# Postnatal Development of the Cerebellar Cortex in the Rat

## III. MATURATION OF THE COMPONENTS OF THE GRANULAR LAYER

JOSEPH ALTMAN
*Laboratory of Developmental Neurobiology, Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907*

*ABSTRACT* The migration of granule cells and the maturation of the various elements of the granular layer were studied in the cerebellar cortex of rats aged 0, 3, 5, 7, 10, 12, 15, 21 and 30 days with histological, histochemical, autoradiographic and electron microscopic techniques. The bulk of the granule cells are formed during the second week, but due to the time required for their migration and the lag in the formation of dendrites, few glomerular synapses are formed with mossy fibers before the beginning of the third week and the process is still in progress at 30 days, long after the dissolution of the external germinal layer. The maturation of Golgi cells is a protracted process. Their axons synapse with granule cell dendrites as soon as the glomeruli begin to mature. Evidence was obtained that mossy fibers synapse with the dendrites of Golgi cells. Towards the end of the second week the Lugaro cells are formed and synapses appear on their somata during the third week. Among these synapses the recurrent collaterals of Purkinje cell axons were identified. The Lugaro cells may be the primary targets of the infra- and supraganglionic plexuses formed by these collaterals. In conclusion it was suggested that there are three major, successive stages in the neurogenesis of the cerebellar cortex, the morphogenic, synaptogenic and gliogenic. However, in the large Purkinje cell the synaptic maturation of one region (the soma) may begin before the morphogenic and synaptogenic maturation of the entire cell (the dendrites) is completed.

The proliferation and differentiation of cells of the external germinal layer and the maturation of Purkinje cells and of the surrounding elements in the molecular layer were described in two preceding papers (Altman, '72a,b). The granule cells in the bipolar phase produce parallel fibers by a stacking process, whereby they become piled up in the molecular layer from the bottom upward in accordance with their time of origin or "age." A similar assembly by stacking is indicated for the non-migratory, indigenous cells of the molecular layer, the basket and stellate cells. Correlated with this growth of the molecular layer from the bottom upward, the Purkinje cell dendritic system gradually invades the maturing elements and eventually synaptic contacts are established with elements, such as parallel fibers, situated higher and higher up in the molecular layer. The synaptic maturation of parallel fibers is, of course, dependent on the migration of granule cells into the granular layer where they establish synapses with mossy fiber rosettes in the glomeruli. In this last paper of the series dealing with the normal development of cerebellar cortex, we describe aspects of the migration and maturation of granule cells and the development of glomerular synapses. In addition, we also deal with the development of two other neuronal elements of the granular layer, the Golgi cells and the Lugaro cells.

### MATERIALS AND METHODS

The material utilized for this study was described in detail in the first paper of this series (Altman, '72a). Cerebella from

several hundred Long-Evans rats were prepared for histological, histochemical, autoradiographic and electron microscopic examination. Over one hundred 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 a single dose of thymidine-$H^3$ at different ages, 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 first paper of this series.)

## RESULTS

### 1. *Migration and maturation of granule cells*

*Light microscopic observations.* In Nissl-stained sections, differentiated granule cells cannot be identified with any certainty in the pyramis of neonates, although in the earlier-maturing nodulus a several cell-deep granular layer is present in the two to three day old rats. Interestingly, with Golgi impregnation, a rare granule cell with parallel fiber and dendrites was seen in the newborn, suggesting that a few early differentiating granule cells are formed before birth.

In the pyramis a two to four cell-deep granular layer is seen at five days of age and thereafter there is a rapid increase in the cell-depth of the granular layer. This increase is associated with the migration of vertically oriented cells that originate in the external germinal layer through the molecular layer. These vertically-oriented cells are often impregnated with the Golgi technique and can be seen to have parallel fibers issuing from them. The soma may be in various depths in the molecular layer, with short or long proximal and distal processes, as previously described by Ramon y Cajal ('60). The dynamic properties of the migration of these cells is best examined with autoradiography. (The transit of cells from the proliferative zone of the external germinal layer to the premigratory zone, and from there into the migratory field of the molecular layer was described in the first paper of this series; Altman, '72a.)

*Autoradiographic results.* In rats injected with two successive daily doses of thymidine-$H^3$ at zero to one days of age and killed at 60 days, intensely-labeled granule cells (that is, granule cells whose precursors ceased to multiply after the injection, presumably because they began to differentiate) are rarely seen in the pyramis, although a few are seen in the earlier-maturing nodulus and uvula. In the animals injected with two successive doses at two to three days of age, an occasional intensely-labeled granule cell can be seen in some sections in the depth of the granular layer. These results indicate that the postnatal differentiation of granule cells during the first few days of life is negligible in the rat. This conclusion is confirmed by examination of the pyramis in rats that were injected with four successive daily doses on days 0–3 and were killed in adulthood.

In the rats injected with two successive doses at four to five days and killed at 60 days of age, a few intensely-labeled cells are seen in the lower half of the granular layer, but the majority of the cells are light- or medium-labeled or unlabeled. There is an appreciable increase in intensely-labeled granule cells in the animals injected with two successive doses on days 6–7 or with four successive doses on days 4–7. These data indicate that the differentiation of granule cells, in terms of exit of their precursors from the proliferative compartment of the external germinal layer, begins on a large scale toward the end of the first week after birth.

Counts of intensely-labeled cells in the lower and upper zone of the internal granular layer in the group that received four successive injections showed (fig. 1) maximal concentration of such cells in the animals injected at 8–11 days; but the concentration of these cells is also high in the

animals injected at 12–15 days, after the differentiation of stellate cells is terminated. In the group that received two successive daily doses of thymidine-$H^3$, the highest concentration of intensely-labeled cells was obtained at 10–11 days, but the concentration of these cells is also high at 8–9, 12–13 and 14–15 days. From this, it is concluded that the bulk of the granule cells are formed between 7–15 days, that is, over the long time span of the second week of life. Interestingly, of the small proportion of granule cells formed during the first week, more were situated in the lower half of the internal granular layer than in the upper half, and the reverse held for the granule cells formed during the third week (fig. 1). This was shown by computing the ratio of the concentration of intensely-labeled cells in the two zones of the internal granular layer (fig. 2).

*Electron microscopic observations.* Vertically-oriented, spindle-shaped cells are seen in the molecular layer in all the animals up to and including 21 days of age, but they are particularly numerous at 7, 10, 12 and 15 days. Typically, these cells have oval nuclei (figs. 4, 5), studded with clumps of coarse chromatin, and there are cytoplasmic enlargements at the upper (trailing) and lower (leading) poles of the cell; on the sides, the cytoplasm is sparse. The cytoplasm of the upper pole is rich in free ribosomes but devoid of other organelles (fig. 5), the cytoplasm of the lower pole is rich also in mitochondria and occasionally has other organelles, such as centrioles (figs. 4, 5). Most of these cells are contiguous with parallel fibers and other cell processes of the molecular layer; occasionally there is a glial separation, but synapses are never seen nor are coated vesicles or junctional dense membranes present in apposition to parallel fibers or other processes. It is assumed that these cells have no synaptic affinity with ele-

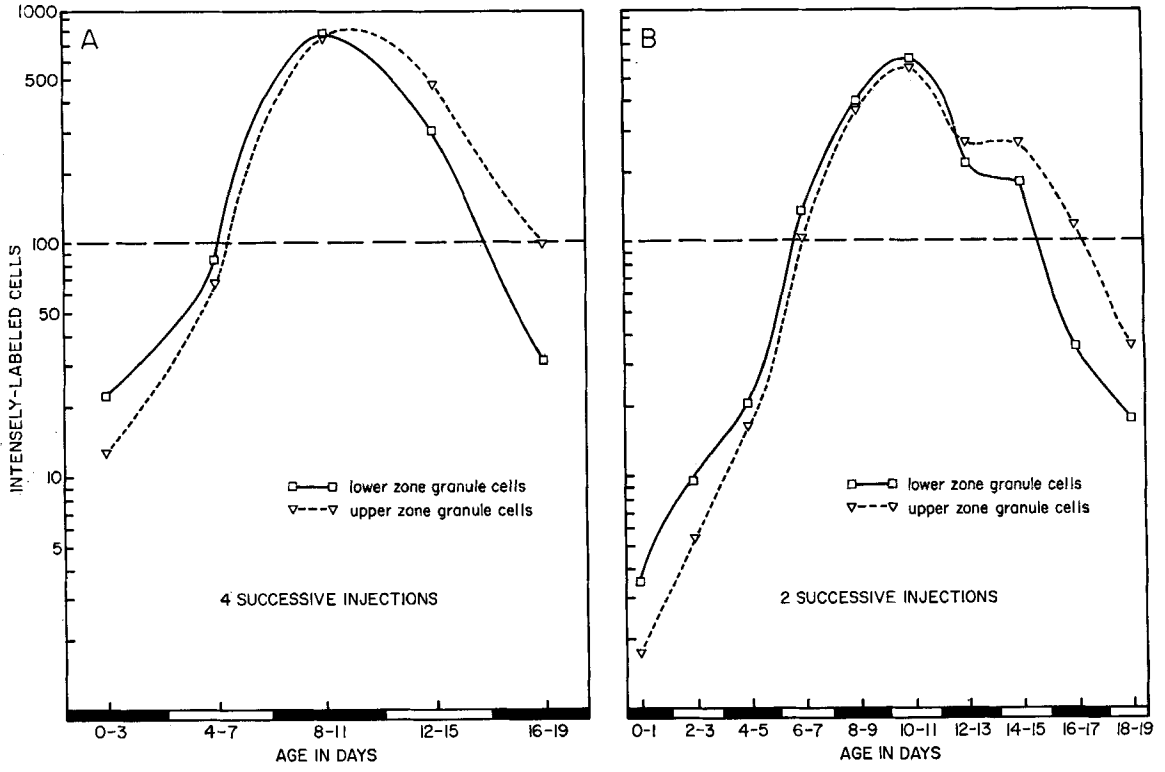


Fig. 1 Concentration of intensely-labeled cells in the granular layer of the pyramis in adult rats that were injected with two or four successive daily doses of thymidine-$H^3$ at different ages.

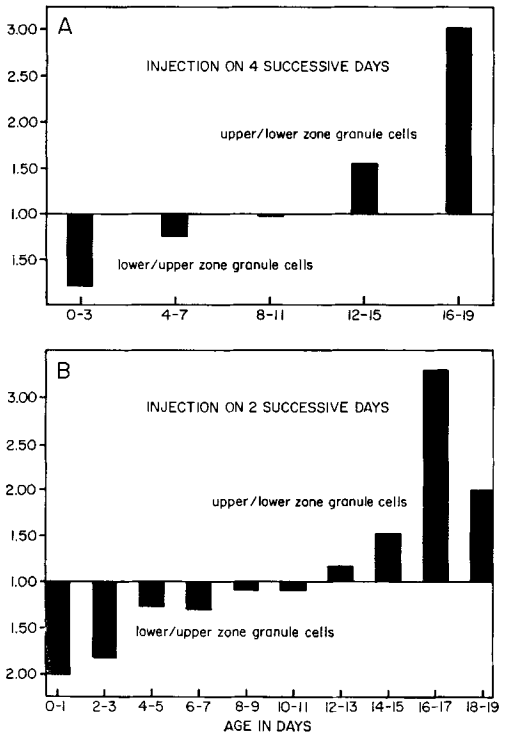


Fig. 2 Ratio of upper/lower zone or lower/upper zone intensely-labeled cells in the granular layer as a function of age at injection.

ments in the molecular layer, and move unhindered through this layer. Often the descending cells are contiguous with one another and appear to move in tandem through apparent channels in the mass of parallel fibers (fig. 4).

In several instances (fig. 5) vertically-oriented cells were cut in a single plane with their leading and trailing cytoplasmic enlargements and their fibrous extensions. Both the trailing and the leading fibers have a tubular organization. This indicates that in the formation of the horizontal portion of the granule cell axon the leading fibers may be extruded ahead of the perikaryon and that the axon-like process leads during descent.

In the newborn and three-day old rats, the cells are extremely loosely packed in the transitional region between the Purkinje cell zone and the medullary layer, the future site of the granular layer (and this is probably not a fixation artifact). At these ages, differentiated granule cells were not encountered in the available material. What were presumed to be primitive granule cells were seen in the five-day old animals. These cells had no dendrites. Neither profiles of granule cell dendrites nor mossy fiber terminals could be identified at five days in the pyramis.

The number of recognizable primitive granule cells increases at seven days, forming a distinct layer beneath the row of Purkinje cells. The layer itself has a loose appearance with wide spaces between the cells and cell processes (this is systematically seen in the infants but not in the older animals). In several instances large processes are seen issuing from the granule cells which are rich in free ribosomes. These processes could be the precursors of granule cell dendrites, but they lack the characteristic accumulation of microtubules (fig. 12). Paralleling the absence of differentiated granule cells at this age in the pyramis, mature mossy fiber terminals cannot be identified either. In the 10–12 day old rats, granule cells have become quite common but still few of them have recognizable dendrites with packed parallel microtubules and claw endings with mossy fiber terminals and synapses (fig. 6). A Golgi axon synapse on the shaft of the granule cell dendrite may also be present.

Well-differentiated granule cells become numerous in the 15-day old rats (fig. 7). Characteristically, several granule cell bodies are aggregated in groups; their membranes are contiguous without glial separation, but occasional dendrites may criss-cross the field. Boutons with synapses never terminate on the soma of granule cells. These dendrites have 6–12 closely-packed microtubules and terminate in claws which often have a serrated edge. In a few instances, the dendrites issuing from the granule cell soma were seen contacting with their serrated edges mossy fiber terminals with multiple synapses (fig. 8) and there may be desmosoid dense membranes (attachment plaques) between the dendrites (fig. 10).

In the 21-day old rats, the majority of granule cells are differentiated (fig. 9). There is an increase in the desmosoid junctions between contiguous granule cell dendrites, but these are as yet not numerous at this age (fig. 10). Relatively few changes

are seen between 21 and 30 days in the internal granular layer. One of these is the increase in the desmosoid junctions between granule cell dendrites in the glomeruli. These are seen between granule cell dendrites, one of which may have a synapse with a mossy fiber terminal (fig. 11) or between a dendrite that is contiguous with a mossy fiber and another which appears some distance away from the latter. These desmosoid junctions are also seen between granule cell dendrites and mossy fibers, although in this position they are difficult to distinguish from true synapses. A single granule cell dendritic ending may have several synapses with a mossy terminal and it may also have desmosoid contact with other dendritic endings. What are probably terminals of Golgi cells are also seen on granule cell dendrites.

In summary, light microscopic observations indicate that apart from a few early granule cells that may be present at birth or are formed soon thereafter, the bulk of them come into existence during the second week with a peak at 10–11 days. At that age, however, differentiated granule cells with mossy fiber synapses are still scarce, indicating some lag between the time these cells are formed, descend through the molecular layer, and have their dendrites formed in the granular layer.

### 2. *Maturation of the mossy fibers and of the glomeruli*

*Light microscopic observations.* As an indicator of the maturation of the mossy fibers, the development of staining for cholinesterase was examined in the lobules in which the mossy fibers stain intensely in the adult (e.g., the nodulus and uvula). The progressive growth of the glomeruli, the cell free islands where the mossy rosettes form synapses with the granule cell dendrites, was studied by following the development of staining in these "islands" with various oxidative enzyme stains (see figs. 7–12 in the second paper of this series.)

In the pyramis, staining reaction for AChE is altogether absent at zero to three days, but some faint staining is seen in the nodulus and uvula (and more marked staining in the fastigial nucleus). This indicates that the cholinergic mossy fibers and Golgi cells have not commenced to differentiate in terms of the production of an enzyme linked to their transmitters. In the nodulus, strong staining reaction is seen at five days in the medullary layer and the Purkinje cells themselves and their immediate surroundings are lightly staining. There is little or no staining at this age in the granular layer. Patchy staining in the granular layer is first seen at seven days (when the Purkinje cells become intensely stained). The staining of the medullary layer for AChE was interpreted in a previous study (Altman and Das, '70) as an indication that the cholinergic mossy fibers reached a certain degree of functional maturity, and the staining of the Purkinje cells (which do not stain in the adult) was thought to reflect a transient cholinoceptive property at these ages. At ten days, diffuse staining is seen throughout the granular layer and the medullary layer is strongly stained in the nodulus. By the 15th day, the adult pattern predominates, with strong staining of the glomeruli and of the Golgi cells; the soma of the granule cells is unstained or only lightly stained. These results indicated that the cholinergic mossy fibers were chemically mature at this age.

Whereas the growing portions of the cytoplasm of Purkinje cells stain from the third day onward with the various mitochondrial oxidative enzymes (LDH, SDH, CYO, etc.), discrete glomerular staining, which is a prominent feature of the adult cerebellar cortex, is not seen during the first ten days (figs. 7–9 in the previous paper of this series). In the ten-day old rat, there is diffuse, light staining in the granular layer, indistinctly outlining the unstained granule cell nuclei, but the glomeruli are not yet made visible by these methods. In the 15-day old rats, the negative image of the granule cells becomes more pronounced due to the light staining of the narrow cytoplasmic rings around the unstained nuclei, and small scattered moderately staining patches appear throughout the layer, heralding the onset of glomerular maturation (fig. 10 in the previous paper). The number of stained glomeruli and their staining intensity increases by the twenty-first day (same, fig. 11). But a comparison with the 30-day old animals

(same, fig. 12), in which the glomeruli become larger and more darkly staining, indicates that glomerular maturation continues for a long time after cessation of cerebellar neurogenesis.

*Electron microscopic observations.* Paralleling the absence of differentiated granule cells and the lack of glomerular staining for oxidative enzymes, mossy fiber rosettes and synapses are not seen during the first week in the pyramis. In the seven-day old rats, in a few rare instances, profiles were seen in contiguity with undifferentiated processes of granule cells which had one characteristic of mossy fiber terminals, the presence of one or several dense core vesicles, but they were lacking in the typical accumulation of vesicles and synaptic dense membranes (fig. 12). Their identification as presumptive mossy fiber terminals must remain uncertain. At ten days in some animals, a few mossy fiber terminals can be identified, but in most animals these are quite rare and apparently immature, with a roundish shape, variable concentration of vesicles and a small number of elongated and serrated dendrites, presumably those of granule cells. In most, though not all of the mossy fiber terminals, dense core, or granular, vesicles are present in variable numbers and there are also a variable number of asymmetrical synapses.

In the 12-day old rats, the number of mossy rosettes begins to increase but most of them are relatively small and simple (roundish) in configuration and are contacted by few (3–6) boutons (fig. 13). Synaptic vesicles are present in the mossy fiber terminals in high concentration and they often form a crystalline lattice. In some of these terminals, dense core vesicles are not seen (figs. 13, 14); in others they are conspicuous in number and in staining intensity (fig. 15). The elongated dendrites making contact with a large surface of the mossy terminals often have a serrated shape and several asymmetrical synaptic junctions (figs. 14, 15). At 15 days, a few simple rosettes (with few granule cell dendrites) are still seen, but the majority have become complex with a large number of dendritic profiles, each with one or several asymmetrical and conspicuous adhesion membranes. The mossy rosette is elongated in shape when cut longitudinally. It appears either as the bulbous terminal of a recognizable axon filled with microtubules and neurofilaments or it may just be an expansion on an axon (fig. 16). The accumulation of synaptic vesicles is usually very high, but the number of dense core vesicles is variable, with many in some glomeruli, none in others.

As described earlier, the desmosoid junctions are still infrequent at 15 and 21 days, but they increase in number and become conspicuous in the 30-day old animals. This change is coupled with the glomeruli becoming more and more complex in organization, presumably due to the increasing number of granule cell dendrites with which synapses are formed.

In summary, the histochemical and electron microscopic examination of the glomeruli has revealed that their maturation begins during the third week, that is, after the bulk of the granule cells have been formed. This delay represents the descent time of granule cells and the onset of growth of their dendrites. The growth of the mossy fiber-granule cell relay system continues for a long time after cessation of cerebellar neurogenesis.

### 3. *Maturation of the Golgi cells and the Lugaro cells*

*The Golgi cells.* According to the autoradiographic evidence, the majority of Golgi cells are formed before birth; labeled Golgi cells are rarely found in adult rats that were injected with thymidine-$H^3$ soon after birth. In neonates, the Golgi cells cannot be distinguished from Purkinje cells in Nissl-stained sections, though this is more readily done in Golgi-impregnated material. In the five to seven day old rats, the Golgi cells have several, very coarse and bulbous descending processes; these are presumably the growing axons. A comparable number of thorny ascending processes are also seen which may penetrate the external germinal layer; these are the maturing dendrites. In the ten-day old animals, the processes become more numerous and thinner, but only in the 15-day old animals are Golgi cells found that have the typical configuration of the adult cell.

Golgi cells do not show staining reaction for AChE during the first week and

the staining is not clearly evident in the ten-day old rats. In the 15-day old animals, moderately stained Golgi cells are common and their stainability increases appreciably in the 21 and 30 day old animals. With electron microscopy, the soma of Golgi cells can be recognized at ten days by virtue of their increased size and position in the granular layer (fig. 17). In the 15- and 21-day old animals, the following characteristics are noted: the nucleus is lobulated, large and lightly staining; the cytoplasm is large and irregular in shape and it is rich in granular endoplasmic reticulum, mitochondria and Golgi apparatus; glial covering is patchy and sparse; granule cell bodies and their dendrites, and other cells and processes are contiguous with the cell membrane of the Golgi cell (fig. 18). Boutons with synapses are rare on the soma of Golgi cells at these ages and also in the older animals. The few boutons that are seen tend to have a clear cytoplasm, a small clutch of vesicles and inconspicuous, symmetrical dense membranes. On the dendrites of Golgi cells at least two types of boutons and synapses are found. One of these synapses is of the conspicuous, symmetrical type. In a fortuitous section a mossy fiber terminal was identified on the dendrite issuing from the soma of a Golgi cell (fig. 18B, C). The identity of the inconspicuous, symmetrical type of synapse is not known (fig. 18D). Golgi axon terminals on granule cell dendrites are seen as early as 12 days (fig. 6) but they become more frequent in the older animals. These boutons have smaller vesicles than the mossy fibers and they form with the granule cell dendrites inconspicuous or conspicuous synapses, either of the symmetrical or asymmetrical type (figs. 19, 20).

*The Lugaro cells.* In the animals injected with two successive daily doses of thymidine-$H^3$ at 8–9, 10–11 or 12–13 days and killed as adults, intensely-labeled cells are seen in moderate numbers above, on the side or below the soma of Purkinje cells (figs. 21–23). It was suspected that these cells may not be granule cells because of their larger size, and that they may not be basket cells because the zone of basket cells (lower molecular layer) no longer has labeled cells at these ages except lightly-labeled endothelial cells; indeed, in the animals injected at 12–13 days, there are few labeled nerve cells present throughout the molecular layer. Accordingly, it was hypothesized that these cells are either Bergmann glia cells or else that they are the intermediate cells of Lugaro (Fox, '59).

In Golgi-impregnated sections of the older animals, presumed Lugaro cells can be found in small numbers situated at the interface of the molecular and granular layers. They are distinguished from basket cells by having a moderately arborized, narrow span of dendritic plexus with most of the branches located in the granular layer and the presence of a single axon which is directed upward and penetrates the lower part of the molecular layer (fig. 3). With electron microscopy, differentiating nerve cells are seen in moderate numbers in the 15-day old and older rats at the level of or just below the soma of Purkinje cells (fig. 24) and in the vicinity of Bergmann glia cells. The nuclei of these cells are usually oval and twice as long as those of granule cells; they tend to stain lightly, but less so than the nuclei of basket cells (fig. 25). Where dendrites are seen issuing from the soma, they are directed downward toward the granular layer, rather than upward as those of basket cells (fig. 27).

Three types of synapses were identified on the soma of these cells. The first type resembles parallel fibers (fig. 24, inset). The possibility that these are not parallel fibers was raised by the type of contact shown in figure 25. This process is cut longitudinally, but unlike parallel fibers, it appears to have some neurofilaments. The second type of terminal is the ending of a myelinated small caliber axon that may reach the molecular layer and is accordingly, assumed to be a recurrent collateral of a Purkinje cell axon (fig. 26). This bouton has a larger collection of round or flat vesicles than the previous one, and probably has conspicuous but symmetrical dense membranes (fig. 28). These may also be present on the descending dendrites of Lugaro cells (fig. 27). A third type of bouton and synapse, an inconspicuous symmetrical one, was seen on the initial portion of the ascending axon (fig. 29).

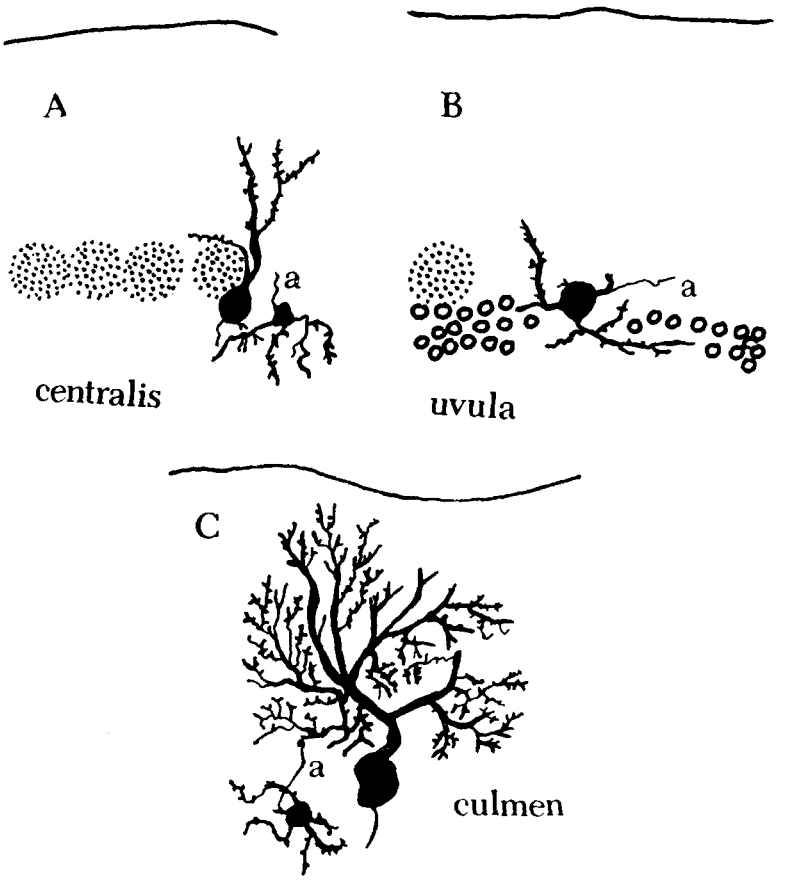


Fig. 3 Drawings of Golgi-impregnated, presumed Lugaro cells. A, from depth of centralis; B, from ventral uvula; C, from culmen dorsalis. Thirty days.

## DISCUSSION

*Migration of differentiating granule cells.* The autoradiographic data from rats injected with two successive doses of thymidine-$H^3$ indicate that very few granule cells are formed during the first five days after birth and that the peak in the formation of granule cells falls between 8–15 days. This is in agreement with the conclusions of Addison ('11) and others. It was noted that of the few granule cells that are formed before this date, more settle in the lower half of the granular layer than in the upper half, and of the few formed afterwards, more are situated in the upper than the lower half of the molecular layer. However, a strict zonal segregation was not observed and, therefore, it would be erroneous to conclude that all parallel fibers located in the lower or upper molecular layers have their soma of origin located correspondingly in the lower or upper granular layer. Presumably, there are forces operating in the organization of the granular layer which interfere with such a strict segregation based on time of origin.

The autoradiographic data relating to the time of origin of granule cells provide information about one aspect of granule cell differentiation, namely, when the precursors of granule cells ceased to multiply, presumably because they "left" the stem cell population (the proliferative zone of the external germinal layer) and "entered" the first differentiating compartment (the premigratory zone of the external germinal layer). The complete differentiation of

granule cells, however, is a protracted process involving first the formation of the horizontal branch of the parallel fiber axon during the horizontal bipolar stage, then the formation of the vertical branch of the parallel fiber while the cell migrates through the molecular layer and, finally, the sprouting of dendrites in the granular layer, and their establishing of synapses with mossy fibers.

The ultrastructure of migrating, vertical bipolar cells was studied in chick embryos by Mugnaini and Forstrønen ('67). They described differences in the superior and inferior poles of the cytoplasm, the former being thin and tubular in organization, the latter thicker and filled with various organelles. This has also been our observation, although in some fortuitous sections, long thin processes with tubular organization could also be seen in the leading position. This agrees with the drawings of Lugaro (1894) based on Golgi material. Mugnaini and Forstrønen also noted that the vertical bipolar cells are narrow in the transverse plane (at a right angle to the long axis of the folium) and wider or flattened in the longitudinal plane. This is also suggested by the drawings of Rakic ('71). Such a change in shape obviously facilitates the descent of the cell, as it requires little displacement of the sheaves of parallel fibers which run in the longitudinal plane (see fig. 7 in Altman, '72a). Mugnaini and Forstrønen also noted that the descending cells are frequently apposed to the vertical processes of Bergmann glia cells. This was confirmed and described in detail by Rakic ('71), who postulated that this relation may be of functional significance. Among the possible functions that he considered was the guidance provided by the vertical fibers, which facilitates the movement of granule cells through a tissue densely packed with cells and processes. In our material the apposition of vertical bipolar cells and Bergmann glial processes was not too frequent; often descending granule cells were directly contiguous with parallel fibers over long distances. But this does not rule out the possibility that such a cell at some other aspect of its surface may be contiguous with Bergmann glial processes. We have been impressed with the frequent association of several descending bipolar cells with one another, as if moving in a tandem, in agreement with the suggestion made by Rakic that several cells may use the same channels in the molecular layer.

*Differentiation of granule cells in the granular layer.* Although the descending bipolar cells come into contact with innumerous parallel fibers and other processes, they do not show synaptogenic activity in the molecular layer, presumably because their "synaptic competence" (Larramendi, '69) matures after the arrival of the soma in the granular layer. Certainly the onset of the formation of parallel fiber synapses in the molecular layer is the last event in the maturation of the molecular layer (Altman, '71b). In the granular layer many presumed granule cells are seen that have a primitive appearance and lack differentiated dendrites by the end of the first week; in general, differentiated dendritic profiles of granule cells, which become so numerous later, are not seen during this period. Likewise, glomeruli with synapses are absent at this time and for several days thereafter, suggesting that the synaptic maturation of granule cells may occur many days after their arrival in the granular layer.

Only a few granule cells have dendrites at 10–12 days, by which time the concentration of these cells has become quite high in the granular layer. But by the fifteenth day many granule cells have dendrites, and dendritic profiles in cross section are quite numerous at this age. Mugnaini and Forstrønen ('67) reported (and illustrated) the formation of mossy fiber synapses in the chick with the soma of granule cells; this was never seen in the rat. From the fifteenth day onward, granule cells are packed densely in groups in the granular layer and their plasma membranes are contiguous over large surfaces (Gray, '61). Specialized desmosoid junctions (or attachment plaques) between granule cell dendrites, which is a frequent feature in the adult cerebellar glomerulus (Gray, '61; Palay, '61), develop gradually and is a sign of advanced maturation; in the chick it is not common until after hatching (Mugnaini and Forstrønen, '67) and in the rat it is not seen in great frequency until after 21 days.

From the beginning when granule cell dendrites begin to form synapses with mossy rosettes in the glomeruli, presumed Golgi axon terminals (Hámori and Szentágothai, '66; Uchizono, '67; Fox et al., '67) are also seen on the granule cell dendrites. It is interesting to note that these Golgi axon synapses, which are presumed to be inhibitory in nature (Eccles et al., '67) may form either the expected inconspicuous, symmetrical synapses or conspicuous, symmetrical (possibly asymmetrical) synapses. This was previously described by Larramendi ('69).

*Maturation of mossy fibers and the glomeruli.* The histochemical staining for oxidative enzymes proved to be a useful method for selectively staining the glomeruli. At 15 days the glomeruli are still small and few in number, which agrees with the histological observations in the rat by Addison ('11). The size, number and staining intensity of the glomeruli increased through 21 to 30 days. Ramon y Cajal ('60) reported that in the dog, glomeruli form in large numbers between 25 and 40 days. In electron micrographs, mossy fiber terminals were seen in small numbers in the rat at 12 days, at which time they tended to be immature in appearance, being roundish in shape and having few synapses, as previously reported (Larramendi, '69; Mugnaini, '69). Larramendi distinguished two stages in the maturation of glomerular synapses, the cup-stage (when the granule cell dendrites formed cup-like contacts, enveloping totally the small mossy rosette) and the claw-stage. These two stages were recognizable in our material and were illustrated. In 12-day old rats the glomeruli can be examined to great advantage and many granule cells are seen with claw-like endings and "serrated edge dovetailing the mossy fiber rosette" (Fox et al., '67). In the older animals, innumerous granule cell dendrites form synapses and they become extremely crowded over the surface of the mossy rosette.

*The maturation of Golgi cells.* The soma of Golgi cells is easily recognized in young and adult rats. In young animals the soma apparently lacks synapses, and even in mature animals, few synapses are seen on the soma as was noted previously by Uchizono ('67). Among these rare synapses, Uchizono noted two types, what he referred to as the S-type and the F-type. Similarly in our material two types of synapses could be distinguished, a conspicuous, asymmetrical one which could be those of climbing fibers (Scheibel and Scheibel, '54) and an inconspicuous, symmetrical synapse which may be those of the recurrent collaterals of Purkinje cell axons (Fox et al., '67; Eccles et al., '67). The dendrites of Golgi cells are difficult to identify, except where they are continuous with the soma. In one such instance, which was illustrated, a mossy terminal could be seen forming a synapse, confirming the previous observation of Hámori and Szentágothai ('66) which had been questioned (Fox et al., '67). Golgi axon synapses in the glomeruli on granule cell dendrites, as previously mentioned, were seen in young rats from the time onward that the granule cells formed synapses with the mossy fibers.

*The maturation of Lugaro cells.* According to Fox ('59) these fusiform horizontal cells have their cell bodies and dendrites elongated in the transverse plane and are located beneath the somata of Purkinje cells where they are contacted by basket cell terminals. The axons of these Lugaro cells are directed upward and enter the lower molecular layer where they are said to terminate on the somata of basket cells. This type of cell was also described by O'Leary et al. ('68) who observed other variants, such as cells that were oriented vertically rather than horizontally, and those with dendrites that were not confined to the region of Ramon y Cajal's ('11) infraganglionic plexus. On the basis of ultrastructural observations, O'Leary and his associates suggested that the recurrent collaterals of the Purkinje cell axon, which retain their myelin sheath close to the site where synapses are formed, form synapses with the somata of Lugaro cells.

Our autoradiographic studies suggested that after the basket cells and the majority of stellate cells are formed in the molecular layer, a special cell type is formed in the vicinity of the soma of Purkinje cells. On the basis of their position the possibility was considered that these late-form-

ing elements are Lugaro cells. In Golgi material, Lugaro cells were identified in small number in older rats and they were seen in appreciable numbers with electron microscopy from the fifteenth day onward. Typically, the Lugaro cells had dendrites directed downward into the granular layer and many synapses were seen on the soma. They are clearly distinguishable from granule cells by their morphology and the presence of synapses on the soma, and from Golgi cells by their much smaller size (although there is some ambiguity in the identification of large Lugaro cells). It is likely that many of the "very low basket" cells (Larramendi and Lemkey-Johnston, '70) are Lugaro cells. Three types of synapses were identified on the soma of Lugaro cells. The first of these were conspicuous, asymmetrical synapses that could not be identified. The second were inconspicuous, symmetrical synapses, perhaps those of basket cells. Finally, there were numerous conspicuous symmetrical synapses which were tentatively identified as those of the recurrent axon collaterals of Purkinje cells. The latter, which often retain their myelin sheath up to the region where synapses are formed, were seen terminating on the soma of Lugaro cells, in accordance with the earlier description of O'Leary et al. ('68).

According to Ramon y Cajal ('11) the recurrent collaterals of Purkinje cell axons form the infraganglionic and supraganglionic plexuses. These collaterals come into existence quite early and may be seen in the newborn cat or dog. Ramon y Cajal suggested that these collaterals terminate directly on the soma and thick branches of Purkinje cells, whereas more recent studies have suggested that Golgi cells and basket cells may be more important targets. Fox et al. ('67) obtained evidence that the fibers that terminate in the granular layer may terminate on the soma of Golgi cells, and this was also suggested by experimental studies of Hámori and Szentágothai (Eccles et al., '67). The termination of collaterals forming the supraganglionic plexus was traced by Lemkey-Johnston and Larramendi ('68) to the soma and dendrites of basket cells. In a recent study, Larramendi and Lemkey-Johnston ('70) attempted to quantify the distribution of Purkinje cell axon collaterals. Their data indicated that about one-third formed the supraganglionic plexus, which terminated on basket cells, and two-thirds formed the infraganglionic plexus which terminated on "very low basket" cells. From this they concluded that both the infra- and supraganglionic plexus terminated on basket cells, although they admitted the possibility that the "very low basket" cells may be Lugaro cells. If one considers the fact that the collaterals of Purkinje cell axons do not lose their myelin until their point of termination, and relates this to the circumstance that there are very few (or no) myelinated fibers in the rat in the molecular layer above the level of the soma of Purkinje cells, it becomes conceivable that the "basket cells" situated at the level of the soma of Purkinje cells are "high Lugaro cells" and that all Purkinje cell collaterals terminate on Lugaro cells.

*Concluding remarks.* This examination of the maturation of the Purkinje, Golgi, basket, stellate, granule and Lugaro cells suggests that there are three major stages in the maturation of the cerebellar cortex, which might be termed the morphogenic, synaptogenic and gliogenic stages. The morphogenic stage consists of cell proliferation, the migration of cells to their final destination, and the establishment of all the major interconnecting pathways. The migration of cells and the formation of pathways are often intimately interrelated events, as exemplified by the location of the germinal matrix of the cerebellar cortex in a subpial position, and the migration of cells from there to the internal granular layer. It is this arrangement that makes possible the assembly of an extremely regular, three dimensional lattice of parallel fibers from the bottom of the molecular layer upward by a stacking process. The morphogenic maturation of a single cell may be a rapid or a protracted process depending on the length of its migration and on the complexity of connections established with other cells. For instance, the maturation of a basket cell is rapid, that of a Purkinje cell, which establishes connections directly or indirectly with all other cells of the cerebellar cortex, is protracted.

The second stage, the formation of synapses, does not start until the cell body

has occupied its terminal position. This may be an early event, as in the case of basket cells (which do not truly migrate) or a very delayed one, as in the case of granule cells which first have to reach the granular layer before their axons (the parallel fibers of the molecular layer) form synapses. But once the cell body has reached its final destination, synaptogenic activity can start in a mature region of the cell while morphogenic activity is still in progress in another part. This was exemplified by the formation of permanent synapses of basket cells on the soma of the Purkinje cell while the dendritic system was still growing, which led to the sequential synaptic maturation of different "domains" of the Purkinje cell. The gliogenic stage is the last event, signalling the termination of synaptogenic activity. Soon after synapses are formed, as between parallel fibers and Purkinje cell dendritic spines, glial sheath covers the contact region, apparently "sealing" the junction. However in a large cell, like the Purkinje cell, the gliogenic stage may be reached in one region when morphogenic and synaptogenic activity is in progress in another. This was exemplified by the gliogenic maturation of the soma of the Purkinje cell by the middle of the second week, when the morphological and synaptic development of the spiny branchlets has just begun.

These conclusions are in agreement with some of the principles formulated by Larramendi ('69), in particular the following: (a) that neuroblasts do not form synapses in their migratory phase; (b) that during an early stage in the development of neuroblasts there is an asynaptogenic period; and (c) that "synaptic competence" develops more or less simultaneously in both dendrites and the axon. In view of the fact that the dendrites are formed in several cells (e.g., Purkinje cell, granule cell) after the axon was formed, this implies that the axon has to mature before synapses can be established. To what extent these principles apply to the neurogenesis of other brain structures remains to be established.

This analysis of the development of the cerebellar cortex will be concluded with a critique of the approach used. Notwithstanding the large number of cerebella examined, at closely spaced intervals of development and with several preparative techniques, relatively little could be added to our existing knowledge of the events constituting, and the mechanisms underlying cerebellar neurogenesis. The highlights of the development of the "wiring" of the cerebellar cortex was established by Ramon y Cajal with the Golgi technique, and the ultrastructural studies of the last decade provided supplementary information about the "connections" formed. But to this day the identity of many of the terminals remains unknown (Bloom et al., '71) or uncertain, and the normative-descriptive approach is not well suited for an analysis of the forces operating during neurogenesis. Perhaps the application of experimental procedures (Altman and Anderson, '72) in combination with newer methods of analysis will make possible further progress in this area.

ACKNOWLEDGMENTS

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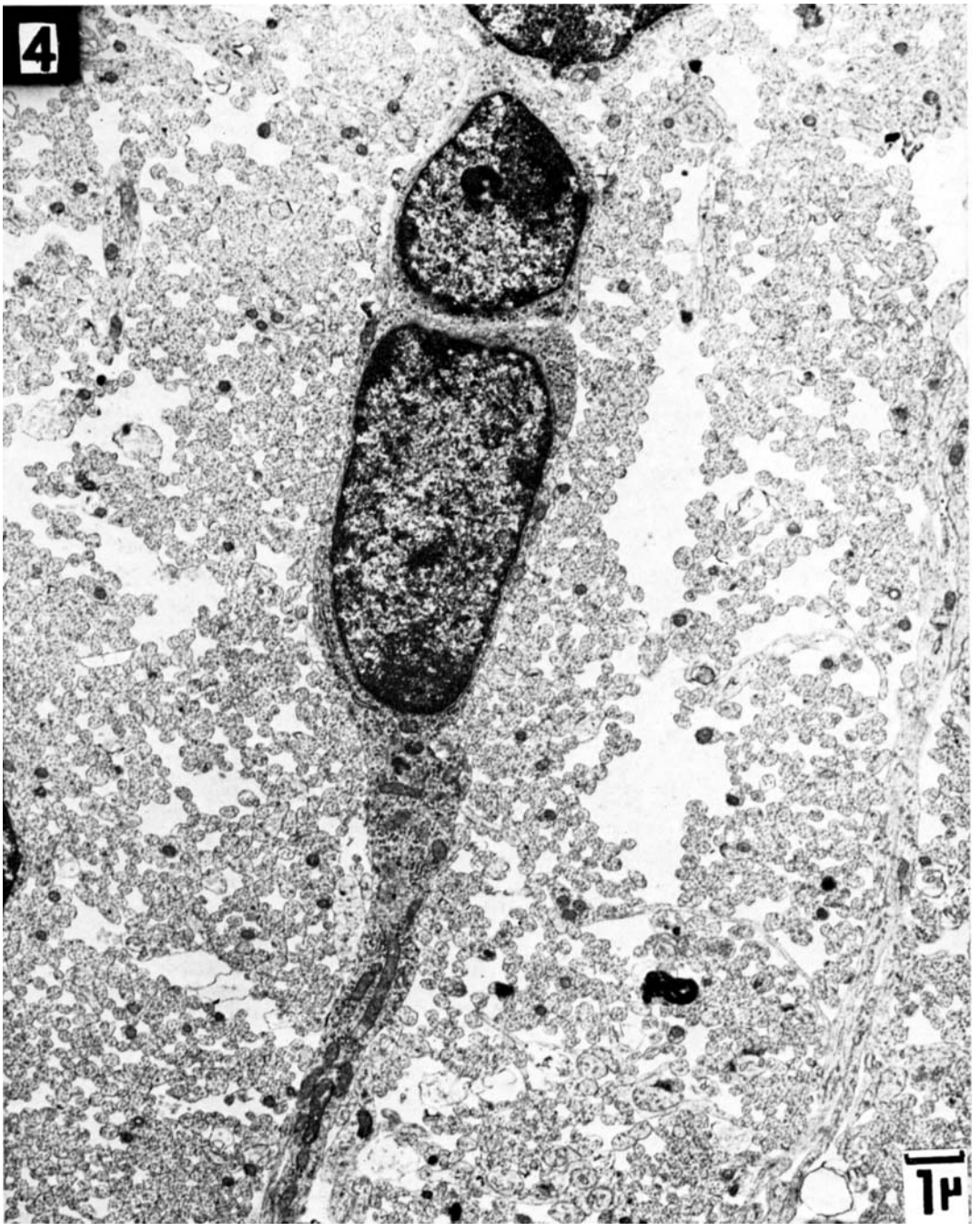
## PLATE 1

### EXPLANATION OF FIGURE

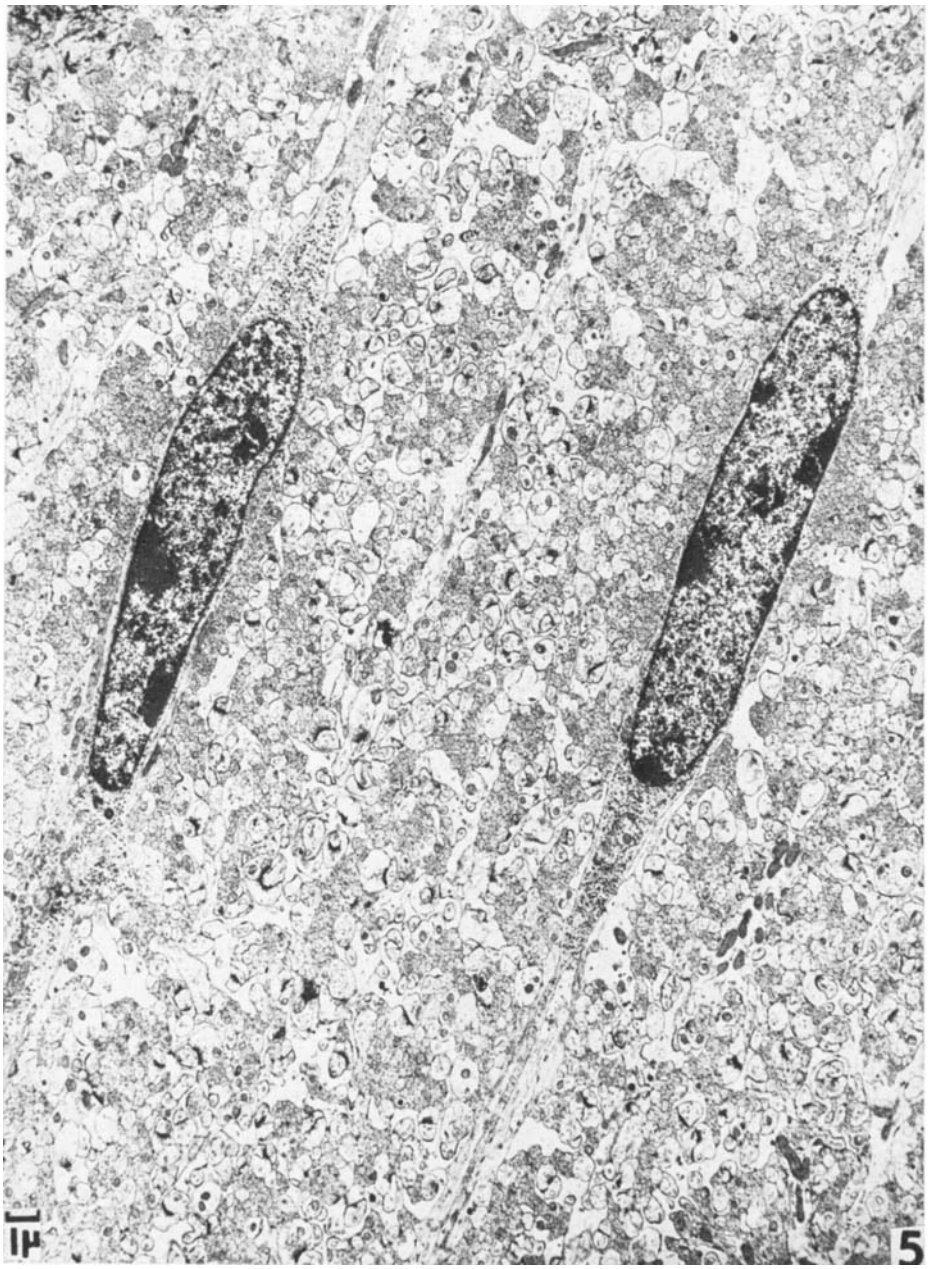
4 Three descending granule cells in tandem. Presumably this reduces the work required in moving through the palisades of parallel fibers and may result in the eventual aggregation of the vertical branches of the granule cell axons into bundles. Pyramis, 12 days.


4
1μ

## PLATE 2

EXPLANATION OF FIGURE

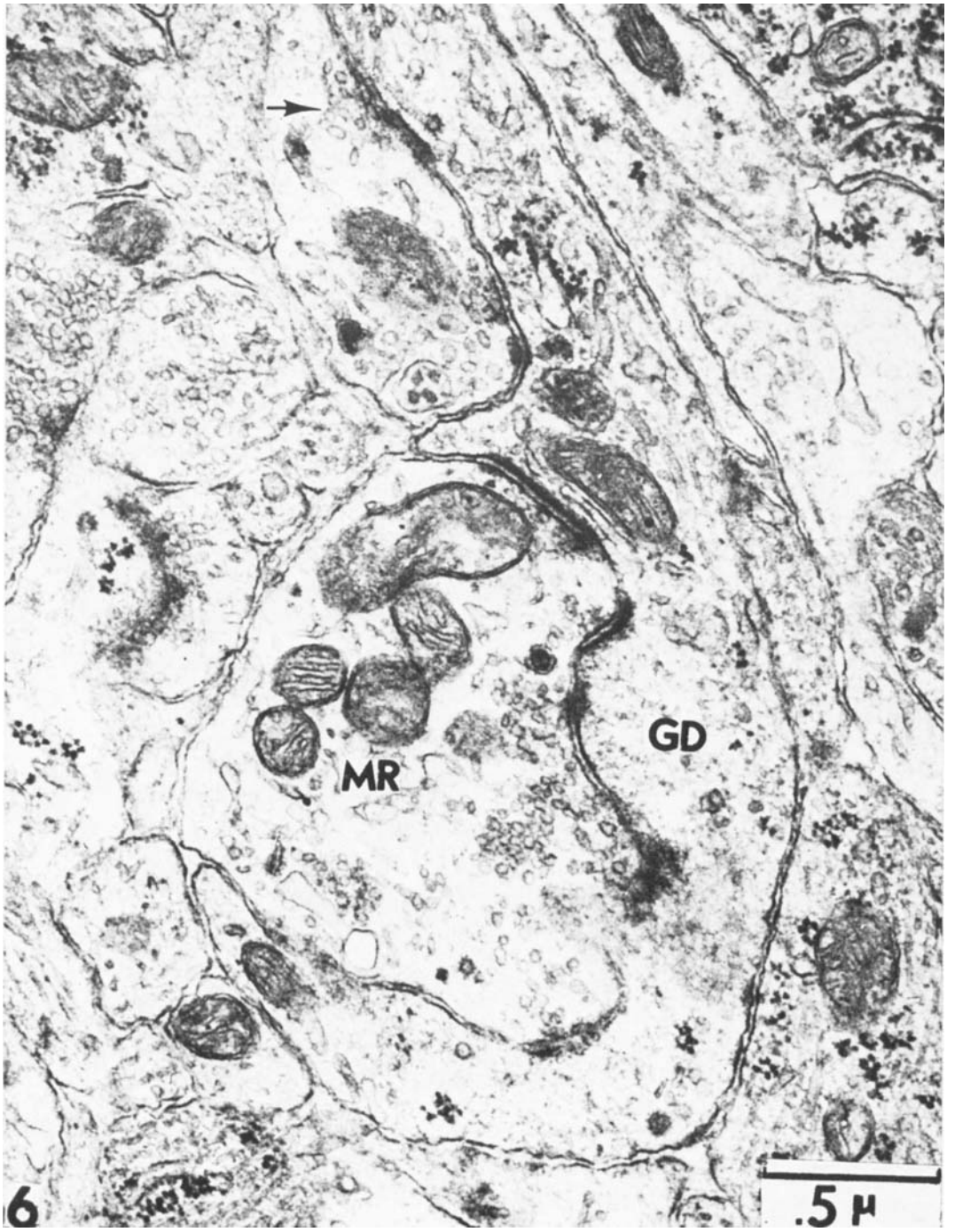
5 Vertically oriented, spindle-shaped cells in the molecular layer, interpreted as descending granule cells. There is minimal cytoplasm on the sides. The two poles have an accumulation of ribosomal cytoplasm, but both the trailing process (cell on the left) and the leading process (cell on the right) have tubular cytoplasms. Pyramis, 21 days.



## PLATE 3

### EXPLANATION OF FIGURE

6 Claw-shaped granule cell dendritic ending (GD) and synapse with immature mossy rosette (MR). The conspicuous, asymmetrical junction is characteristic of mossy fiber-granule cell synapses. The inconspicuous, symmetrical synapse, with flattened vesicles, on the shaft of the granule cell dendrite (arrow) is probably that of a Golgi cell. Pyramis, 12 days.

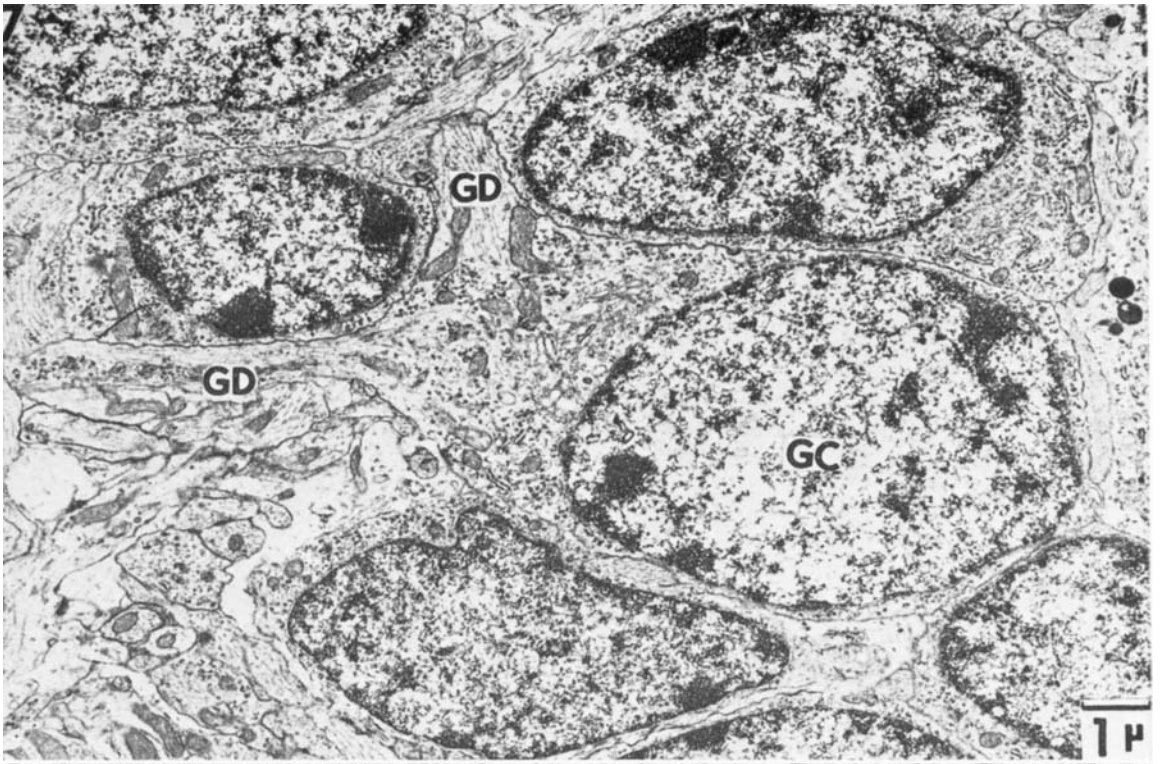

MR
GD
6
.5 μ

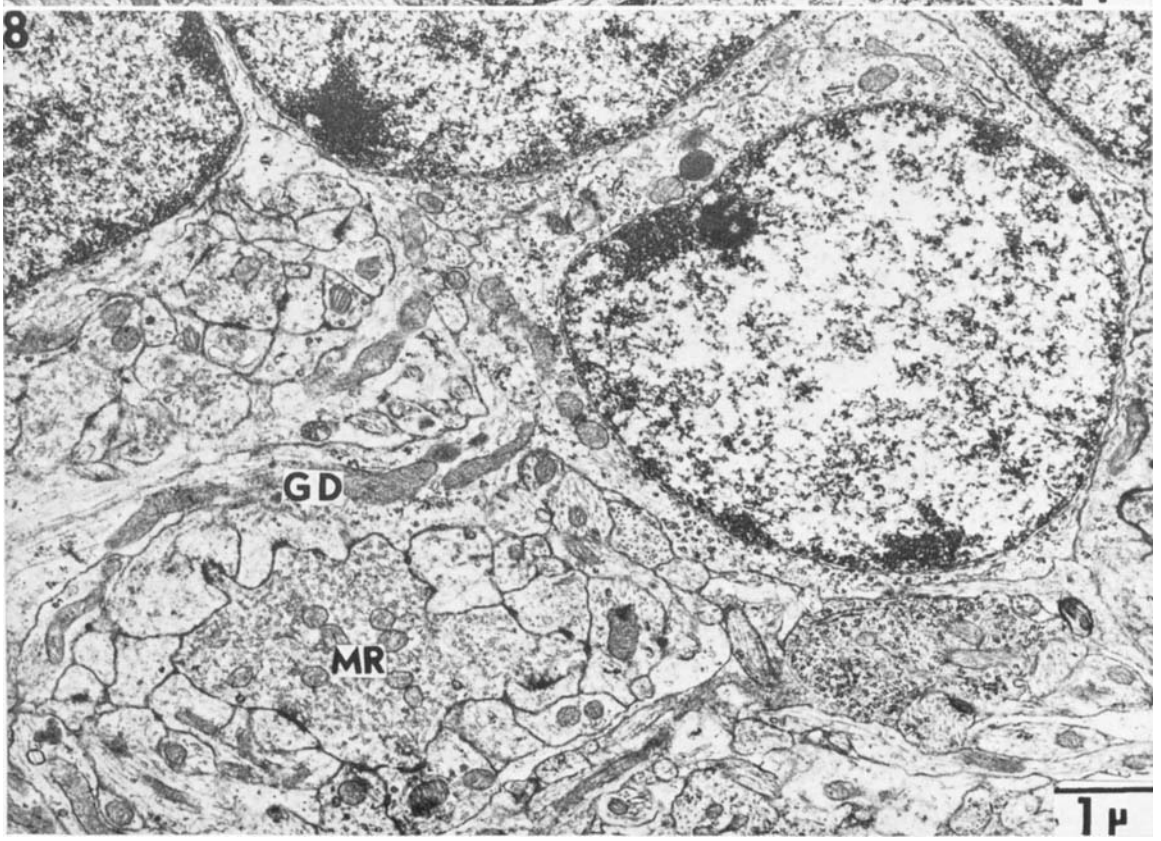
## PLATE 4

### EXPLANATION OF FIGURES

7 Granule cell (GC) with two dendrites (GD), one of them relatively long. Granule cell cytoplasm is minimal except where dendrites are issuing. Here, mitochondria, Golgi apparatus and ribosomes are present. Some clusters of ribosomes are seen in the dendrites which are characterized by numerous microtubules. Synapses are never seen on the perikaryon of granule cells. Pyramis, 15 days.

8 Granule cell with a short claw-shaped, serrated dendrite (GD) that synapses with a mossy rosette (MR). Pyramis, 15 days.

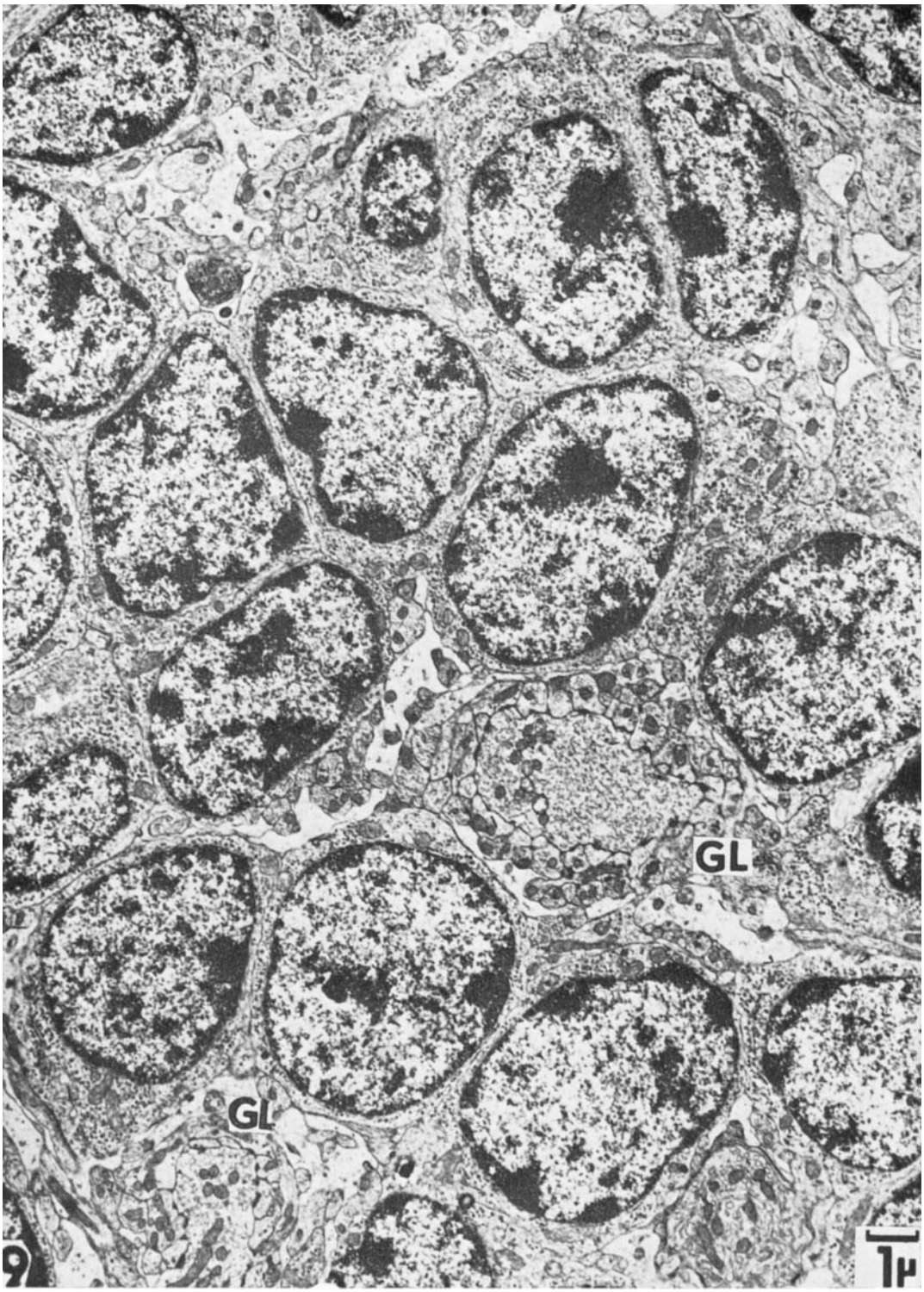

7
GD
GD
GC
1 μ


8
GD
MR
1 μ

## PLATE 5

### EXPLANATION OF FIGURE

9 Mature granule cells surrounding a glomerulus (GL). The high concentration of small dendritic endings on the mossy rosette is typical of the mature glomerulus. Pyramis, 21 days.

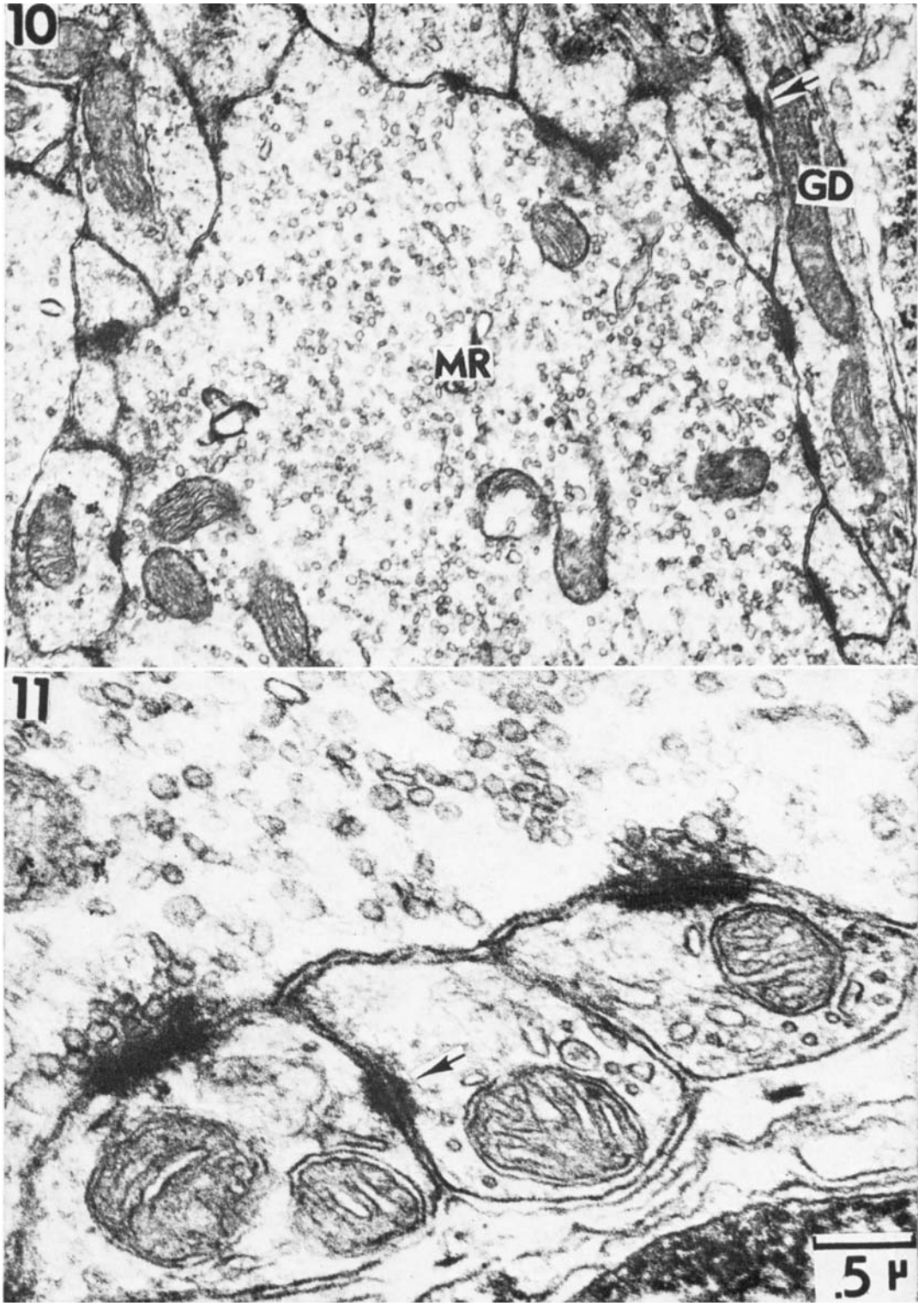

GL
GL
9
1μ

## PLATE 6

### EXPLANATION OF FIGURES

10 Granule cell dendrite synapses with mossy rosette. The granule cell dendrite (GD) in the upper right hand corner has two synapses with the mossy rosette (MR) and desmosoid dense membrane junction (arrow) with another granule cell dendrite which, in turn, synapses with the mossy rosette. Such desmosoid junctions are still scarce at this age. Pyramis, 15 days.

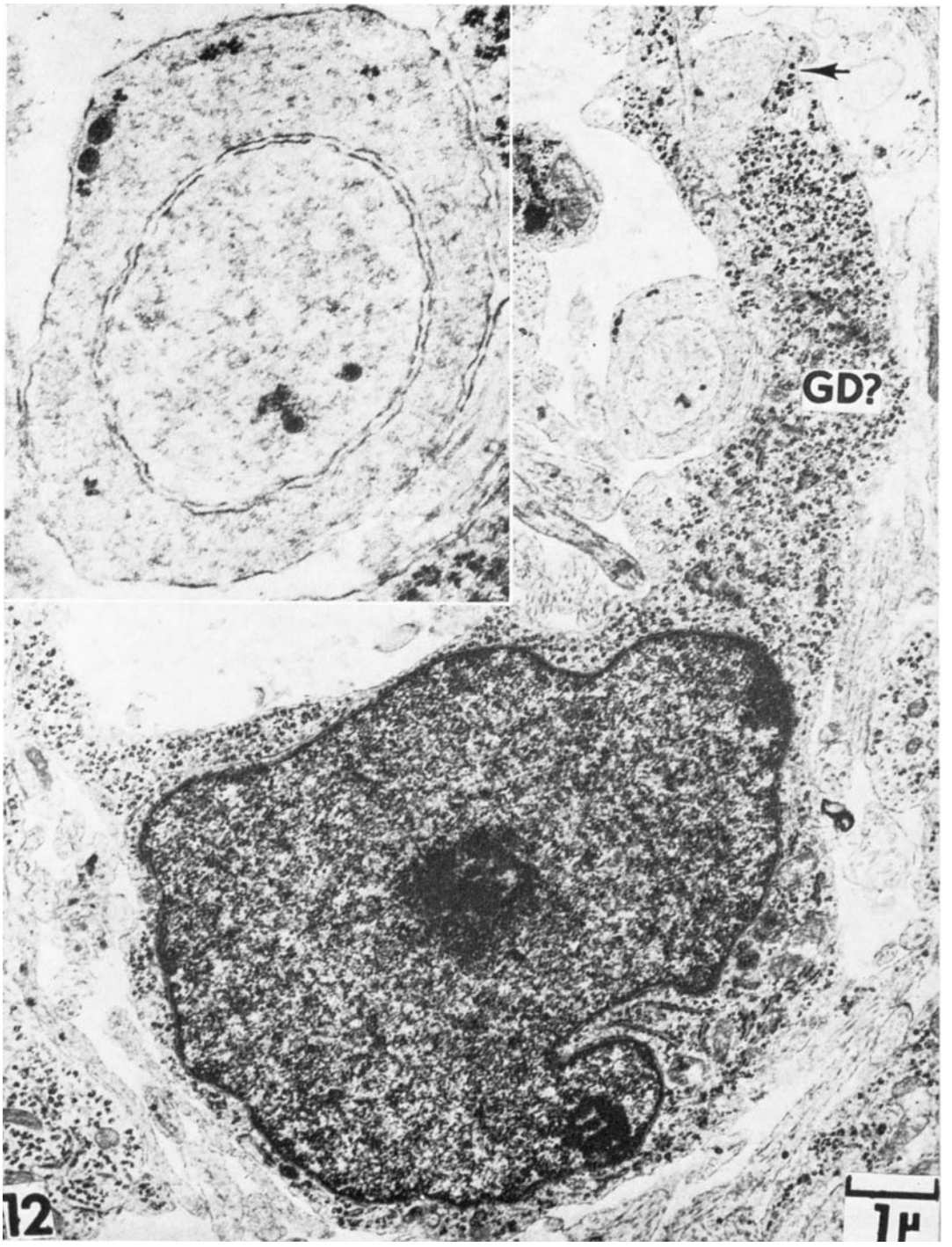
11 Granule cell dendrite synapses and desmosoid dense membranes (arrow) at higher magnification. Pyramis, 30 days.


10
GD
MR
11
.5 μ

## PLATE 7

### EXPLANATION OF FIGURE

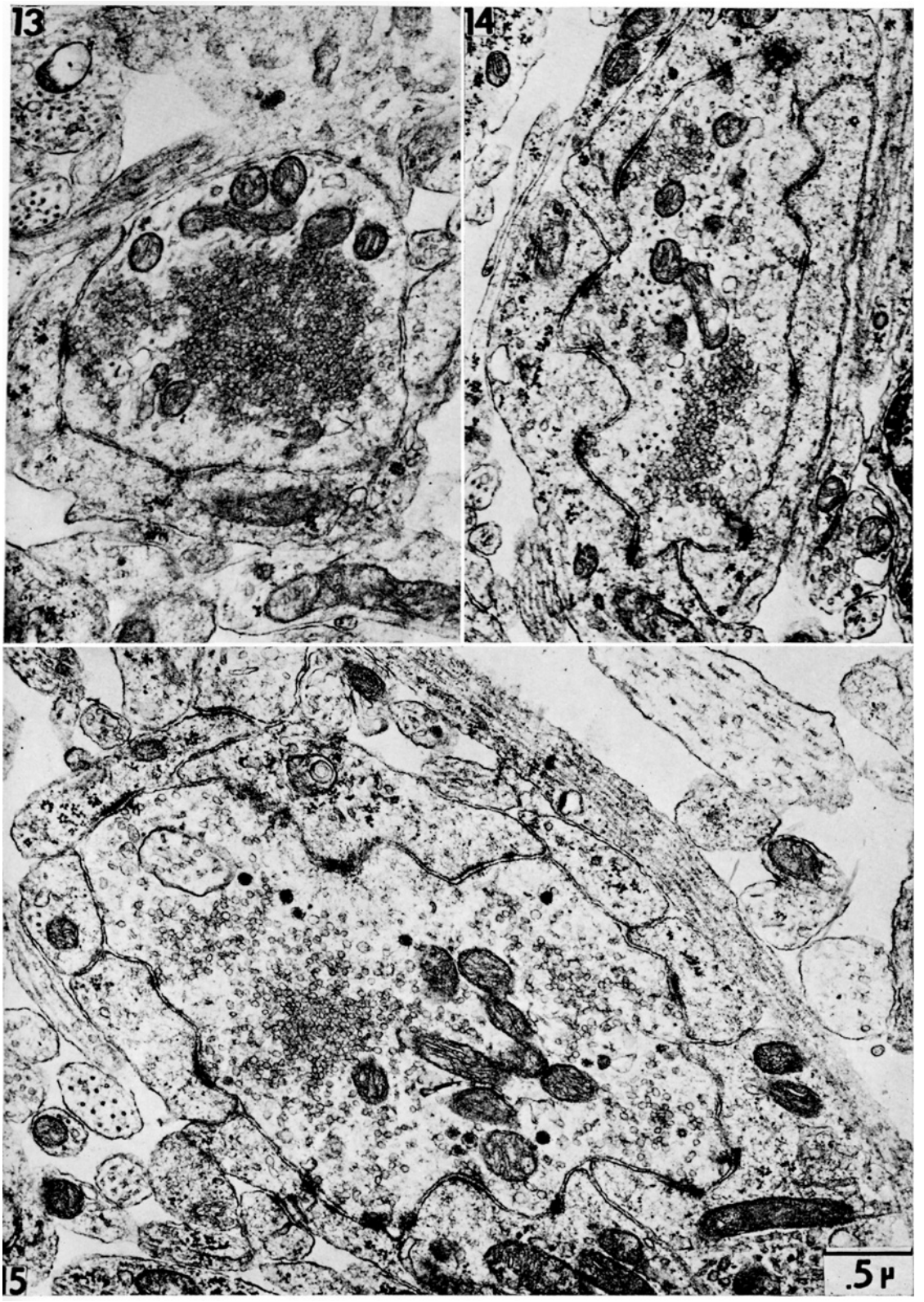
12 Presumed maturing granule cell with an immature dendrite (GD?) that may surround an immature mossy terminal (arrow). The junctional density may be indicative of early synaptogenic activity. Another immature rosette (inset) is surrounded by an unidentified process, but there is no synapse present. Pyramis, seven days.


GD?
12
1μ

## PLATE 8

### EXPLANATION OF FIGURES

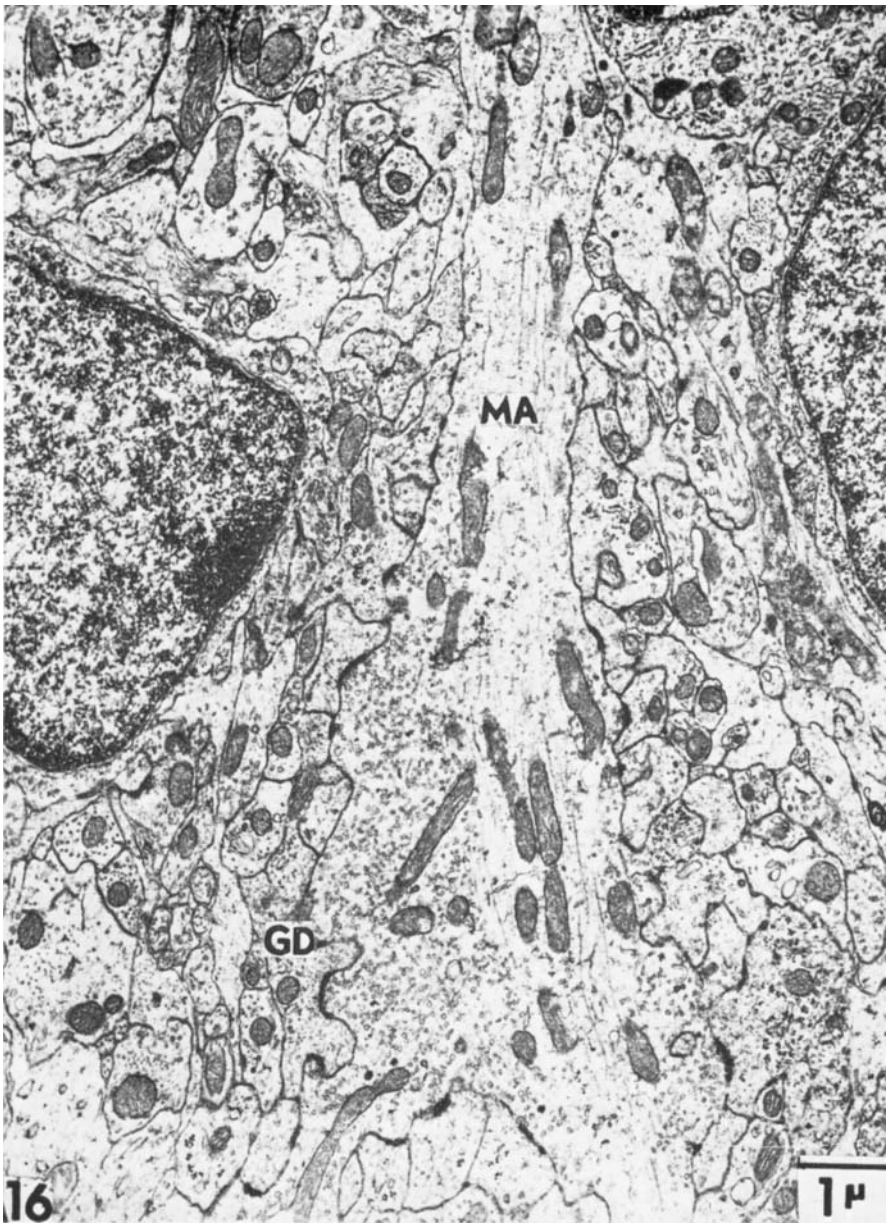
13–15 Mossy rosettes and granule cell dendrites in the pyramis of a 12-day old rat suggesting different stages in the development of this synapse. In figure 13, the synaptic vesicles form a dense crystalline lattice and synapse formation is just commencing. In figure 14 the serrated surfaces of three dendrites are seen, each forming multiple but not very conspicuous junctions with the rosette. The rosette in figure 15 forms more synapses and it is studded with several granular vesicles.


13
14
15
.5 μ

## PLATE 9

EXPLANATION OF FIGURE

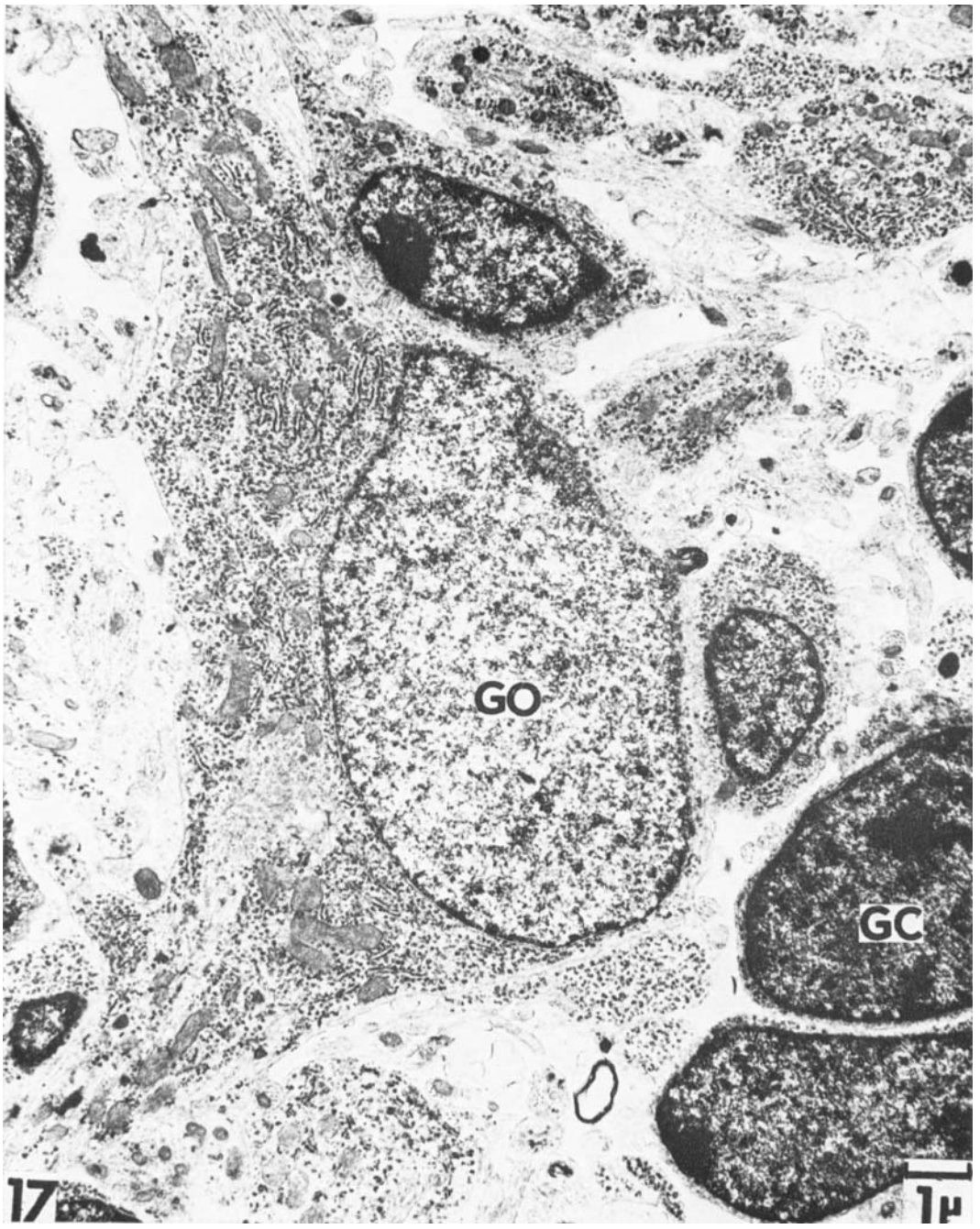
16 Mossy axon (MA). It has microtubules and neurofilaments with synaptic enlargements on both sides (bottom). Granule dendrite (GD) synapses are also seen in regions where the concentration of synaptic vesicles is low (center). Pyramis, 15 days.


MA
GD
16
1μ

## PLATE 10

EXPLANATION OF FIGURE

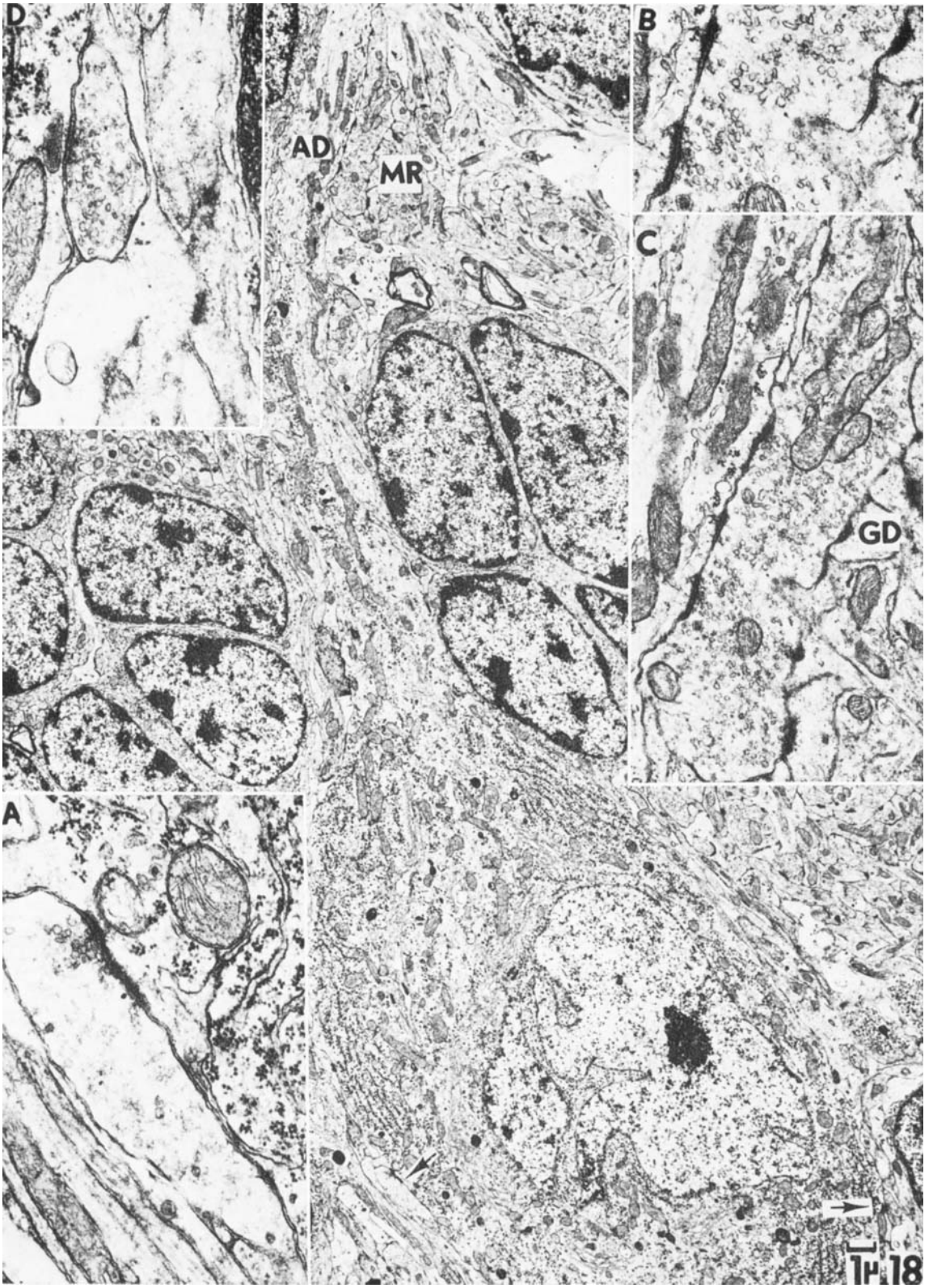
17 Maturing Golgi cell (GO) surrounded by maturing granule cells (GC). Synapses are not seen on the soma or dendrites of the Golgi cell. Pyramis, ten days.


GO
GC
17
1μ

## PLATE 11

### EXPLANATION OF FIGURE

18 A Golgi cell with an ascending dendrite (AD) in the pyramis of a 21-day old rat. On the dendrite there is a mossy fiber (MR) synapse (enlarged in insets B, C). This mossy fiber also has synapses with granule cell dendrites (GD). On the soma of the Golgi cell, two types of synapses are seen. On the right side (arrow) a bouton forms an inconspicuous symmetrical synapse (inset D); this may be a Purkinje cell axon recurrent collateral synapse. On the left side (arrow) a bouton forms a conspicuous asymmetrical synapse (inset A); this may be a climbing fiber synapse.

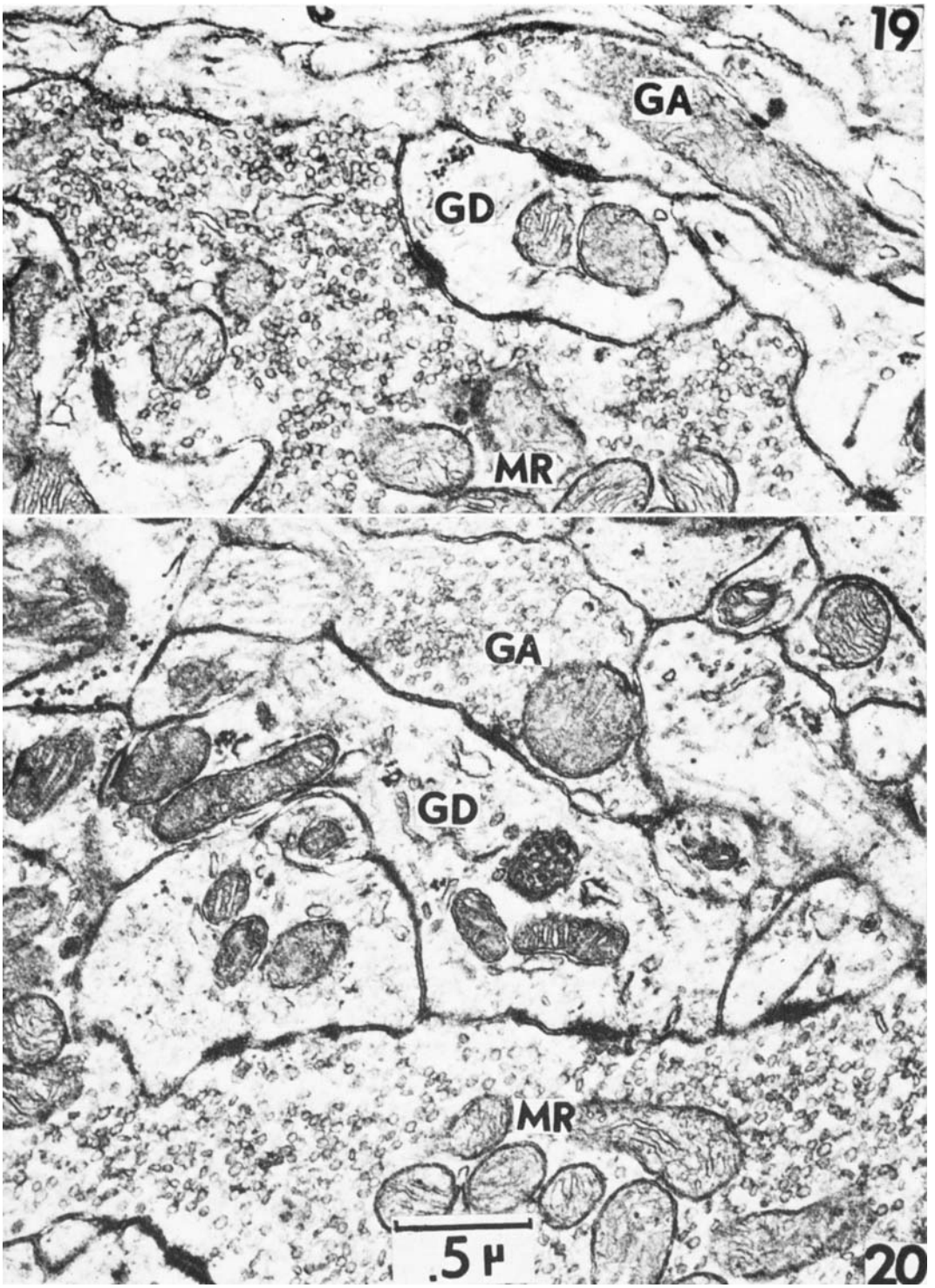

D
AD
MR
B
C
GD
A
1μ 18

## PLATE 12

### EXPLANATION OF FIGURES

19 Bouton and synapse of a presumed Golgi axon terminal (GA) on a granule cell dendrite (GD) which, in turn, synapses with a mossy rosette (MR). This Golgi synapse is of the conspicuous, asymmetrical type, although the subsynaptic density is less pronounced than in the synapse formed with the mossy fiber terminal. Pyramis, 21 days.

20 Bouton and synapse of a presumed Golgi axon terminal on a granule cell dendrite which synapses with a mossy rosette. This Golgi synapse is of the inconspicuous, symmetrical type. Pyramis, 21 days.


19
GA
GD
MR
GA
GD
MR
.5 μ
20

## PLATE 13

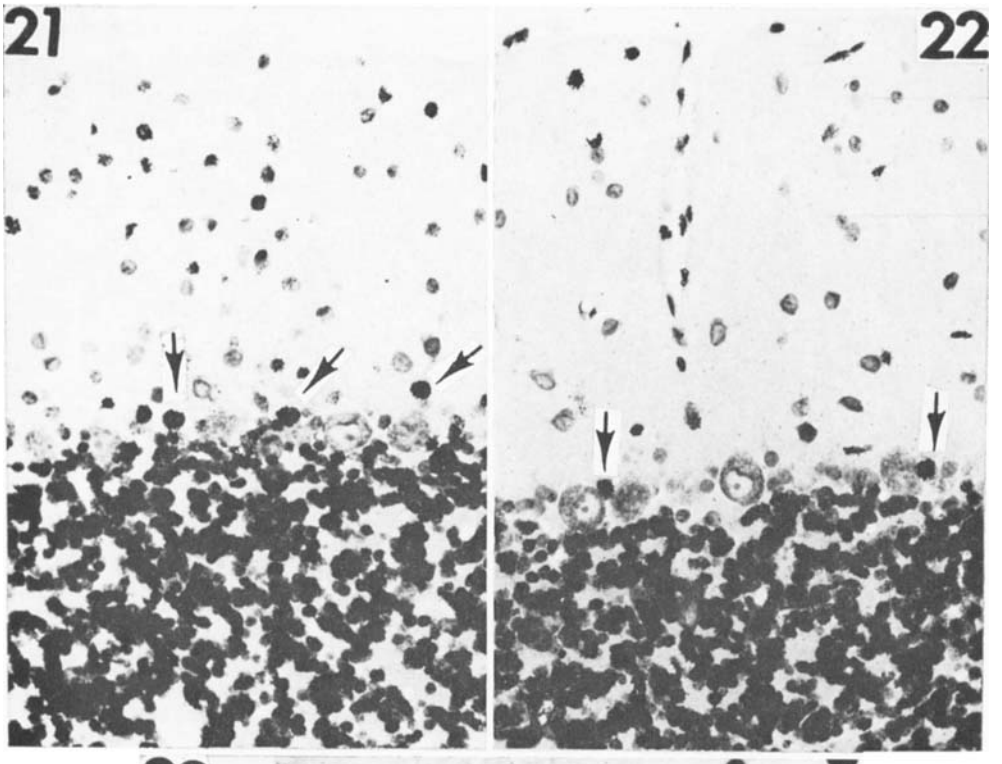
### EXPLANATION OF FIGURES

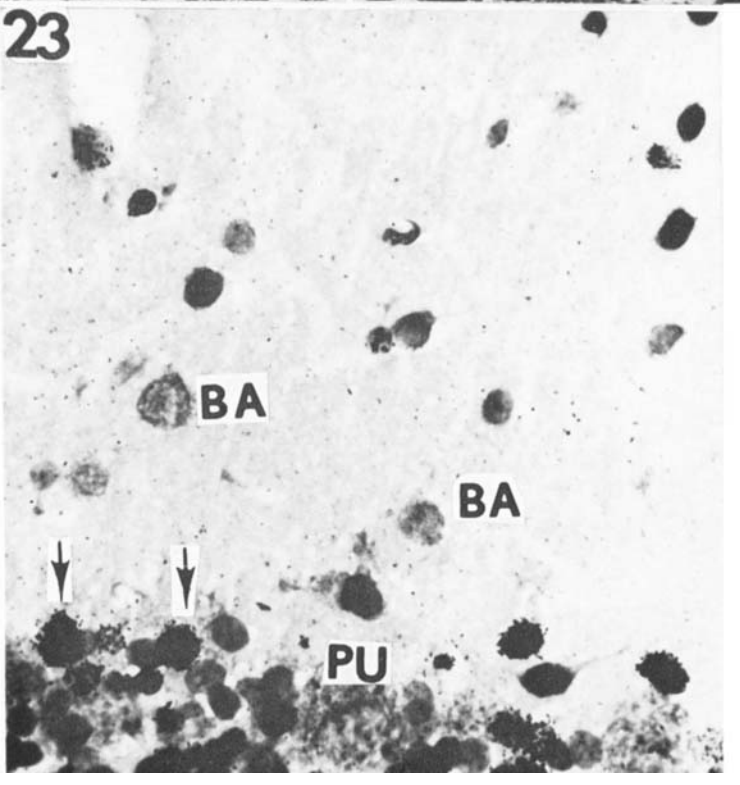
Autoradiograms of the pyramis of adult rats that were injected with two successive daily doses of thymidine-$H^3$ on different days. Figures 21–22, × 256. Figure 23, × 640.

21 This animal was injected on days 8–9. Many of the cells in the upper molecular layer are labeled (which include stellate cells). Most of the cells in the lower half of the molecular layer (which include basket cells) are unlabeled because these cells were formed before the injection. However, several cells around or just above the Purkinje cells (arrows) are intensely labeled. These are presumed to be Lugaro cells. The majority of the granule cells are also labeled.

22 This animal was injected on days 12–13. Few cells are labeled in the molecular layer, except some near the surface, and endothelial cells throughout the molecular layer. Evidently the basket cells and most of the stellate cells were formed before these days. However, some labeled cells are seen in the vicinity of Purkinje cells (arrows). These are presumed to be Lugaro cells. The majority of granule cells are also labeled.

23 This animal was injected on days 10–11. Basket cells are not labeled. It is in this group that the highest number of intensely labeled presumed Lugaro cells were seen (arrows) in the vicinity of Purkinje cells (PU).

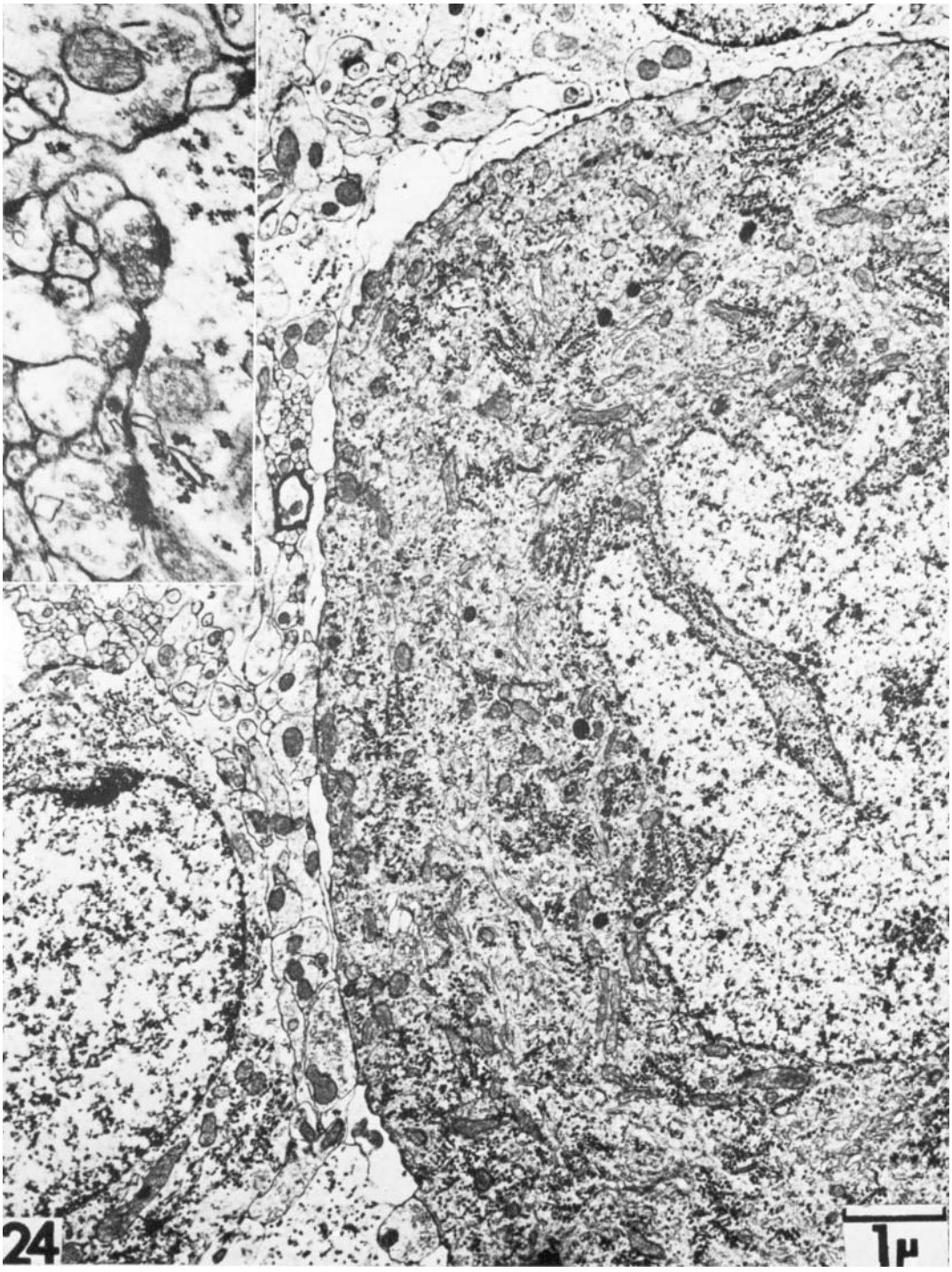

21
22


23
BA
BA
PU

## PLATE 14

### EXPLANATION OF FIGURE

24 The cell in the lower left quadrant, situated at the base of the Purkinje cell is presumed to be a Lugaro cell. One of the type of synapses seen on its soma (inset) resembles those of parallel fibers. Pyramis, 30 days.

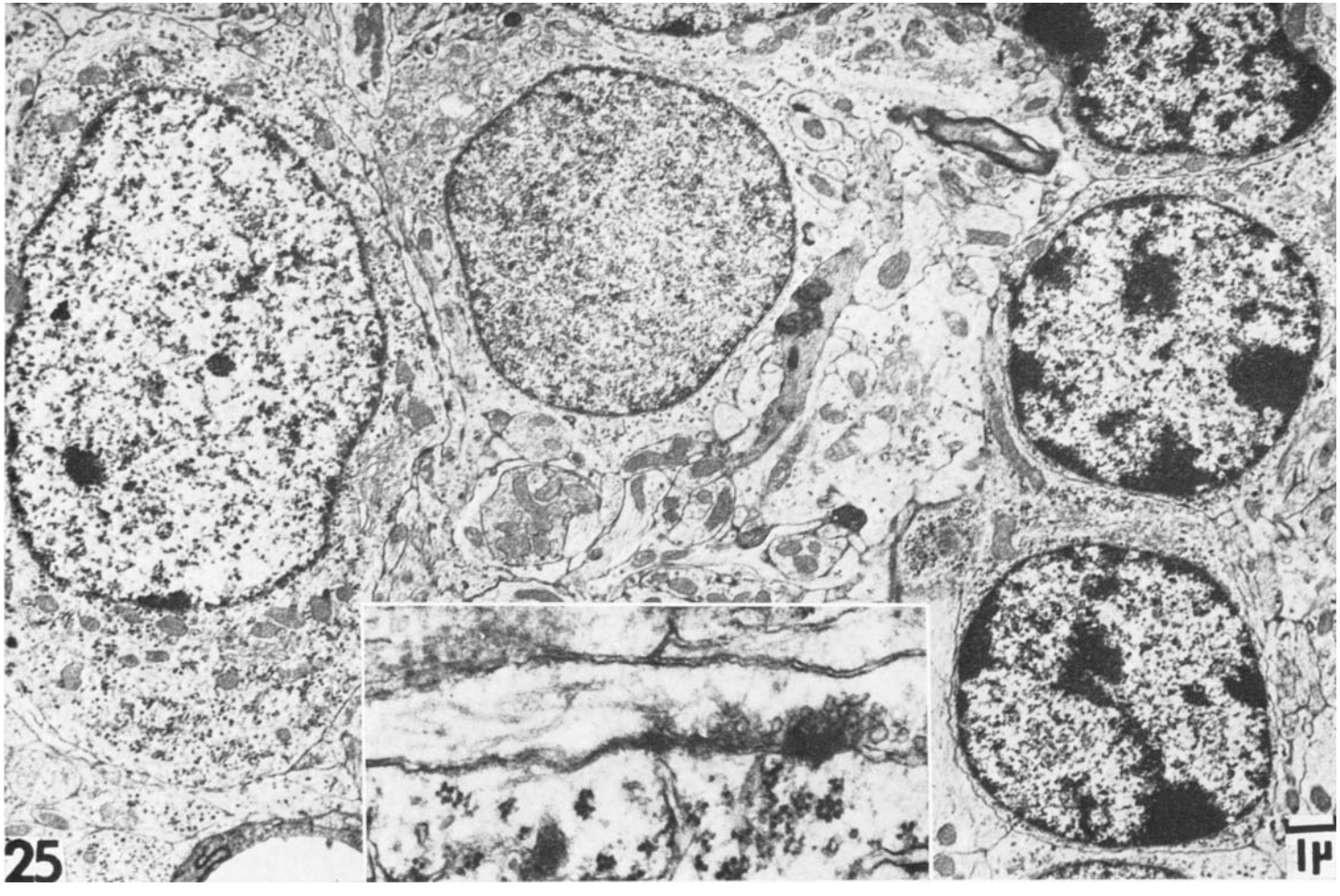

24
1μ

## PLATE 15

### EXPLANATION OF FIGURE

25 This Lugaro cell (left) is situated in the vicinity of a Bergmann glia cell (center) and granule cells. One of the axon terminals, which has a synapse similar to that shown in figure 24, has microtubules and also neurofilaments in it (inset, arrow) and is, accordingly, probably not a parallel fiber. Pyramis, 30 days.

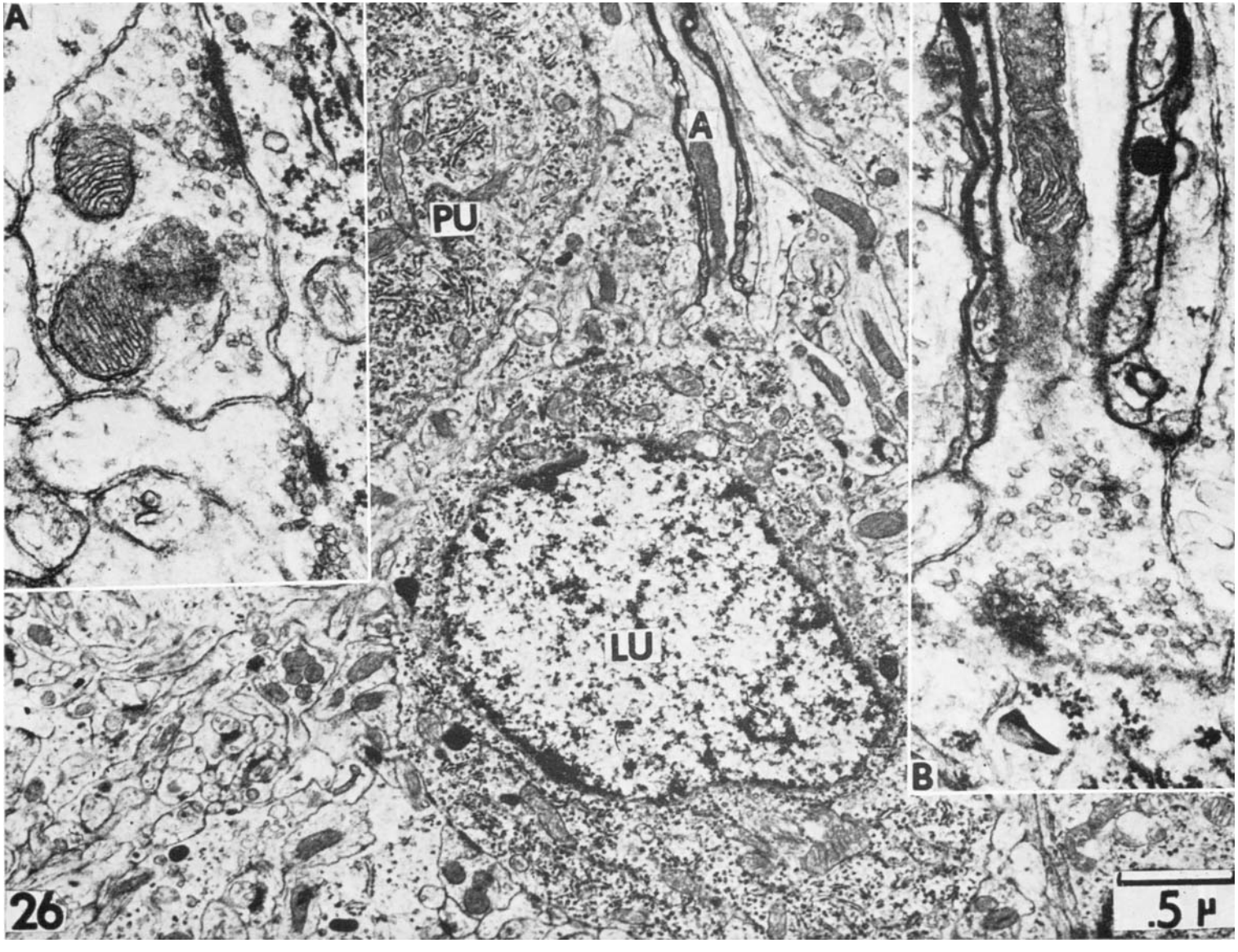
DEVELOPING CEREBELLUM, III.
Joseph Altman



## PLATE 16

### EXPLANATION OF FIGURE

26 A Lugaro cell (LU) at the base of the soma of a Purkinje cell (PU). A myelinated axon (A) which is presumed to be a Purkinje cell axon collateral, is seen terminating on it. The synaptic vesicles are flattened, the characteristic of the synaptic densities are not discernible (inset B). Inset A shows one of the boutons on the soma of this Lugaro cell. It has conspicuous, but symmetrical junctional dense membranes. This may be a Golgi cell terminal or the terminal of a Purkinje cell recurrent axon collateral. Pyramis, 30 days.

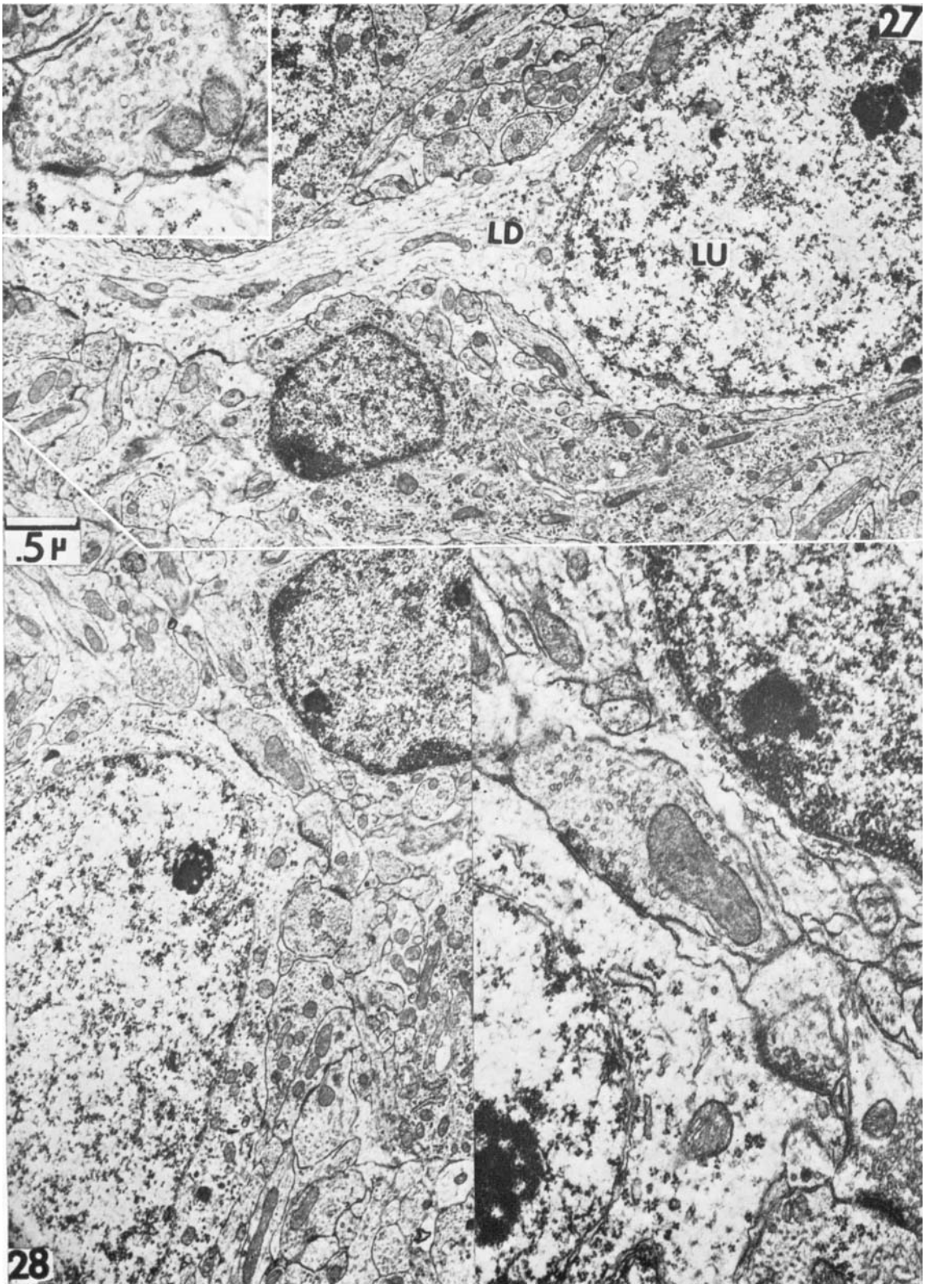

A
PU
A
LU
B
26
.5 μ

## PLATE 17

### EXPLANATION OF FIGURES

27 A Lugaro cell (LU) with downward directed dendrite (LD) and several boutons with synapses on it. The synapses have conspicuous, but symmetrical junctional densities (inset). Pyramis, 15 days.

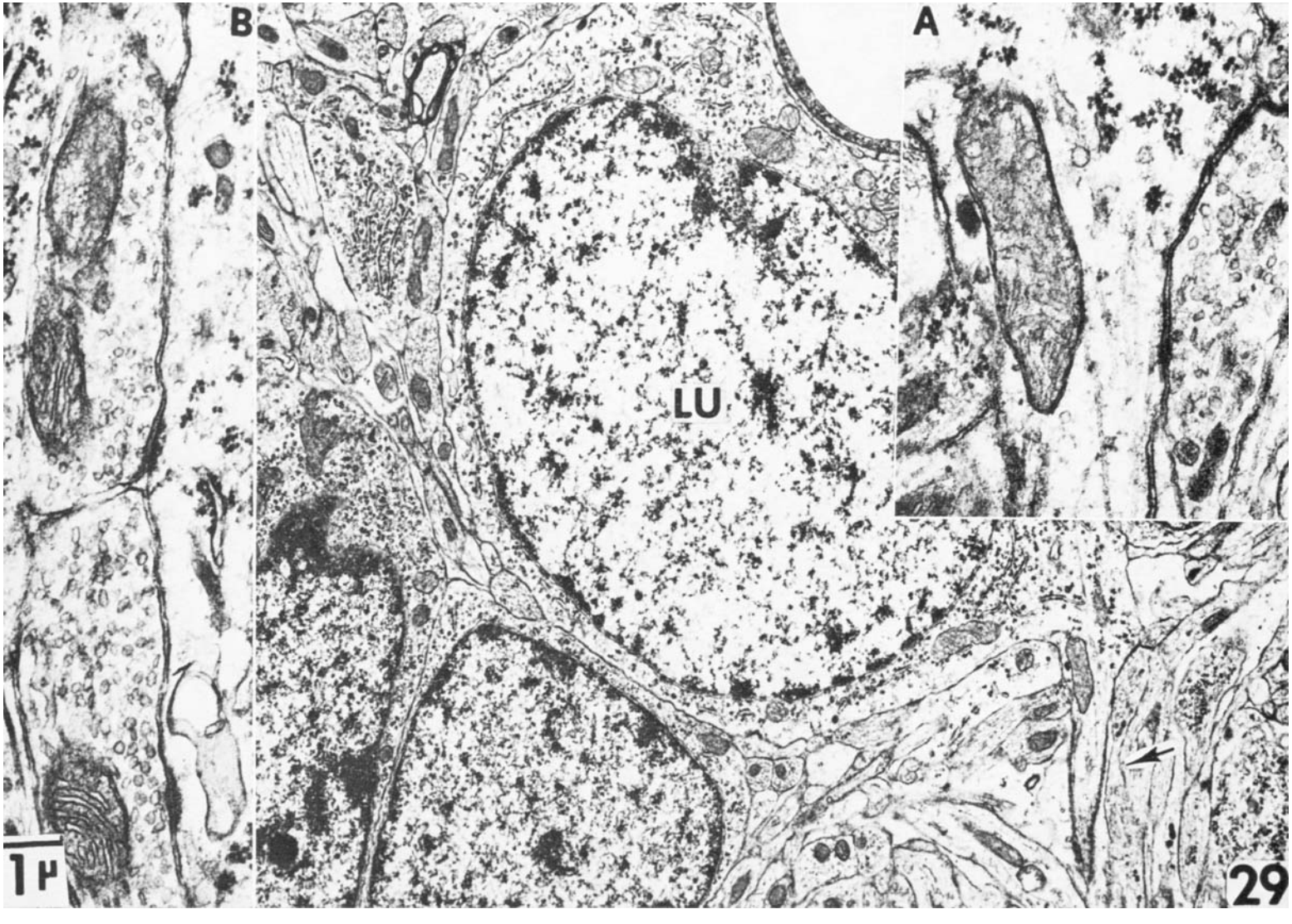
28 A Lugaro cell which has several boutons on or in the vicinity of its soma. These boutons are characterized by the accumulation of many flattened vesicles and conspicuous, but symmetrical junctional densities (inset, left). Pyramis, 15 days.


27
LD
LU
.5 μ
28

## PLATE 18

### EXPLANATION OF FIGURE

29 Lugaro cell (LU) with axon (arrow) and boutons and synapses on the soma (insert B) and the initial portion of the axon (insert A). The boutons on the soma are of the conspicuous, asymmetrical type; the one on the axon is inconspicuous, symmetrical type. Pyramis, 30 days.


B
LU
A
1 μ
29