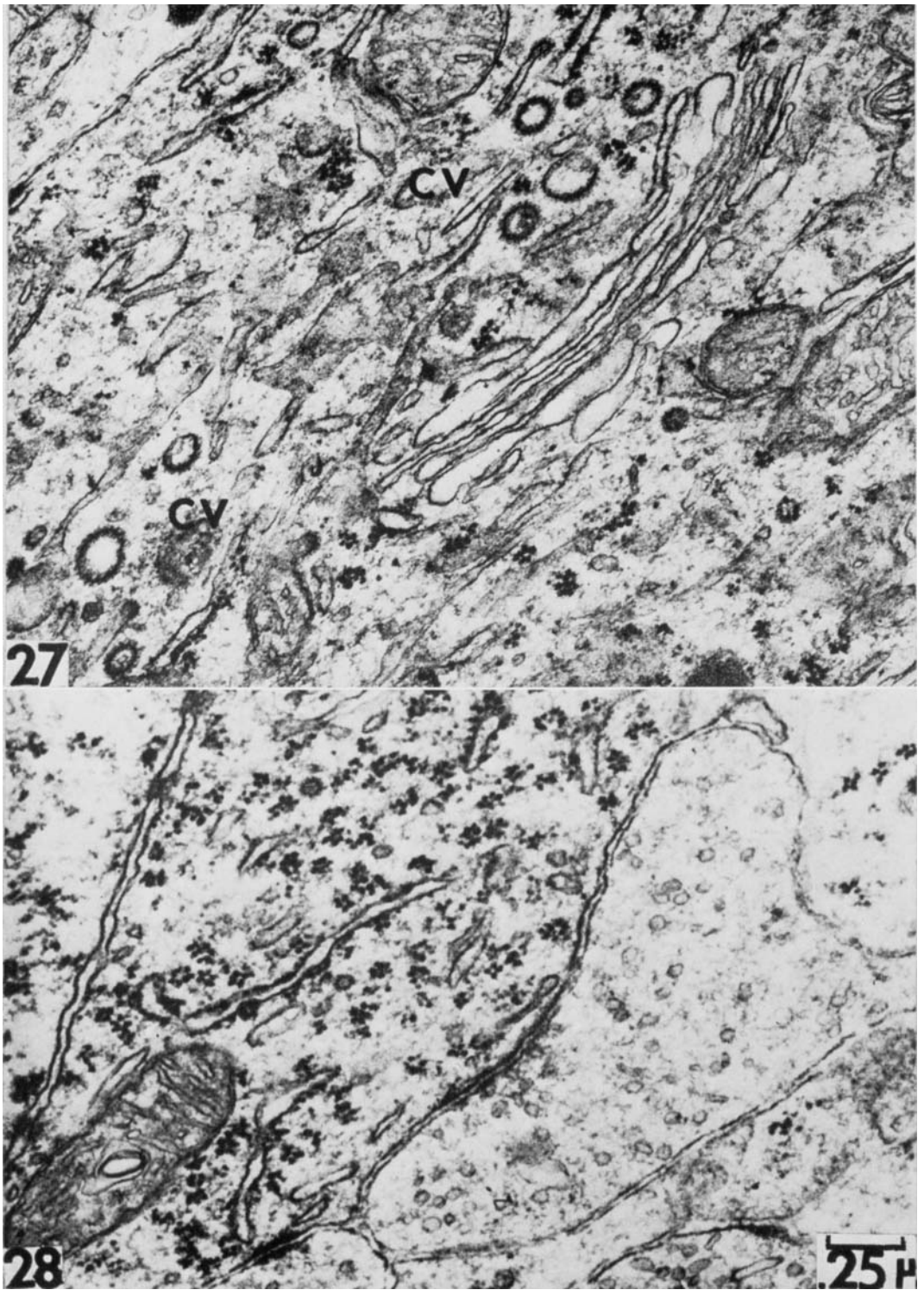

CV
CV
27
28
.25 μ

PLATE 13

EXPLANATION OF FIGURES

29 High concentration of open coated vesicles opposite parallel fibers on the primary dendrite of a Purkinje cell. Because parallel fibers do not form synapses in the adult with smooth dendrites, these are either transient junctions or else, following the outgrowth of spiny branchlets, the adhering junctions are translocated together. Pyramis, 12 days.

30 The outgrowth of the secondary and tertiary dendrites begins coincidentally with the commencement of synaptogenic interaction between the lower domain of the Purkinje cell dendrite. The cytoplasm of these processes has the typical reticular organization of growing presynaptogenic regions. Pyramis, 12 days.

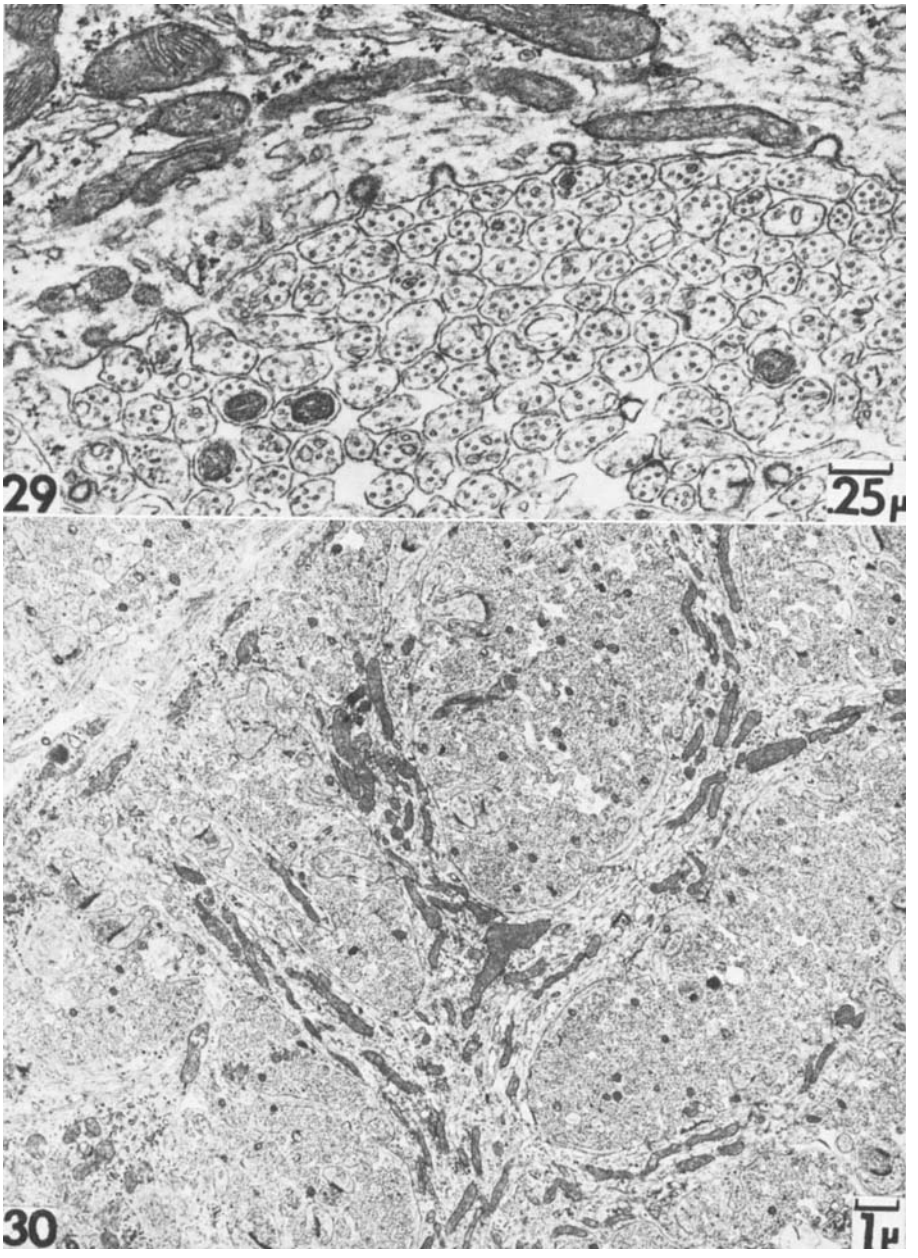

29
25μ
30
1μ

PLATE 14

EXPLANATION OF FIGURES

31 Glial process (GP) interposed between the primary dendrite of a Purkinje cell and parallel fibers. Note the presence of an open coated vesicle (OV), suggesting synaptogenic activity, where the glial barrier is absent. Pyramis, 12 days.

32 Bergmann glia (BG) cell adjacent to the soma of a Purkinje cell. The cytoplasm of the Bergmann glia cell can be seen to form a sheath around the soma of the Purkinje cell (PC). Pyramis, 12 days.

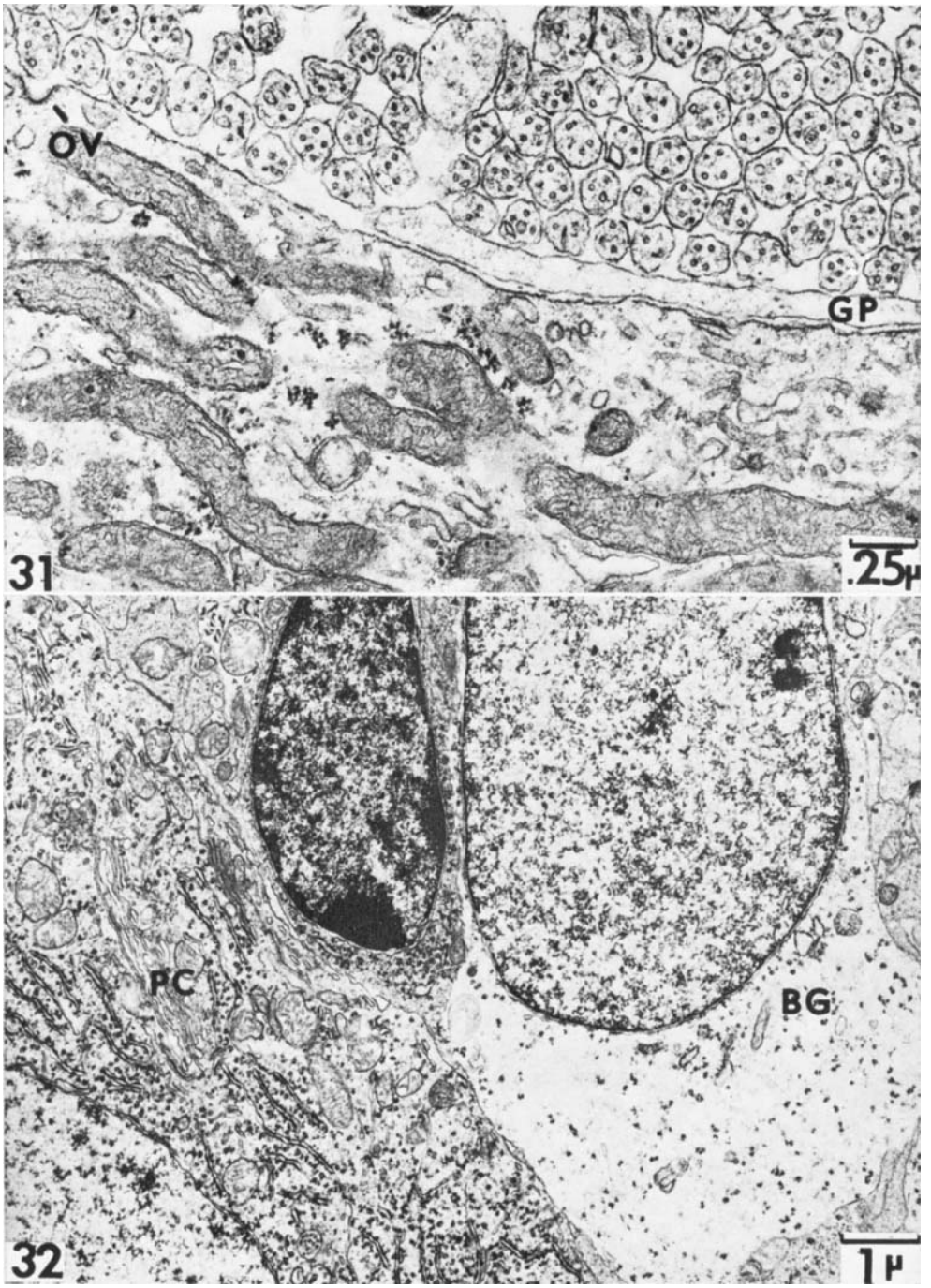

OV
GP
31
.25μ
PC
BG
32
1μ

PLATE 15

EXPLANATION OF FIGURES

33–36 Primary and secondary dendrites of Purkinje cells with open coated vesicles into which protrusions of parallel fibers are apparently sucked. Some of the latter may be in a process of being pinched off and incorporated (fig. 35, arrow). Pyramis, 12 days.

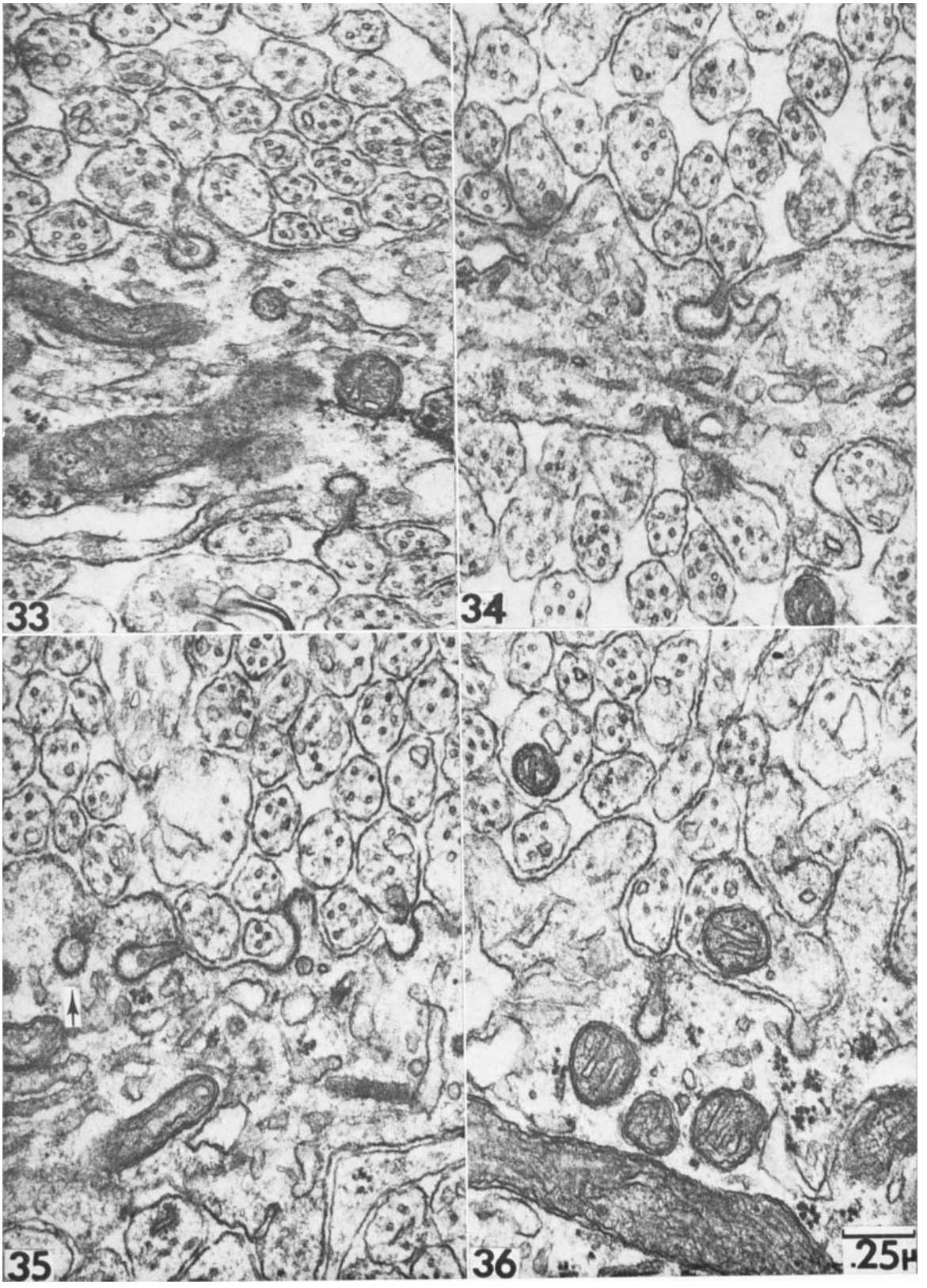

33
34
35
36
.25μ

PLATE 16

EXPLANATION OF FIGURE

37 Mature Purkinje cell soma in the pyramis of a 15-day old rat. Mitochondria and Golgi apparatus are present in moderate concentration, granular endoplasmic reticulum is abundant. Several basket cell synapses are seen on the soma of which one is shown enlarged.

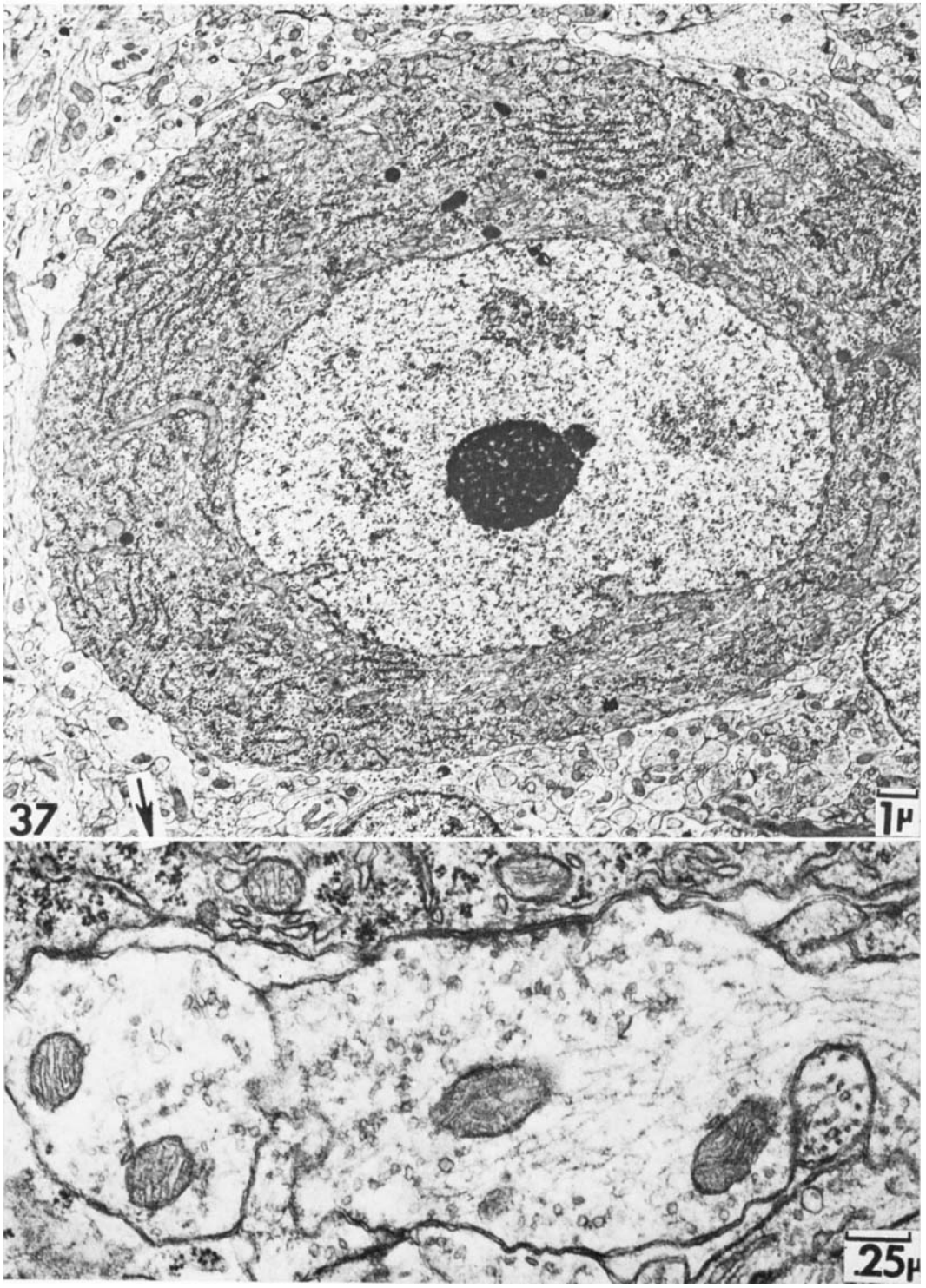

37
1μ
.25μ

PLATE 17

EXPLANATION OF FIGURES

The appearance of boutons of basket cell axons on the perikarya of Purkinje cells (PS) in two different planes of sectioning.

38 Basket cell terminal cut longitudinally. The axon contains microtubules and neurofilaments and *en passant* type of junctions are seen at irregular intervals (arrows). Pyramis, 15 days.

39 Basket cell terminals cut transversely. Their clustering, as seen here, is not uncommon. The characteristic of basket cell synapses with inconspicuous, symmetrical junctional membranes, is evident. Pyramis, 15 days.

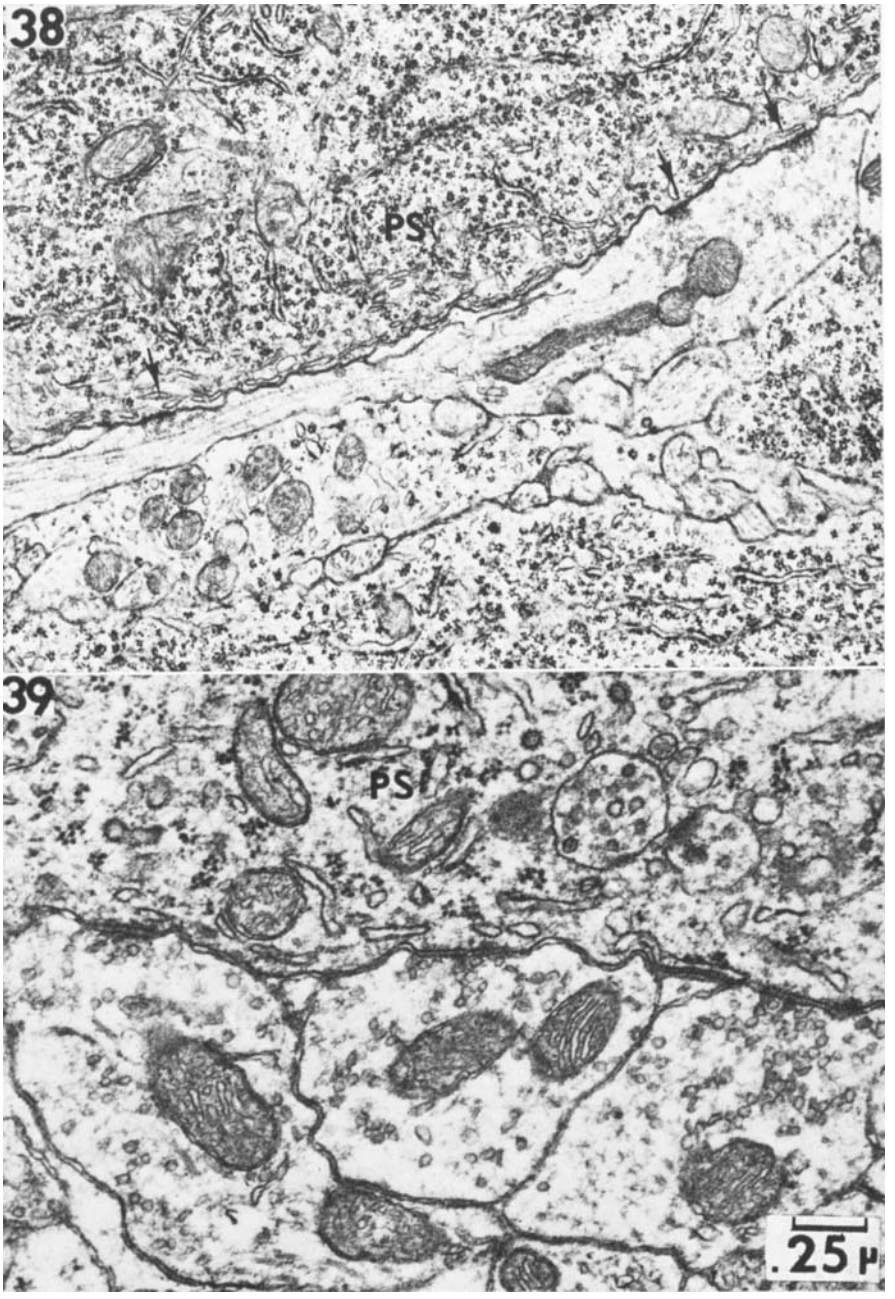

38
PS
39
PS
.25 μ

PLATE 18

EXPLANATION OF FIGURES

40 Large smooth dendrite of a Purkinje cell cut longitudinally. Glial sheath (GS) separates the dendrite from neighboring parallel fibers. Note the reduction in the concentration of mitochondria when compared with the growing dendrite (figs. 22, 26) and the increase in the concentration of microtubules. Pyramis, 21 days.

41 Purkinje cell smooth dendrite in cross section. Note peripheral position of mitochondria and the regular spacing of microtubules. Pyramis, 30 days.

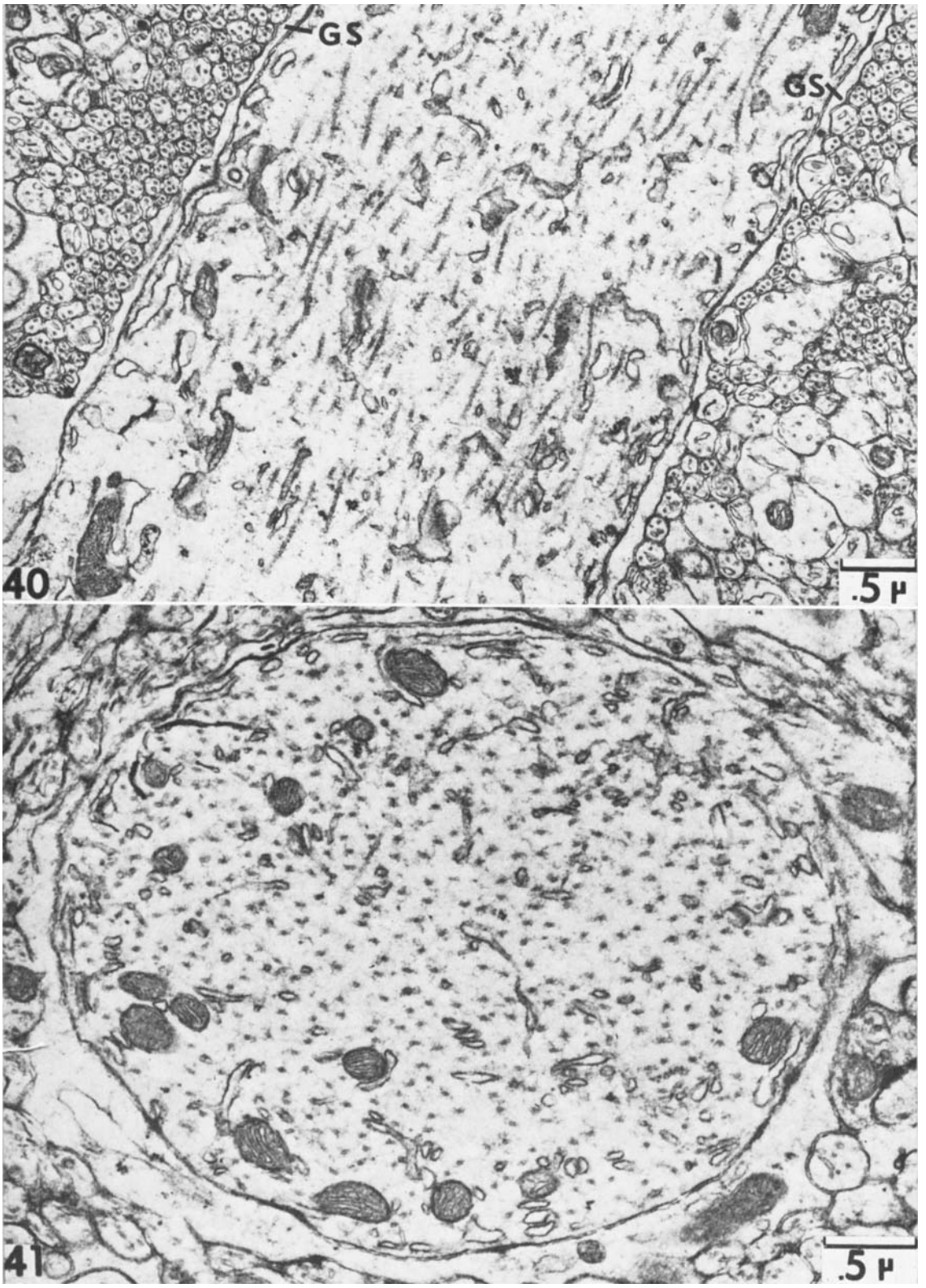

GS
GS
40
.5 μ
41
.5 μ

PLATE 19

EXPLANATION OF FIGURE

42 Branching Purkinje cell smooth dendrites with several boutons (arrows). The synapses are of the basket axon type (arrows) and may be those of basket or stellate cells. One bouton has dense core vesicles and possibly a conspicuous synapse (double arrows). Pyramis, 21 days.

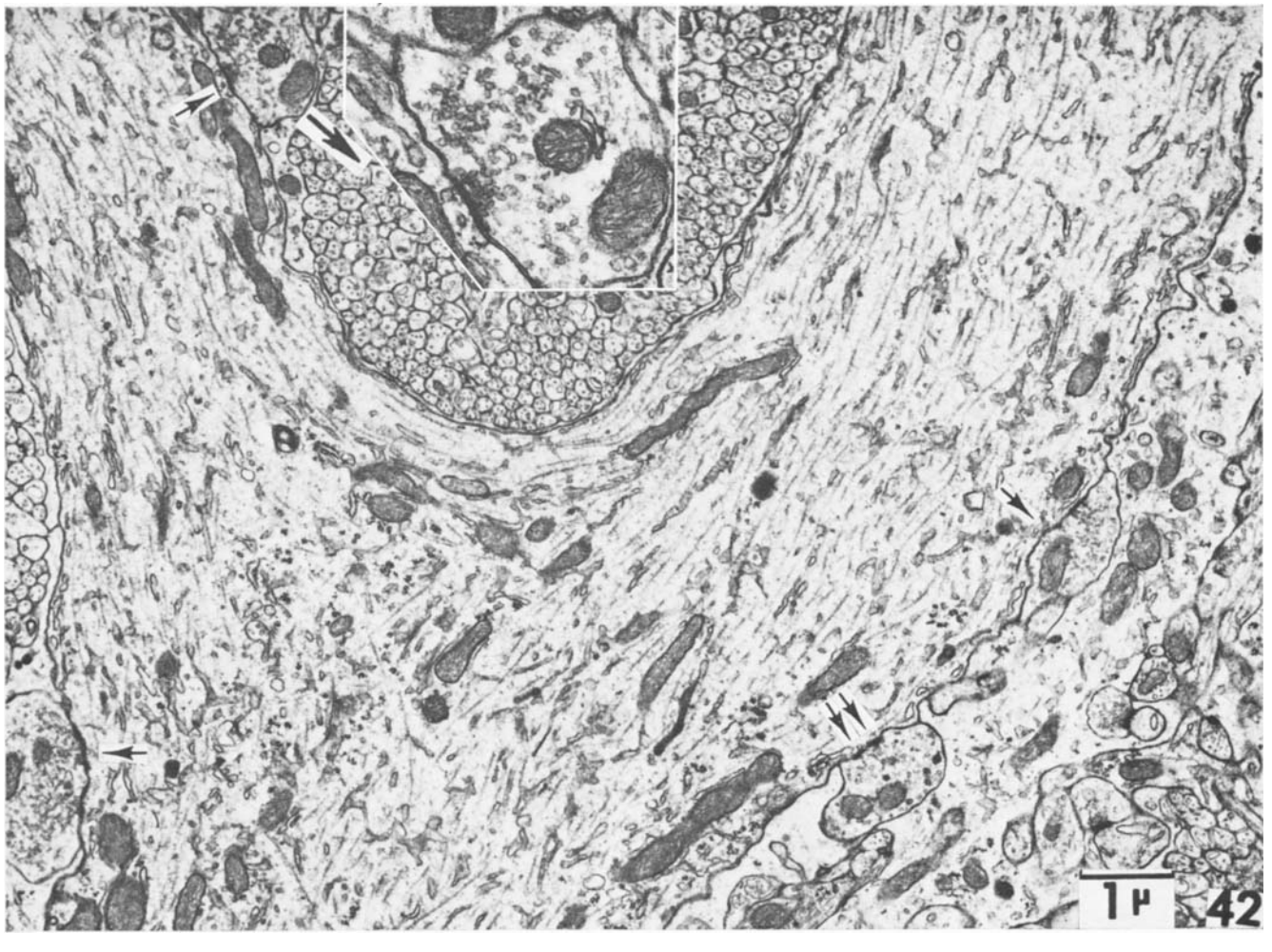

1μ
42

PLATE 20

EXPLANATION OF FIGURES

43 An axon adjacent to a Purkinje cell smooth dendrite (PSD) in the molecular layer, presumed to be a climbing fiber (CF), forming synapses with profiles that may be "thorny" outpouchings of a Purkinje cell smooth dendrite. Pyramis, 30 days.

44 Enlarged view of the conspicuous asymmetrical synapses.

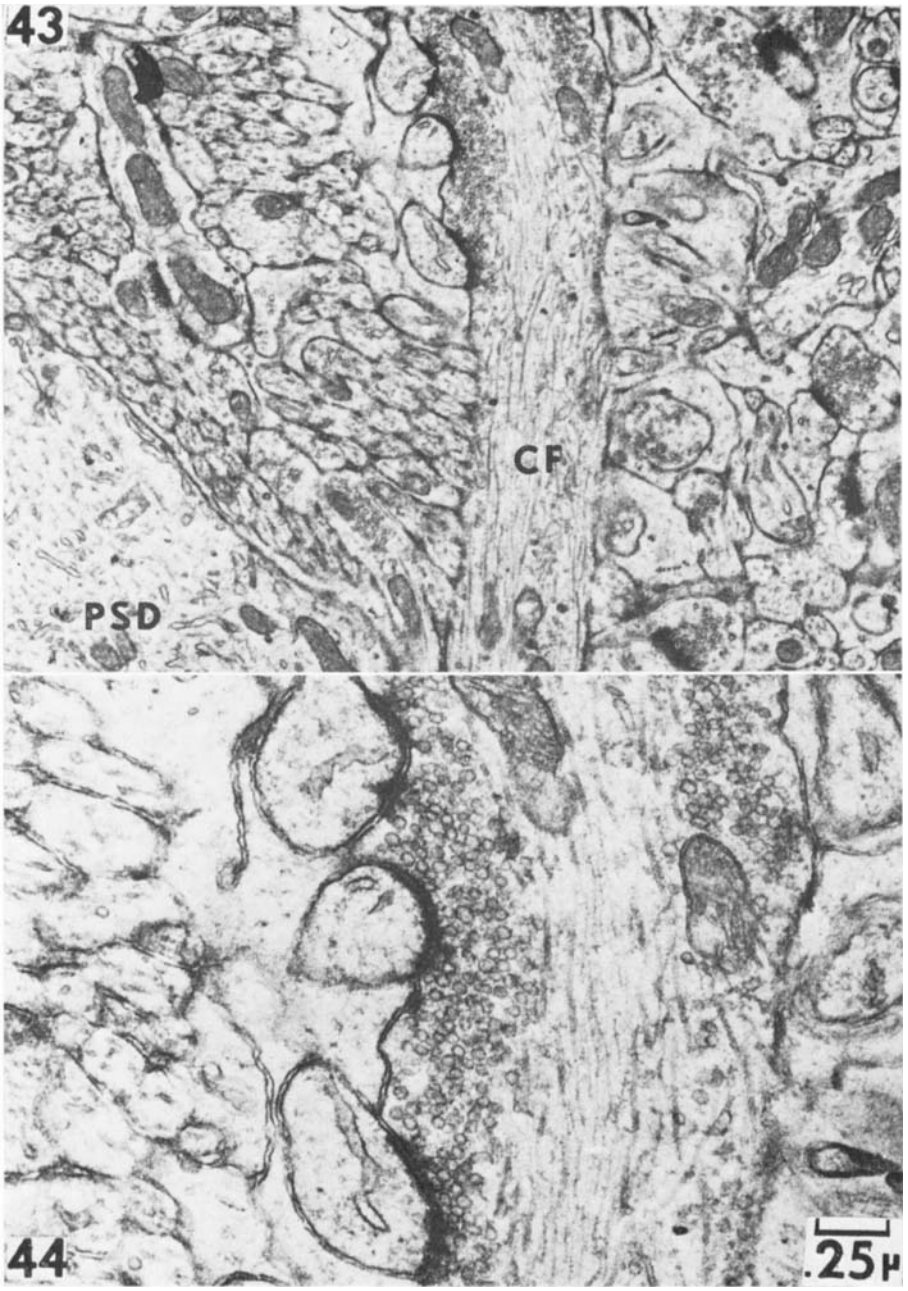

43
CF
PSD
44
.25μ

PLATE 21

EXPLANATION OF FIGURES

45–47 Changing proportion of parallel fiber profiles, and of parallel fiber varicosities and synapses with dendritic spines in the upper (fig. 45), middle (fig. 46) and lower portions (fig. 47) of the molecular layer in the pyramis of a 21-day old rat. The lower aspect of the molecular layer with decreased parallel fiber profiles appears mature, whereas the upper portion with few dendritic processes and synapses is quite immature at this age. Compare these figures with figures 10–12 showing oxidative enzyme staining in different parts of the molecular layer as a function of age.

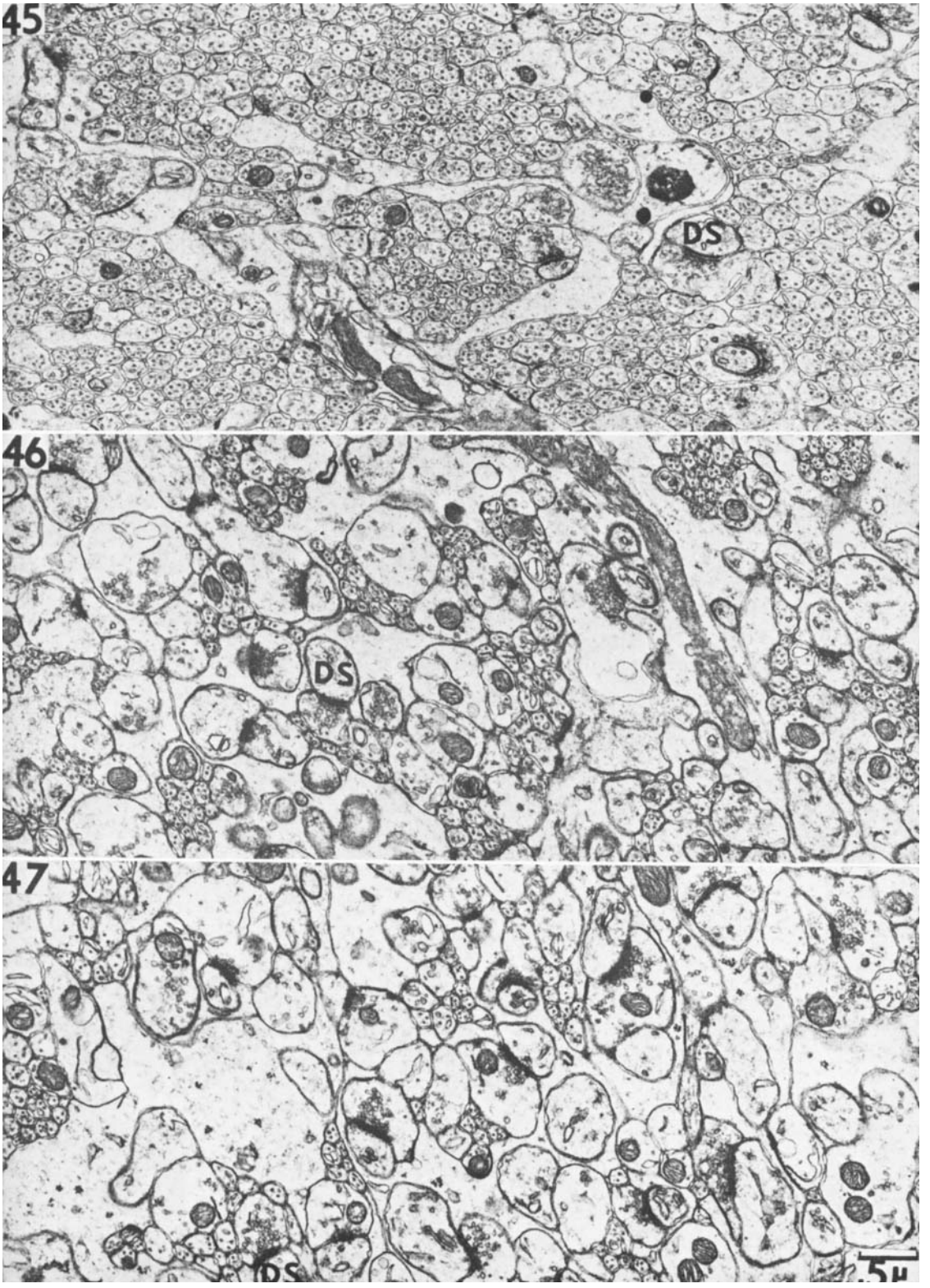
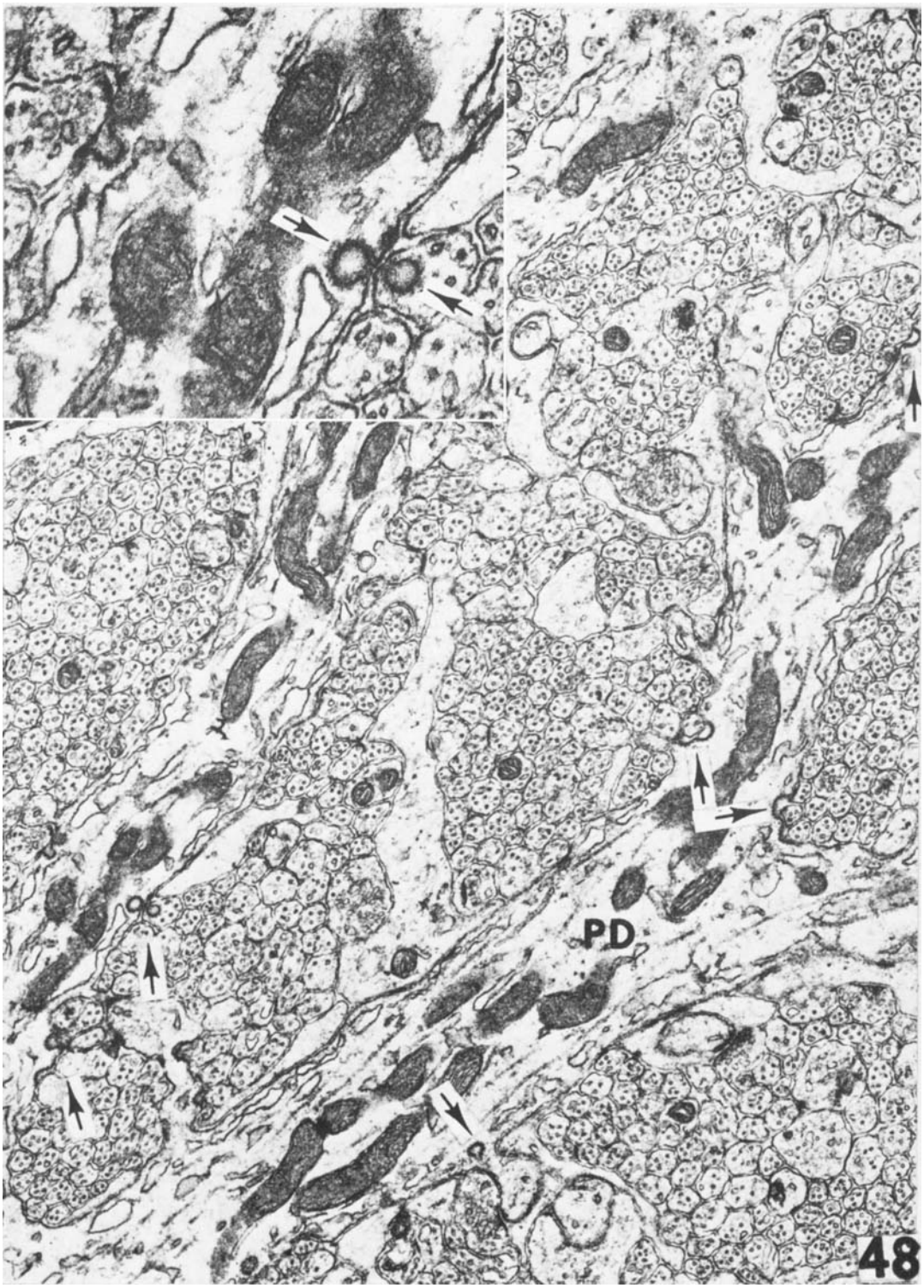

45
DS
46
DS
47
DS
.5μ

PLATE 22

EXPLANATION OF FIGURE

48 Purkinje cell dendritic branchlets (PD) in the upper molecular layer in the pyramis of a 21-day old rat. Except in the lower regions (left corner), there is direct contiguity between the dendrites and parallel fibers and synaptogenic activity is indicated by the presence of many open coated vesicles (arrows) opposite parallel fibers. Inset shows the apposition of two coated vesicles in a Purkinje cell dendrite and a parallel fiber.


PD
48