ANATOMICAL, PHYSIOLOGICAL AND BIOCHEMICAL STUDIES OF THE CEREBELLUM FROM *REELER* MUTANT MOUSE

By J. MARIANI,†¶ F. CREPEL,† K. MIKOSHIBA,‡
J. P. CHANGEUX,‡ AND C. SOTELO§
† *Laboratoire de Physiologie Comparée, Université Pierre et Marie Curie,*
9 *Quai Saint-Bernard, Paris,*
‡ *Laboratoire de Neurobiologie Moléculaire, Institut Pasteur,*
25 *rue du Docteur Roux,* 75015 *Paris, and*
§ *Laboratoire de Neuromorphologie, U-*106 *INSERM, C.M.C. Foch,* 92150 *Suresnes*

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¶ Present address: Laboratoire de Neurobiologie Moléculaire, Institut Pasteur, 25 rue du Dr Roux, 75015 Paris.

The cerebellum of the homozygous *reeler* mouse shows a marked reduction in size and in the number of fissures, its dry mass and DNA content are respectively $\frac{1}{3}$–$\frac{1}{4}$ and $\frac{1}{7}$–$\frac{1}{8}$ of those of normal animals. Its high content in the P_{400} protein, which is abundant in the Purkinje cell, indicates that the decrease in cell number associated with the fall in DNA affects primarily the granular cells.

The anatomy of the *reeler* cerebellum is rather unique: a thin cortex with almost normal molecular, granular and Purkinje cell layers embracing a central mass of closely packed large neurons, mostly Purkinje cells. Purkinje cells may therefore be found in four different cellular environments: (1) at their normal position in the superficial cortical structure; (2) within the granular layer; (3) intermingled with white matter in the central mass; (4) overlapping with neurons of the deep cerebellar nuclei. The *reeler* cerebellum therefore offers a model to study to what extent local cellular interactions are required to achieve the planar organization of the Purkinje cell dendrites and the normal synaptic investment of these cells.

Concerning the three-dimensional shape of Purkinje cells, only the rare ones located at their normal position and receiving a normal ratio of all their synaptic afferences succeed to develop a characteristic dendritic pattern. Purkinje cells within the granular layer show three distinct patterns of dendritic arrangements. The variation in shape of the Purkinje cell dendrites located in the central agranular mass mimics that described in other agranular cerebella: in particular they show randomly oriented dendrites devoid of spiny branchlets.

Concerning the cerebellar circuitry, the specificity of most of the synaptic connections is preserved, despite important disorders in Purkinje cell distribution. Several important differences with the normal cerebellum have, however, been observed at the level of the Purkinje cell: (i) The density of climbing fibre varicosities increases in the central cerebellar mass, where Purkinje cells are deprived of parallel fibre afferences. In addition, electrophysiological studies reveal that, at this position, the response of the Purkinje cells to climbing fibre stimulation is graded by steps as a function of stimulus intensity instead of being all-or-none as found in the superficial cortex or in normal cerebellum. These deep Purkinje cells receive therefore several climbing fibres instead of only one as in normal adult cerebellum. (ii) Ectopic synapses (somato-dendritic and dendro-dendritic) between the soma and/or the dendrite of the granule cell as presynaptic element and mainly the Purkinje cell dendrites as postsynaptic element may form. (iii) Heterologous synapses between mossy fibres and Purkinje cell spines are found in the granular layer and within the central mass. The electrophysiological studies show that these synapses are functional.

Introduction

The development of the synapse is a long process which lasts for days or even weeks and results from a cascade of complex molecular events. With the ultimate, but still remote, goal of identifying the factors, genetic or not, which govern the establishment of the adult connectivity in vertebrate nervous system, the cerebellum was selected as particularly suitable for a multidisciplinary attack of this question. Its anatomy is rather well understood (Palay & Chan-Palay 1973); the unitary activity of single neurons can be recorded by electrophysiological techniques (Eccles, Ito & Szentagothai 1967) and it contains a few classes of cells repeated many times, giving biochemical simplicity. In addition, in the mouse, several point mutations have been mapped which lead to profound disturbances of cerebellum anatomy (Sidman, Green & Appel 1965; Sidman 1974; Sotelo 1975*b*), physiology (Crepel, Mariani, Korn & Changeux 1973; Crepel & Mariani 1975; Crepel & Mariani 1976) and biochemistry (Mallet, Huchet, Shelanski & Changeux 1974; Mallet, Huchet, Pougeois & Changeux 1976; Beckingham Smith 1976).

During the past few years the collective research developed in our laboratories on the cerebellum from mutant mice has concerned primarily the *staggerer* (Crepel *et al.* 1973; Sotelo & Changeux 1974; Mallet *et al.* 1974, 1976; Crepel & Mariani 1975), *weaver* (Sotelo 1973, 1975*a*; Mallet *et al.* 1974, 1976; Crepel & Mariani 1976) and *nervous* (Mallet *et al.* 1974, 1975) mutations. The present study is devoted to the much studied *reeler* mutation (Falconer 1951, 1952). This autosomal recessive mutation causes extensive perturbations in the development of the cerebellum (Sidman 1968; Rakic & Sidman 1972; Rakic 1976; Steindler 1975) but also affects the cerebral cortex and the hippocampal formation (Meier & Hoag 1962; Hamburgh 1960, 1963; Caviness & Sidman 1972, 1973*a*, *b*; Caviness 1973; Devor, Caviness & Derer 1975; Colwell 1976; Steindler 1976). It leads to a relative malposition of different neuronal classes in many laminated structures and, in particular, of the Purkinje cells in the cerebellum. Some of them are located at their normal position in a zone of cerebellar cortex with a shallow but distinct molecular layer and an internal granular layer; most of the Purkinje cells, however, do not reach their normal position and are dispersed throughout the granular layer, the white matter and the deep cerebellar nuclei. According to their location, the various categories of Purkinje cells grow and develop synaptic connections in different cellular environments. The *reeler* cerebellum therefore offers an interesting 'natural experiment' to characterize the factors which determine or modulate the shape of the Purkinje cells and of their synaptic afferences.

A particular emphasis will be given to the formation of contacts between partners or at sites different from those found in normal cerebellum. Also, it has been recently shown that the establishment of the one-to-one relation between climbing fibre and Purkinje cell (Ramōn y Cajal 1911; Eccles, Llinas & Sasaki 1966*a*) results from the evolution of a transient state of Purkinje cell innervation by *several* independent climbing fibres (Delhaye-Bouchaud, Crepel & Mariani 1975; Crepel, Mariani & Delhaye-Bouchaud 1976*b*). This multiple innervation of the Purkinje cell persists until the adult in the agranular cerebella from rodents (Woodward, Hoffer & Altman 1974; Crepel, Delhaye-Bouchaud & Legrand 1976*a*; Crepel & Mariani 1976). It will therefore be investigated to what extent in *reeler* cerebellum the state of multiple innervation varies with the position of the Purkinje cell. Preliminary reports of this work have been published (Sotelo 1977; Mariani & Crepel 1976).

Material and methods

(*a*) *Breeding of mutant mice*

The *reeler* (*rl*) mutation was maintained on inbred strains of mice with two different backgrounds: (*a*) C_{57}Bl/6J originating from the Jackson laboratory, Bar Harbor, Maine, U.S.A. (*b*) Balb/C originating from the laboratory of the C.N.R.S., Orléans La Source (Pr. Sabourdy, director). Only the homozygous *rl/rl* mice have been studied. Litters containing *rl/rl* individuals were obtained by intercrossing heterozygous (*rl*/+) fertile animals. Some of the homozygous Balb/C *rl/rl* animals being fertile, homozygous individuals were also generated by inter-crossing *rl/rl* males and *rl*/+ females.

The *rl/rl* animals on C_{57}Bl/6J background are generally poorly and do not survive after the end of the first postnatal month. They were used for electrophysiology on postnatal day 19 to 21 and for some biochemical analysis. The electrophysiological studies were carried out with Balb/C *rl/rl* on postnatal day 90 to 100 as well as many biochemical experiments. All the morphological observations were made on Balb/C *rl/rl* mice from 21 to 226 days of age.

(*b*) *Morphological studies*

The mice were anaesthetized with ether and fixed by intracardiac perfusion of 500 ml of a warm solution of 1% paraformaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer (pH 7.3). After fixation, the cerebellum was detached from the brain stem and divided by mid-saggital section. One half was impregnated by the rapid Golgi method (double impregnation) or by the Golgi–Rio Hortega method (Ramōn y Cajal & de Castro 1972). Blocks obtained in the sagittal plane from the other half of the cerebellum were postfixed by immersion for 4 hours in solution of 2% osmic acid and 0.12 M phosphate buffer with 7% glucose. After staining *en bloc* with uranyl acetate, the blocks containing the vermis or the hemisphere were dehydrated in graded solutions of ethanol and embedded in Araldite. Normal mice (+/+), each one taken from the same litter that the corresponding *rl/rl*, were also perfused and prepared for Golgi impregnation and for electron microscopic examination.

The brains of two homozygous *reeler* mice were fixed in 10% formolsaline, embedded in paraffin, serially sectioned (one in the frontal and the other in the sagittal plane) and stained with Luxol fast blue and/or cresyl-violet. The brain of one month old homozygous *rl/rl* was also fixed in formol-saline, sectioned with a freezing microtome in the sagittal plane and stained with reduced silver according to a modification of the Bielschowsky method (Ramōn y Cajal & de Castro 1972).

(*c*) *Electrophysiological experiments*

After Nembutal anaesthesia (30 mg/kg, i.p.), the animals were paralysed with Gallamine triethiodide (Flaxedil 60 mg/kg, i.p.), kept under artificial respiration, locally anaesthetized with xylocaïne and the cerebellar vermis was exposed. During the experiments, the physiological state of the animals was controlled by monitoring the electrocardiogram, and the body temperature was kept at 37 °C by mild warming. Electrical stimulation of the inferior olive (IO) was performed with a concentric bipolar electrode and juxtafastigial (JF) stimulation was performed with fine bipolar electrodes of two silver wires (each 50 μm in diameter) insulated except at their tips and gained side by side with Epon resin. The localization of the sites of stimulation were confirmed by the histology as previously reported (Crepel 1971).

Unitary responses were recorded with glass micropipettes filled with potassium acetate (1 M) and methyl blue or with KCl (3 M) (the d.c. resistance was 1.5–5 MΩ for extracellular records and 10–15 MΩ for intracellular ones). The position of the tip of the microelectrode was determined by iontophoretic injection of methyl blue (Thomas & Wilson 1966). Movements of the tissues were minimized by covering the exposed cerebellum with 4% Agar in 10% sucrose solution.

(d) Biochemical studies

The mice were killed by decapitation without anaesthesia and the cerebella dissected, weighed and immediately frozen in liquid nitrogen where they were stored until use. Samples were mixed with approximately 10 times their volume of 10^{-3} M sodium phosphate buffer pH 7.0 at 4 °C and homogenized in a motor-driven glass-teflon Potter homogenizer with 50 up-and-down strokes at 100 rev min^{-1}. The homogenate was then centrifuged for 20 min in a Beckman 152 microfuge at maximum speed (about 10000 rev min^{-1}). The supernatant was collected and the pellet resuspended in the same volume of the aforementioned buffer and centrifuged again at the same speed yielding a pellet that we shall refer to as low speed pellet (l.s.p.).

The first supernatant was then centrifuged at 30000 rev min^{-1} in a rotor type 40 Beckman for 75 min in a Beckman model L-2-65 ultracentrifuge yielding a pellet that we shall refer to as a high speed pellet (h.s.p.) and a supernatant (s). L.s.p. and h.s.p. were dissolved in 3% solution of sodium dodecyl sulphate (SDS) containing 1% β-mercaptoethanol and kept in a boiling water bath for 3 min.

Slab gel electrophoresis were carried out in linear gradients of 5–15% polyacrylamide in the presence of 1% SDS using the procedure of Ames (1974). Each sample contained 20–30 μg protein. The electrophoresis was run at 4 °C at a constant current of 15 mA. Then the gels were fixed in 25% isopropanol, 10% acetic acid to remove SDS, were stained with Coomassie brilliant blue (2.5 g/l) in methanol:acetic acid:water (65:10:45) for 75 min, and finally destained in methanol:acetic acid:water (65:10:45).

DNA and RNA extraction was performed by a modification of the method of Schmidt & Thanhauser (1945). To the cerebellum stored in liquid nitrogen, ten times the volume of ice-cold 10% trichloroacetic acid (by mass) was added and homogenized in Potter homogenizer of 0.5 ml and centrifuged at 3000 rev min^{-1} in a Sorval centrifuge. The pellet was resuspended in the same volume of 10% TCA and centrifuged again. The pellets were extracted by adding 20 volumes of chloroform:ethanol (1:3, by volume) and the residue again extracted with ether and dried for 2 hours at 37 °C or overnight at room temperature.

0.3 M KOH was added to the dried residue (1.0 ml/100 mg original tissue), homogenized with a Potter glass homogenizer and incubated for 90 min at 37 °C for alkaline hydrolysis. 6 M HCl was added to the mixture (0.5 ml/3.0 ml of 0.3 M KOH) and centrifuged at 3000 rev min^{-1} for 30 min, then 1 M HCl was added to the pellet (1 ml/50 mg tissue) which was then resuspended and centrifuged at 3000 rev min^{-1} for 20 min. The combined supernatant was used for assay of RNA.

5% TCA was added (1 ml/25 mg tissue) to the pellet and boiled for 15 min to hydrolyse the DNA. The supernatant after centrifugation at 3000 rev min^{-1} for 15 min was used for assay of DNA by the diphenylamine procedure as described by Burton.

In the DNA assay 2′-deoxyadenosine (from Sigma, D-5875) (molecular mass 269.2) was used as standard. It was boiled routinely in 5% TCA for 15 min in parallel with the DNA

samples. Pyrimidine sugar linkage was not hydrolysed under these conditions. Taking 311 as an average molecular mass for DNA nucleotides, a correction factor of $311/269.2 \times 2 = 2.31$ was used to calculate masses of DNA from the densitometric readings.

In the RNA assay, RNA purified from yeast (Sigma, no. R-6750) was used as standard. Protein content was measured according to the method of Lowry, Rosebrough, Farr & Randall (1951) by using bovine serum albumine (Sigma, fraction V) as a standard.

Results I: Morphological analysis of the changes of shape and synaptic investment of the Purkinje cells with spatial distribution in adult *rl/rl* cerebellum

(*a*) *General anatomy and histology of homozygous* reeler *cerebellum*

The cerebellum from a homozygous *reeler* mouse differs markedly from that of its littermates (heterozygous and normal) by a gross reduction of size and its almost smooth surface (figure 1, plate 1). Measurements done on midsagittal 1 μm thick plastic sections from 226-day-old *rl/rl* individual show a decrease by about a factor of 3 of the surface of its sagittal section through the vermis (figure 2*a* and *b*, plate 1). Fissures and their consequent folia are almost absent in *rl/rl* cerebella with the exception of one constant fissure present at the posterior vermis and most likely corresponding to the *fissura secunda* (figures 1, 2*b*, plate 1).

At first sight the histology of *rl/rl* cerebellum reveals a rather unique organization. It shows an *emboîtement* of two distinct elements: a quasi-normal cerebellar cortex which encloses a central mass consisting mostly of Purkinje cells and deep cerebellar nuclei (figures 1, 2*b*, plate 1).

At the pial surface a broad band of cortex shows the typical molecular, Purkinje and granular layers and therefore resembles the normal cerebellar cortex. A few differences, however, exist: variable thickness of the molecular layer in all instances thinner than in control animals, the horizontal extension of the granular layer is constantly interrupted by a narrow gap of white matter, which probably marks the position of a vestigial *fissura prima* separating the anterior from the posterior vermis (figures 1, 2*b*, plate 1). In between the molecular and the granular layer lies the layer of Purkinje cells whose number varies from animal to animal and is less than that of control mice. Their density also varies greatly from one region of the vermis to another (figure 1, plate 1). In some areas they form rows of from 3 to 10 Purkinje cells separated between them by an almost normal distance. More often, one or two cells are scattered here and there and in some regions those neurons are absent. Some Purkinje cells are also dispersed within the granular layer. There, they tend to form small clusters or rows invading the granular layer from the white matter up till the molecular layer (figure 1, plate 1).

This typical though somewhat modified cortical structure encloses a dense mass of large neuronal bodies which occupies the central region of the vermis (in its anterior part a shallow band of pure white matter underlies its granular layer) and can be subdivided into three different areas: (*a*) an area immediately adjacent to the pure white matter or to the granular layer, in which some granule cells and stellate cells are intermingled with numerous Purkinje cells and heavily myelinated fibres; (*b*) an area in which Purkinje cells and their dendritic arborizations are tightly packed; in this area few myelinated fibres and few interneurons are present. Part of this area corresponds to the ventricular surface; (*c*) an area mostly occupied by

the neurons of the 'deep cerebellar nuclei': there, some of the Purkinje cell bodies surrounding the deep nuclei project part of their dendritic tree into these non-cortical structures.

As a consequence of this exceptional histological organization, Purkinje cell perikarya and/or dendrites may be found in four different 'environments': (1) at their normal position in the superficial cortical structure; (2) within the granular layer; (3) intermingled with white matter in the central mass; (4) overlapping with neurons of the deep cerebellar nuclei. Interestingly the shape and synaptic investment of the Purkinje cell differ in these four different positions.

(*b*) *The variation of the shape of the Purkinje cell and of its dendritic arborization with different positions*

The morphology of Purkinje cells distributed in the four previously mentioned compartments was examined both by light microscopy on regular or Golgi-impregnated material and by electron microscopy.

(*b* 1) *The Purkinje cells normally positioned in the superficial cortex*

The Purkinje cells which succeed in reaching their normal positions, exhibit all the morphological features of the same cells in control (+/+) mice. Because of the reduced thickness of the molecular layer, the dendritic arborizations of most of these Purkinje cells extend laterally, always in the sagittal plane, for longer distances than in the normal mouse. In 1 μm thick plastic sections (figure 3, plate 2) the secondary dendrites can be seen running horizontally, parallel with the pial surface. In Golgi-impregnated material, the dendritic arborizations of these Purkinje cells keep their typical espalier arrangement (figure 7, plate 3). From a qualitative view point, secondary and tertiary thick dendritic trunks, as well as the spiny branchlets, are present. However, the ratio of spiny branchlets to thicker branches seems to be higher in *reeler* than in control animals. The spiny branchlets are on average larger than in control mice, but the density of spines appears similar in both normal and *reeler* cerebellum. Clusters of stubby spines, the postsynaptic partners for climbing fibres, keep their normal position at the large dendritic trunks.

The electron microscopic study confirms the scarceness of the Purkinje cells at their normal position. As a consequence the molecular and granular layers are not sharply separated by a clearcut border. In some instances, clusters of granule cells are seen in the lower half of the molecular layer (figure 18, plate 5); but such figures are frequently observed in normal mouse cerebellum (Landis 1973*a*) and cannot be considered as typical of the *reeler* conditions. In other instances, molecular and granular layers are delimited by glial cells, probably Golgi-epithelial cells, intermingled with granule cells (figure 20, plate 5). In any case, there is no hyperplasia of glial cell bodies and processes at this level, as is the case in the homozygous *nervous* mouse (Landis 1973*b*) in which Purkinje cells degenerate after a normal development, inducing a local hyperplasia of Golgi-epithelial cells (C. Sotelo, unpublished observations). The perikarya of the few Purkinje cells which succeed in their migration have a smooth contour and a normal polarity. The hillock and the initial segment of the axon (figure 24, plate 7) emerge from the basal pole of the cell opposite to the origin of the main dendritic trunk. Hypolemmal cisternae (figure 24, plate 7), subsurface cisterns associated with mitochondria and other characteristic features of the Purkinje cells are commonly present in these perikarya.

(*b* 2) *The Purkinje cells dispersed within the granular layer of the superficial cortex*

Rows of Purkinje cell perikarya are observed in the inner part of the granular layer and in the white matter as if they were 'invading' the cortex from the white matter. As a consequence the broad horizontal band of the granular layer is partitioned by vertical septa distributed at irregular intervals (figures 1, 4, plates 1, 2). Golgi impregnation of Purkinje cells lying within the granular layer discloses three main types of Purkinje cells differing by the shape of their dendritic aborizations.

(i) The Purkinje cells *en parapluie* (figure 10, plate 3): the perikarya of this type of Purkinje cell lie at different depths of the granular layer. A main dendritic branch emerges from the apical pole and ascends in a vertical direction without branching up till the molecular layer, where it branches profusely (figure 10, plate 3). Clusters of stubby spines are present at the main vertical branch. In the molecular layer, the dendritic arborizations evoke that of normal Purkinje cells and even spiny branchlets are developed.

(ii) The 'multipolar' Purkinje cells (figure 11, plate 3): besides the presence of a main dendritic trunk, from one to three thinner dendritic branches arise from the perikarya of this type of cell. Shortly after its emergence, the main dendritic trunk branches profusely. The whole dendritic arborization of these multipolar Purkinje cells ends within the granular layer (figure 11, plate 3). The medium-sized and the thin branches of this dendritic arborization are studded with spines.

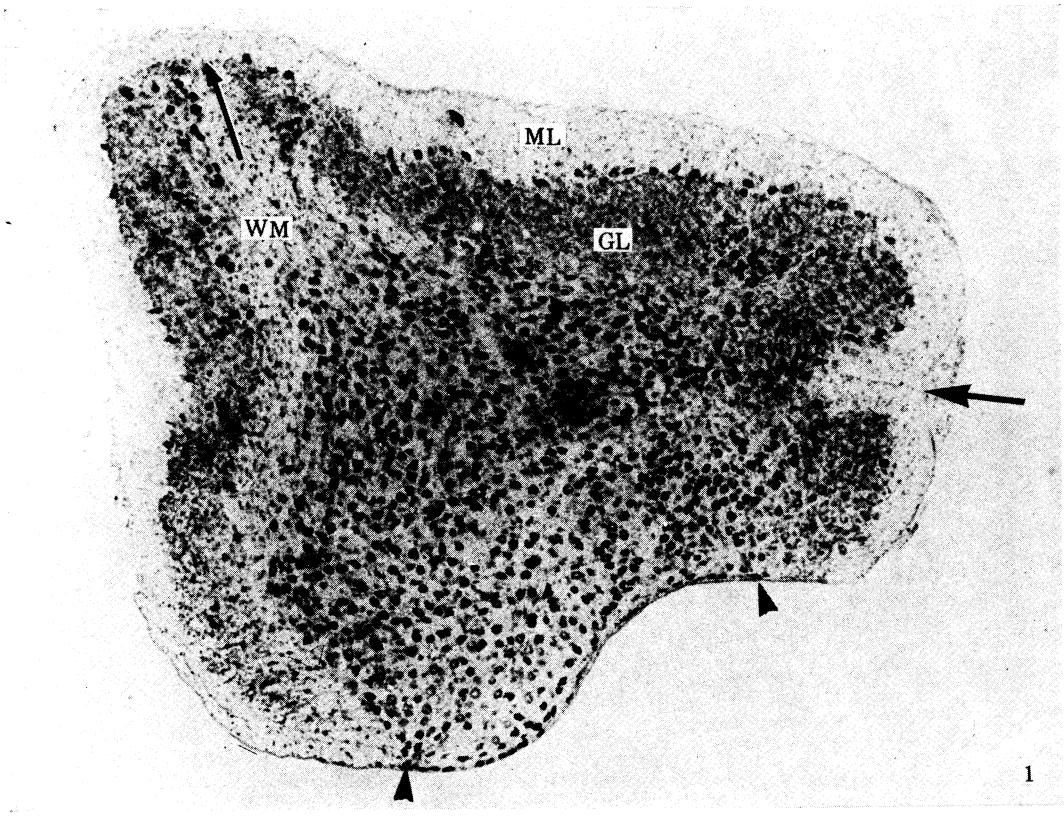
(iii) This third and last type of Purkinje cells is a mixed type of the two previous ones. Generally the dendritic tree emerges from one apical trunk, which during this ascension in the granular layer branches in a dichotomous manner giving off medium-sized dendritic trunks, intermingled with the granular cells. The branches which succeed in reaching the molecular layer, ramify profusely, in a similar way as they do in the first type of Purkinje cells (figure 9, plate 3).

(*b* 3) *The Purkinje cells intermingled with the white matter in the central mass*

We shall consider together the two already defined areas: (i) the one underlying the narrow zone of pure white matter or the granular layer, and (ii) the other, located more deeply in the central mass, in which there is an almost pure population of Purkinje cells (figure 6, plate 2). The majority of the Purkinje cells of the *rl/rl* cerebellum are concentrated in these two areas. Few granule cells are scattered throughout the area beneath the cortical region and they are practically absent in the deeper regions. In addition, some interneurons, which in Golgi-impregnated material appear as multipolar neurons of the stellate type are intermixed with the Purkinje cells. Basket cells, with their typical basket formations around Purkinje cells, have never been observed in any of these areas.

In 1 μm thick plastic sections, the central areas of the cerebellum appear as compact masses of cells in which some small to medium-size myelinated axons are scattered. Some Purkinje cells beneath the white matter send dendritic branches across the white matter up to the granular layer (figure 5, plate 2). The neuropil mainly formed by Purkinje cell dendritic profiles rich in mitochondria, which give, after toluidine blue staining, a characteristic appearance of dense profiles surrounded by lighter ones as illustrated in figures 5 and 6, plate 2.

In Golgi-impregnated material the Purkinje cells exhibit a large variety of shapes (Rakic 1976), all of them resembling those already described for the *weaver* cerebellum (Rakic &



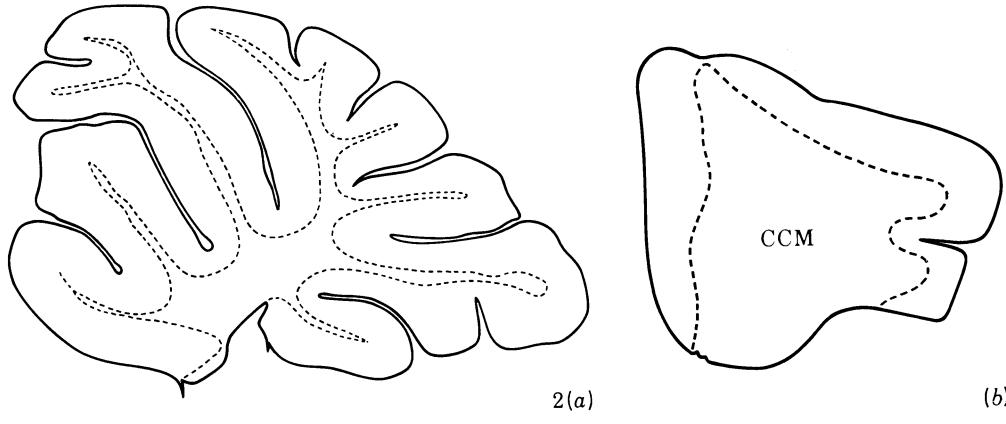
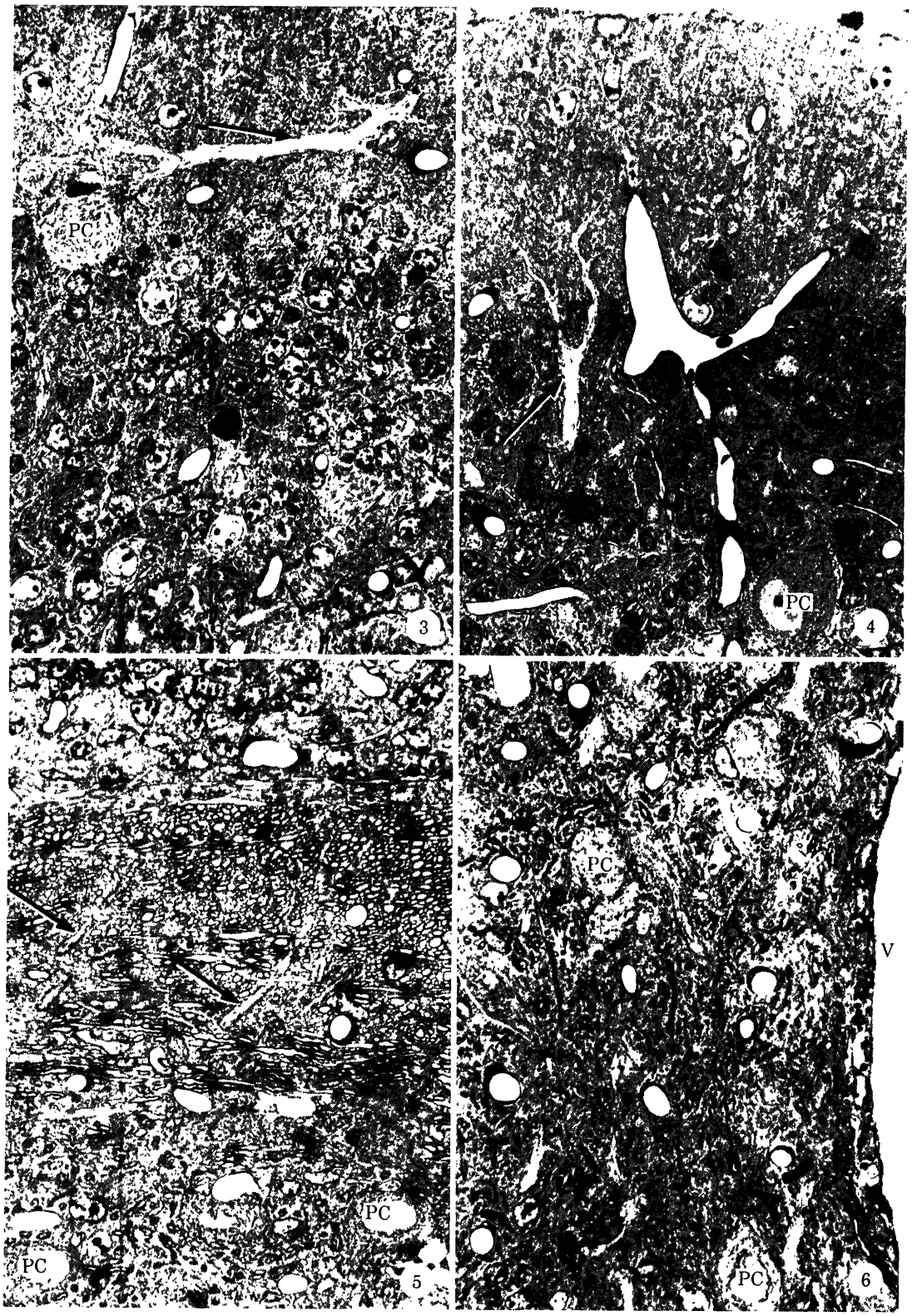


FIGURE 1. Midsagittal section of the vermis of a 226-day old *rl/rl* mouse. The periphery is occupied by a quasi-normal cerebellar cortex, composed by a molecular layer (ML), a Purkinje cell layer and a granular layer (GL), underlayed by a white matter (WM) of variable thickness. This peripheral cortex covers a central cellular mass, almost entirely formed by mispositioned Purkinje cells. The large arrow points to the *fissura secunda*. The small arrow indicates the site of interruption of the granular layer by the white matter, which probably marks the position of a vestigial *fissura prima*. The two arrow heads delimits the ventricular surface. (Magn. × 70.)

FIGURE 2. Schematic outline of a 1 μm thick plastic sagittal section passing through the vermis of a 226-day old *rl/rl* mouse (2*b*) and the vermis of its normal littermate (2*a*). Most of the *reeler* cerebellum is occupied by a central cerebellar mass (CCM), where is located the bulk of the Purkinje cell population. (Magn. × 27.)

(*Facing p.* 8)



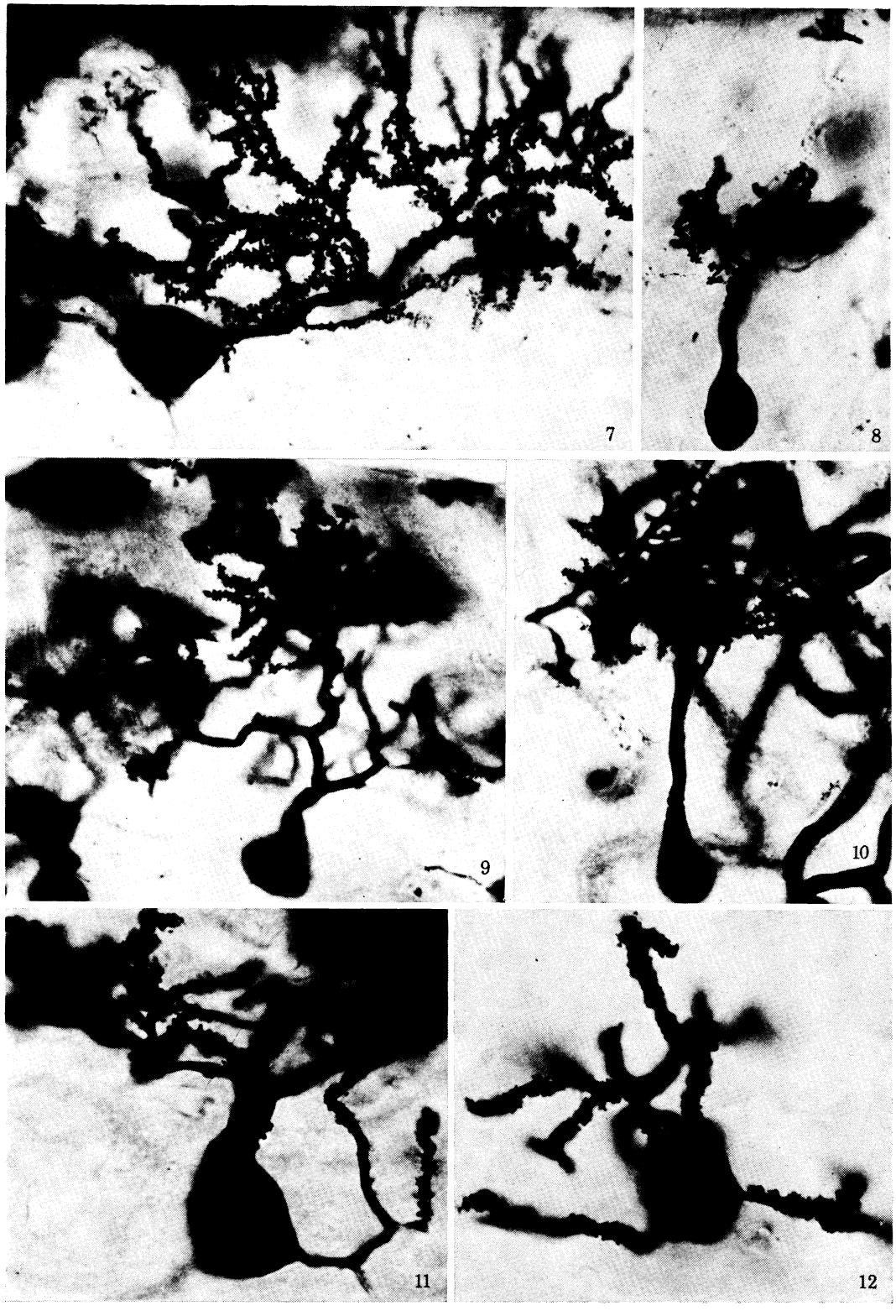
1 μm thick plastic sections stained with toluidine blue, illustrating Purkinje cells at different positions within the cerebellum.

FIGURE 3. Purkinje cell (PC) located at its normal position between the molecular and the granular layers. The arrow points to the secondary dendrite spreading in the molecular layer in an almost parallel direction to the pial surface. (Magn. × 640.)

FIGURE 4. Purkinje cell (PC) within the granular layer. The arrow points to a thick dendritic trunk, ascending in a vertical direction through the granular layer and branching at the molecular layer level. (Magn. × 640.)

FIGURE 5. Purkinje cells (PC) at the periphery of the central cerebellar mass. These cell bodies are just beneath the white matter of the superficial cerebellar cortex. Some of the thick dendritic branches of these Purkinje cells cross the white matter (arrows) and spread within the granular layer. (Magn. × 640.)

FIGURE 6. Purkinje cells (PC) at the central cerebellar mass, close to the ventricular surface (V). (Magn. × 640.)



Golgi impregnation of Purkinje cells located at different levels of the *rl/rl* cerebellum.

FIGURE 7. Purkinje cell which succeeded to reach its normal position. Its dendritic tree spreads within the molecular layer keeping its normal planar orientation. (Magn. × 640.)

FIGURE 8. Purkinje cell at the periphery of the central cerebellar mass. Its main dendrite, studded with spines, crosses the white matter and branches within the granular layer. (Magn. × 540.)

FIGURE 9. Purkinje cell within the granular layer. Its main dendrite branches close to the perikaryon, given rise to large dendritic trunks which spread within the granular layer. One of these trunks ascends towards the molecular layer, where it branches profusely. It therefore corresponds to the mixed type. (Magn. × 590.)

FIGURE 10. Purkinje cell within the granular layer, illustrating the *en parapluie* type. (Magn. × 570.)

FIGURE 11. 'Multipolar' Purkinje cell, which perikaryon is located within the granular layer. (Magn. × 880.)

FIGURE 12. 'Multipolar' Purkinje cell located at the central cerebellar mass. This cell has randomly oriented dendrites, which are studded with spines. (Magn. × 800.)

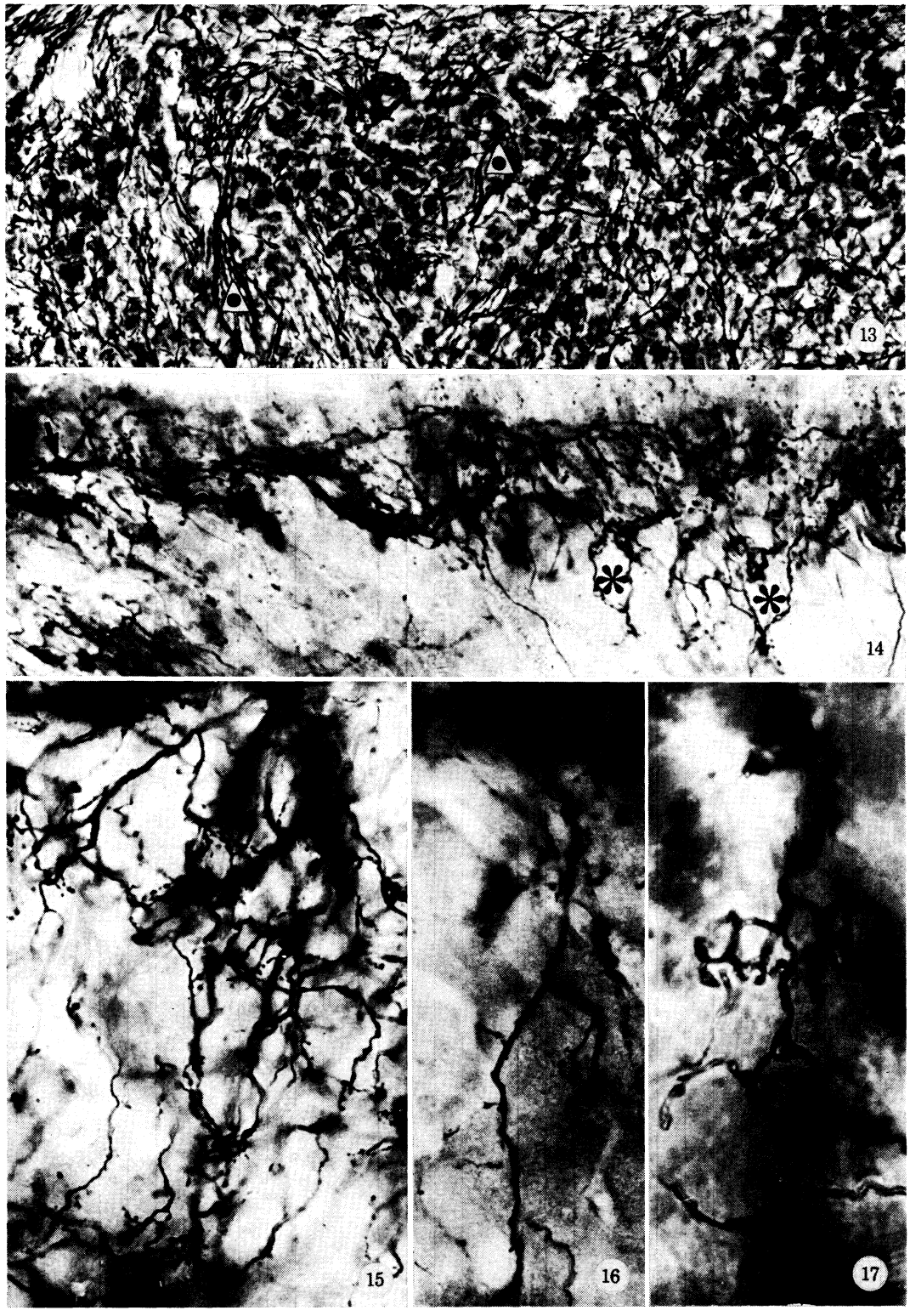


FIGURE 13. Reduced silver impregnation of basket fibres around two Purkinje cell perikarya (white triangle with a black spot) dispersed within the granular layer. (Magn. × 500.)

FIGURES 14–17. Golgi impregnation of a basket cell and numerous basket fibre terminals.

FIGURE 14. The arrow points to a basket cell perikaryon located at the deep region of the superficial molecular layer. Its axon gives rise to a prominent plexus which invades the granular layer to invest some of the mal-positioned Purkinje cell perikarya (asterisks). (Magn. × 330.)

FIGURE 15. Pericellular plexus formed by basket fibres investing Purkinje cell perikarya within the granular layer. (Magn. × 600.)

FIGURES 16 and 17. Terminal axonic arborizations of individual basket fibres embracing Purkinje cell perikarya in the granular layer. (Figure 16: Magn. × 600; figure 17: Magn. × 750.)

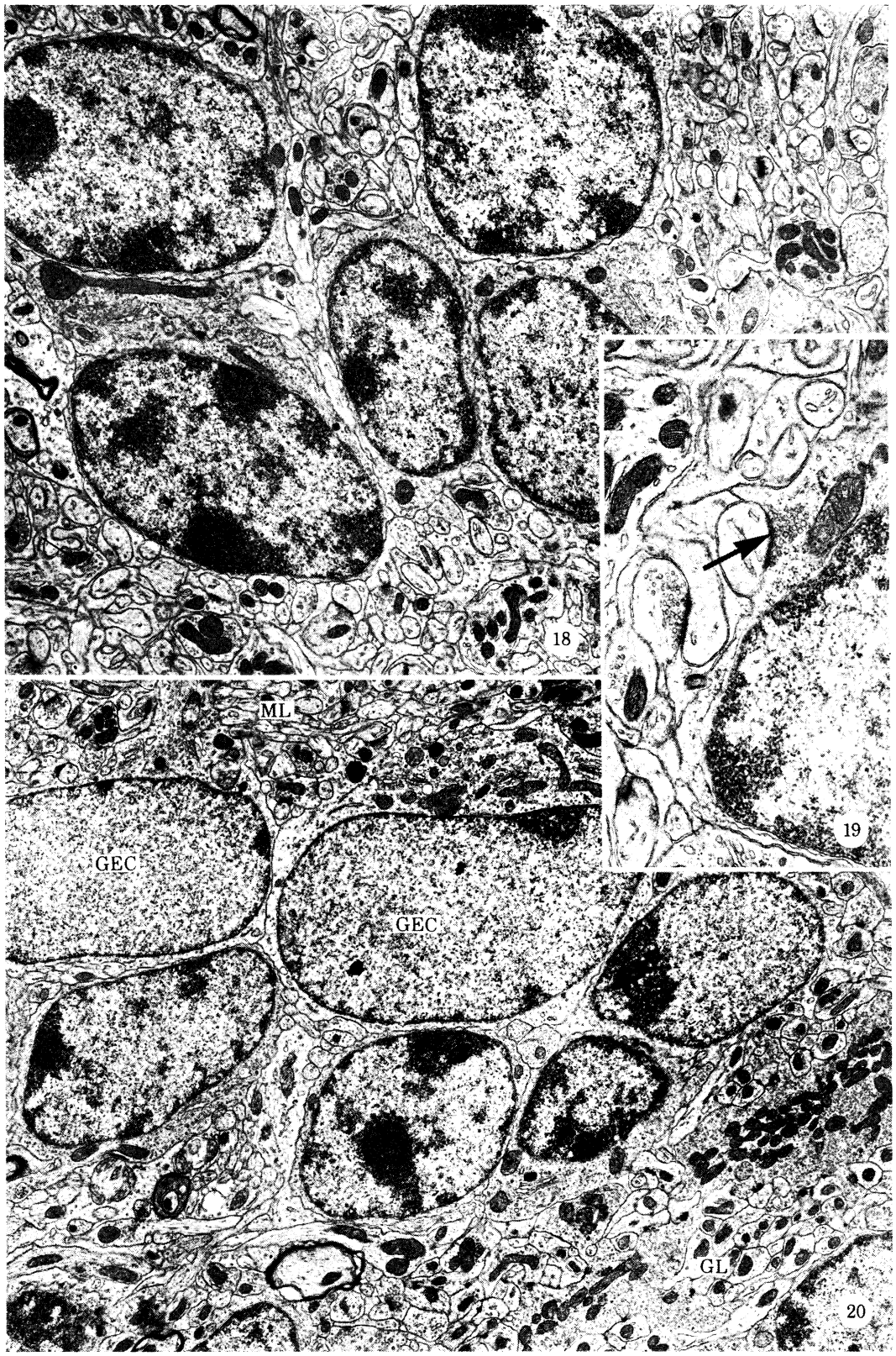


FIGURE 18. Ectopic granule cells in the molecular layer. A cluster of five granule cells is surrounded by a neuropil characteristic of the molecular layer. (Magn. × 8000.)

FIGURE 19. Somato-dendritic synapse (arrow) between an ectopic granule cell and a spine of a Purkinje cell dendrite. (Magn. × 18000.)

FIGURE 20. Border region between the molecular layer (ML) and the granular layer (GL). At this region there are not Purkinje cell perikarya, but Golgi epithelial cells (GEC) separate the molecular from the granular layer. (Magn. × 7000.)

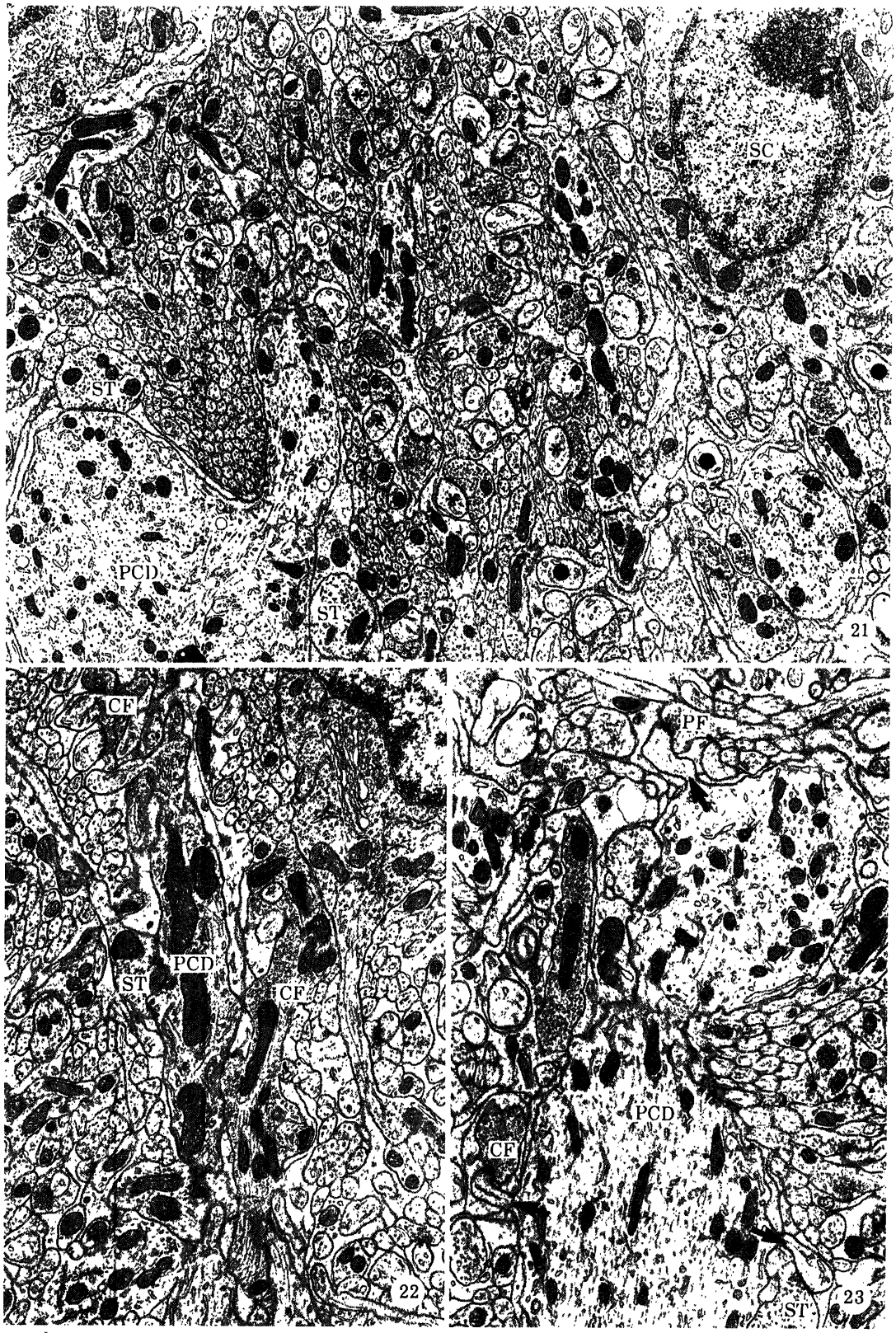


FIGURE 21. Molecular layer. The appearance of this layer is normal. At the right high corner of the micrograph there is a stellate cell perikaryon (SC). A large profile of a Purkinje cell dendrite (PCD) receives stellate axon terminals (ST) on its surface. Many Purkinje cell spines (asterisks) are postsynaptic to parallel fibres; whereas, some of them (black spots) are free of innervation, but do not exhibit postsynaptic-like differentiations. (Magn. × 10000.)

FIGURE 22. Medium-sized Purkinje cell dendritic profile (PCD) contacted on its spines by climbing varicosities (CF) and on its shaft by a stellate axon terminal (ST). (Magn. × 11000.)

FIGURE 23. Three spines (arrows) emerge from a large Purkinje cell dendritic profile (PCD) in the molecular layer. One of them is contacted by a parallel fibre (PF), another by a climbing varicosity (CF) and the third one by a stellate axon terminal (ST). (Magn. × 12000.)

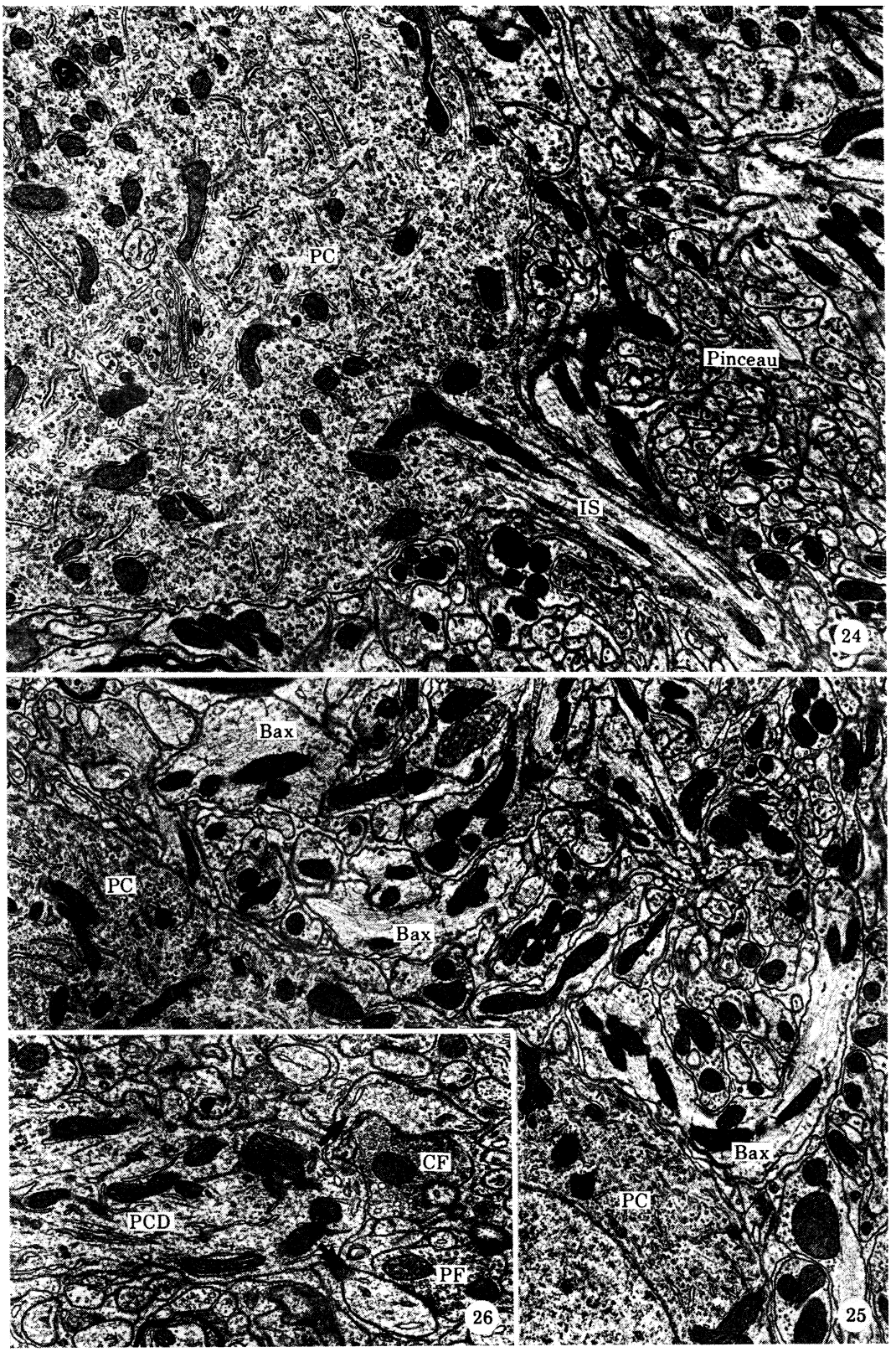


FIGURE 24. A Purkinje cell perikaryon (PC) at its usual location. Note the fine basket processes forming the pinceau around the initial segment (IS) of the Purkinje cell axon. (Magn. × 14000.)

FIGURE 25. Basket fibre (Bax) investment of a Purkinje cell perikaryon (PC) located within the granular layer. (Magn. × 13000.)

FIGURE 26. Electron micrograph of an ascending Purkinje cell dendrite (PCD) within the granular layer. Two spines (arrows) emerge from this dendritic profile. One is contacted by a climbing fibre varicosity (CF) and the other by a parallel fibre (PF). (Magn. × 14000.)

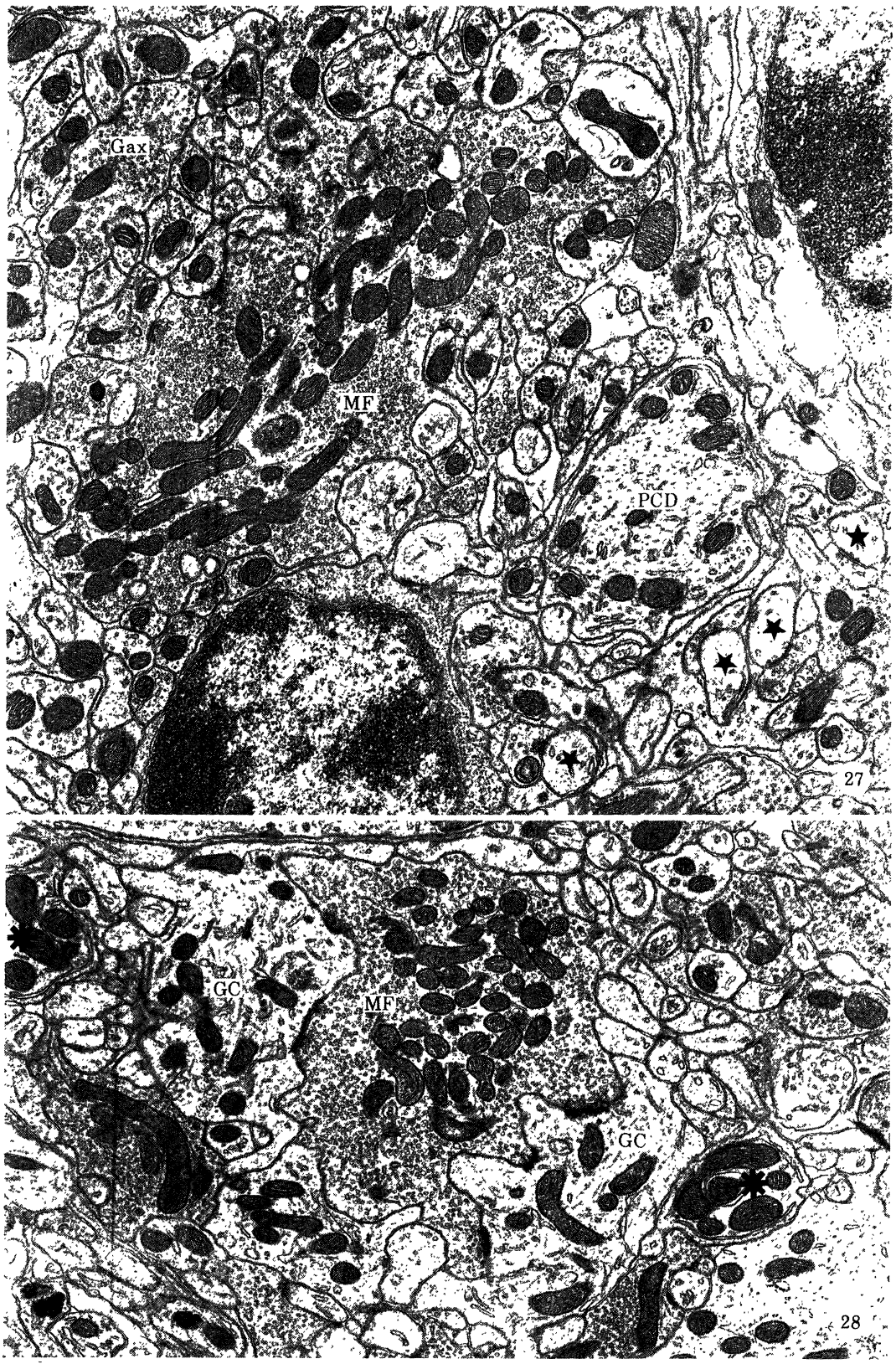


FIGURE 27. Typical appearance of the granular layer in *reeler*. A cerebellar glomerulus composed by a central mossy rosette (MF) and peripheral Golgi axons (Gax) is nearby a Purkinje cell dendritic profile (PCD). The spines (asterisks) of this dendrite are contacted by parallel fibres. (Magn. × 14000.)

FIGURE 28. Atypical glomerulus formed by mossy fibre (MF) synapsing on Golgi cell dendrites (GC). Small Purkinje cell dendritic profiles (large asterisks) are nearby the glomerulus. (Magn. × 15000.)

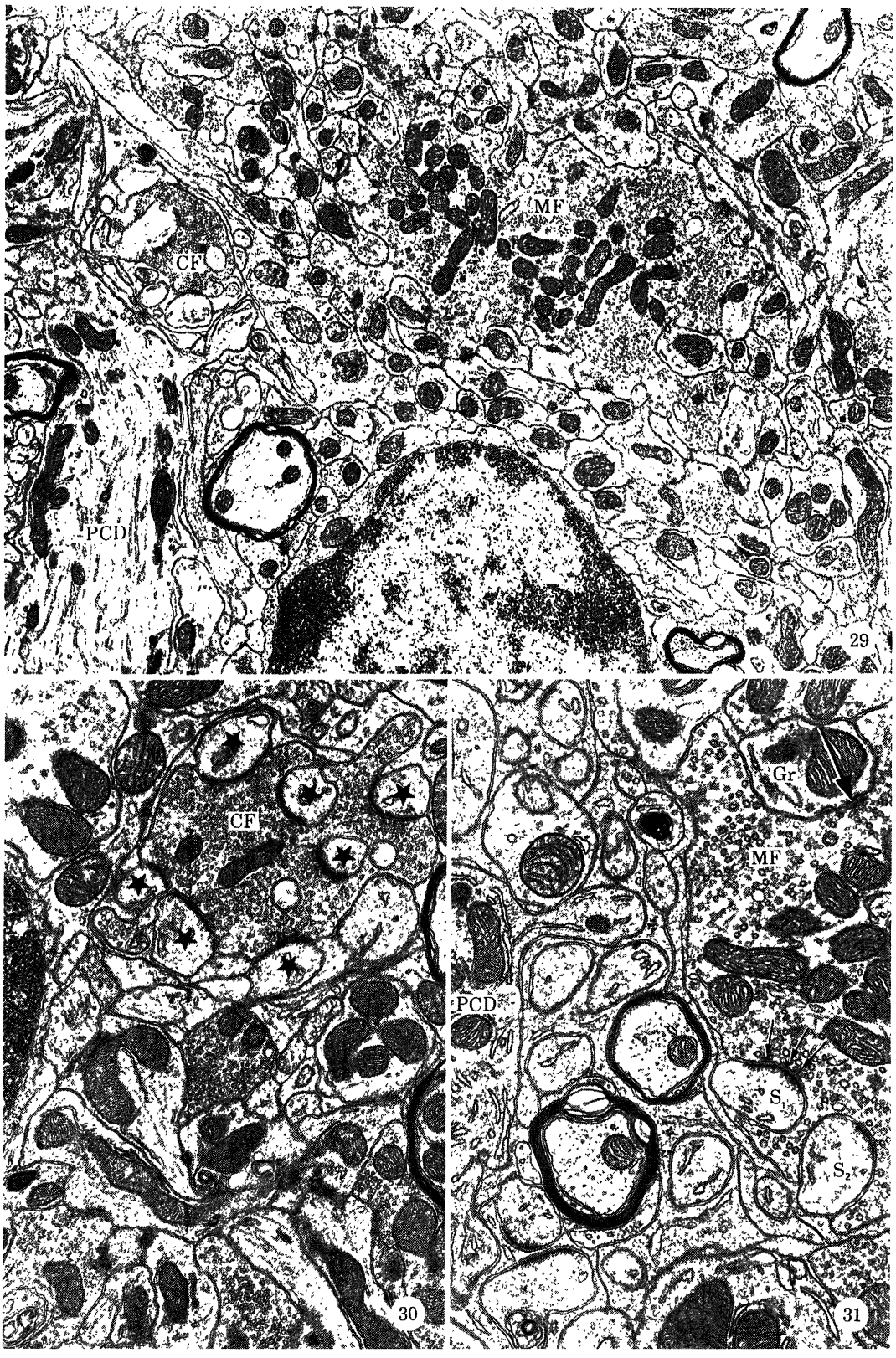
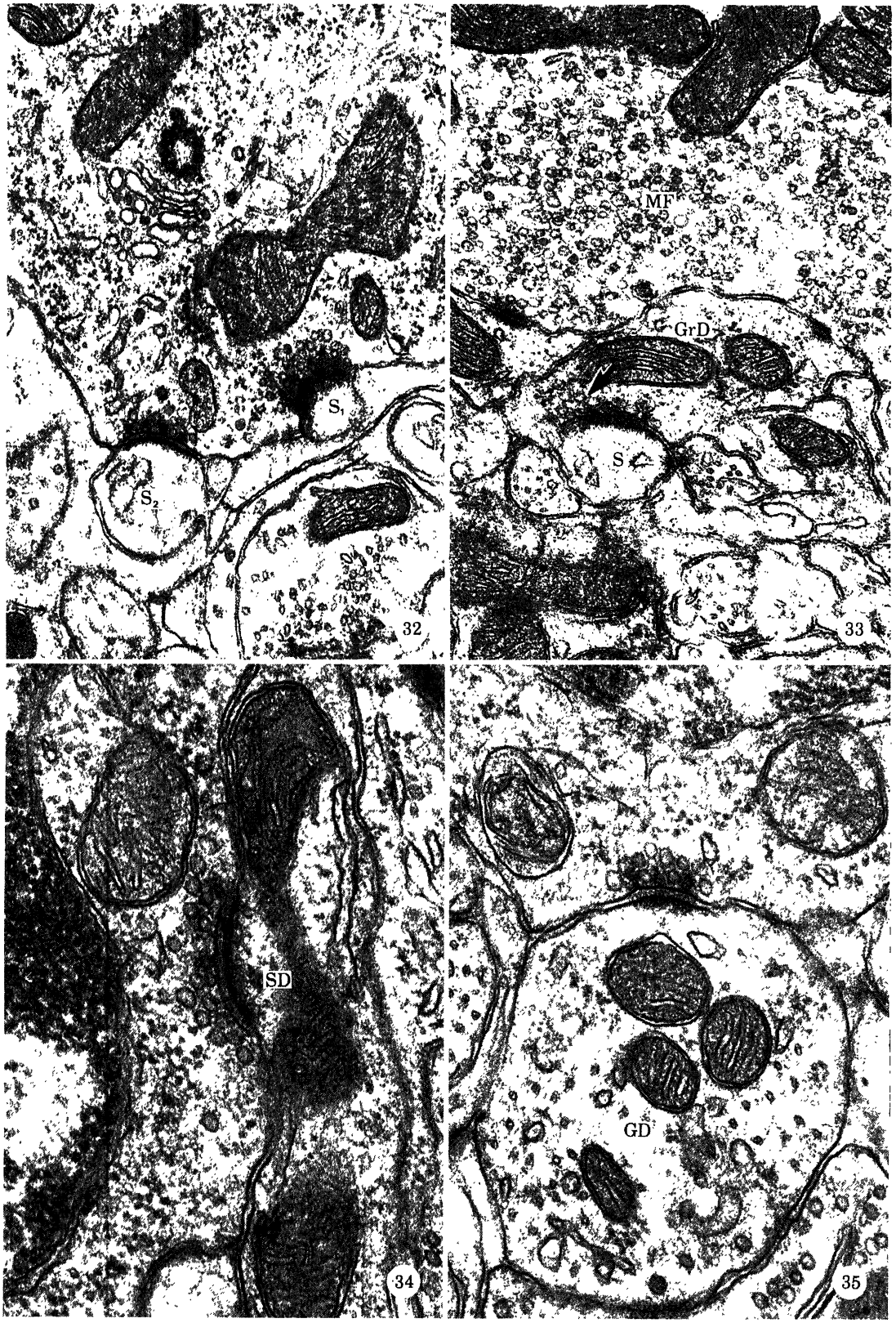


FIGURE 29. The left area of the micrograph is occupied by an ascending Purkinje cell dendrite (PCD) which receives a climbing varicosity (CF). The rest of the micrograph is occupied by neuronal elements characteristic of a granular layer. The two afferent systems, climbing fibres (CF) and mossy fibres (MF) are very close, but each one preserves its synaptic specificity. (Magn. × 12000.)

FIGURE 30. Climbing fibre varicosity (CF), within the granular layer, synapsing on numerous Purkinje spines (asterisks). (Magn. × 16000.)

FIGURE 31. Mossy fibre rosette (MF) synapsing on a granule cell (Gr) dendrite (large arrow). Two Purkinje cell dendritic spines (S_1 and S_2) are directly apposed to the mossy fibre. The mossy fibre has only developed a presynaptic vesicular grid (small arrows), morphological counterpart of a synaptic contact, facing one of the spines (S_1). (Magn. × 21000.)



Somato-dendritic and dendro-dendritic ectopic synapses in the granular layer.

FIGURE 32. Two Purkinje cell dendritic spines (S_1, S_2) are postsynaptic to a granule cell perikaryon. (Magn. × 36000.)

FIGURE 33. Cerebellar glomerulus. The mossy fibre (MF) is in synaptic contact with a granule cell dendrite (Gr D). The cytoplasm of this dendritic profile contains synaptic vesicles (arrow) forming an 'active' zone with a Purkinje cell dendritic spine (S). (Magn. × 39000.)

FIGURE 34. Granule cell perikaryon forming an 'active' zone of the Gray type I on the surface of a small dendritic profile (SD), identified as belonging to an interneuron. (Magn. × 60000.)

FIGURE 35. Granule cell perikaryon synapsing on a large dendritic profile probably belonging to a Golgi cell (GD). (Magn. × 54000.)

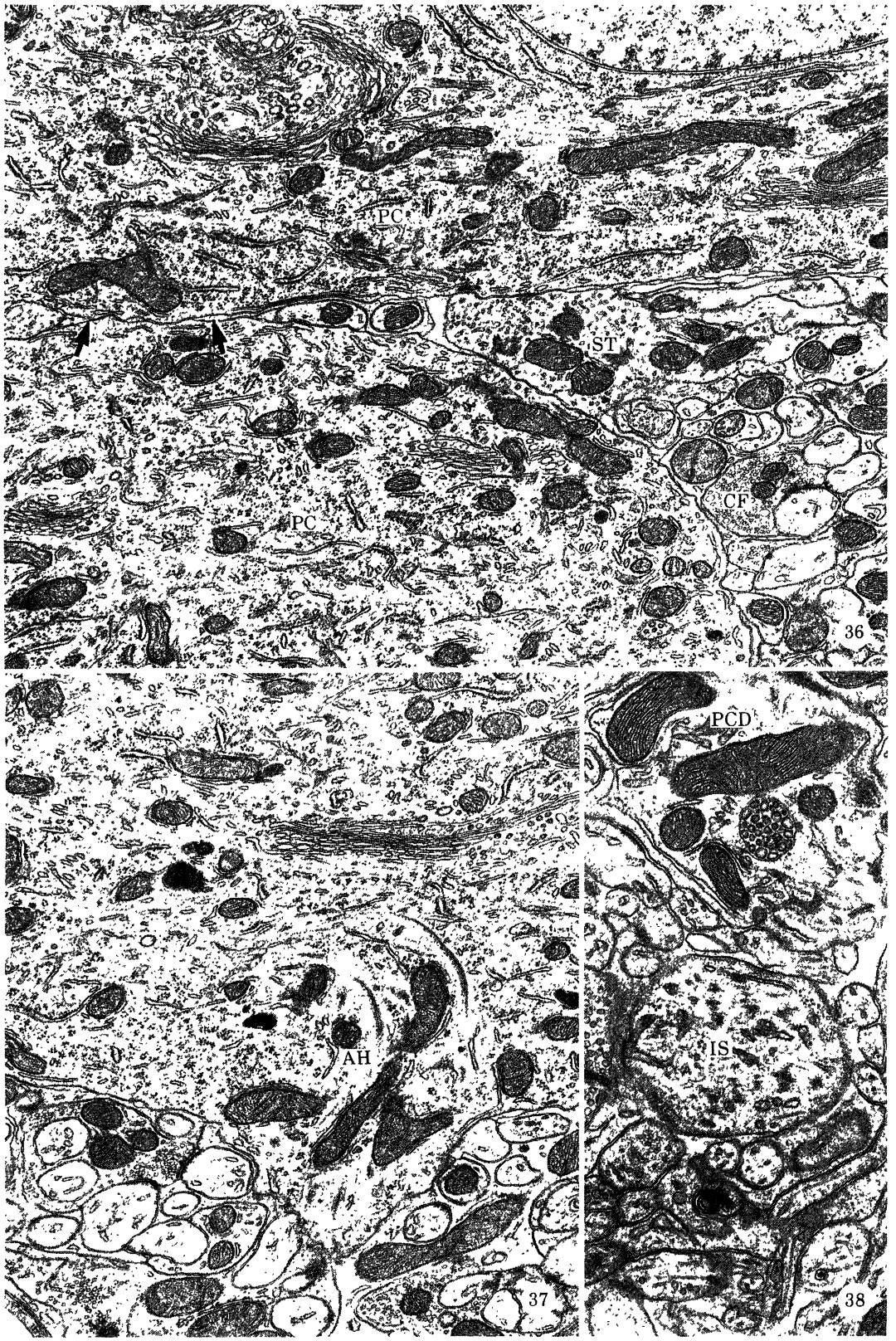


FIGURE 36. Two Purkinje cell perikarya (PC) in the central cerebellar mass. The two perikarya are very close. The arrows indicate a small area in which both perikarya are directly apposed. An axon terminal, probably belonging to a stellate-like interneuron (ST) establishes synaptic contacts on both perikarya. A climbing fibre varicosity (CF) is synapsing on a Purkinje cell spine. (Magn. × 14000.)

FIGURE 37. Axon hillock (AH) of a Purkinje cell located in the central cerebellar mass. Note that these neurons are not invested by basket fibres. (Magn. × 15000.)

FIGURE 38. Oblique section of the initial segment (IS) of a Purkinje cell axon located in the central cerebellar mass. Note the absence of a pinceau formation. (Magn. × 24000.)

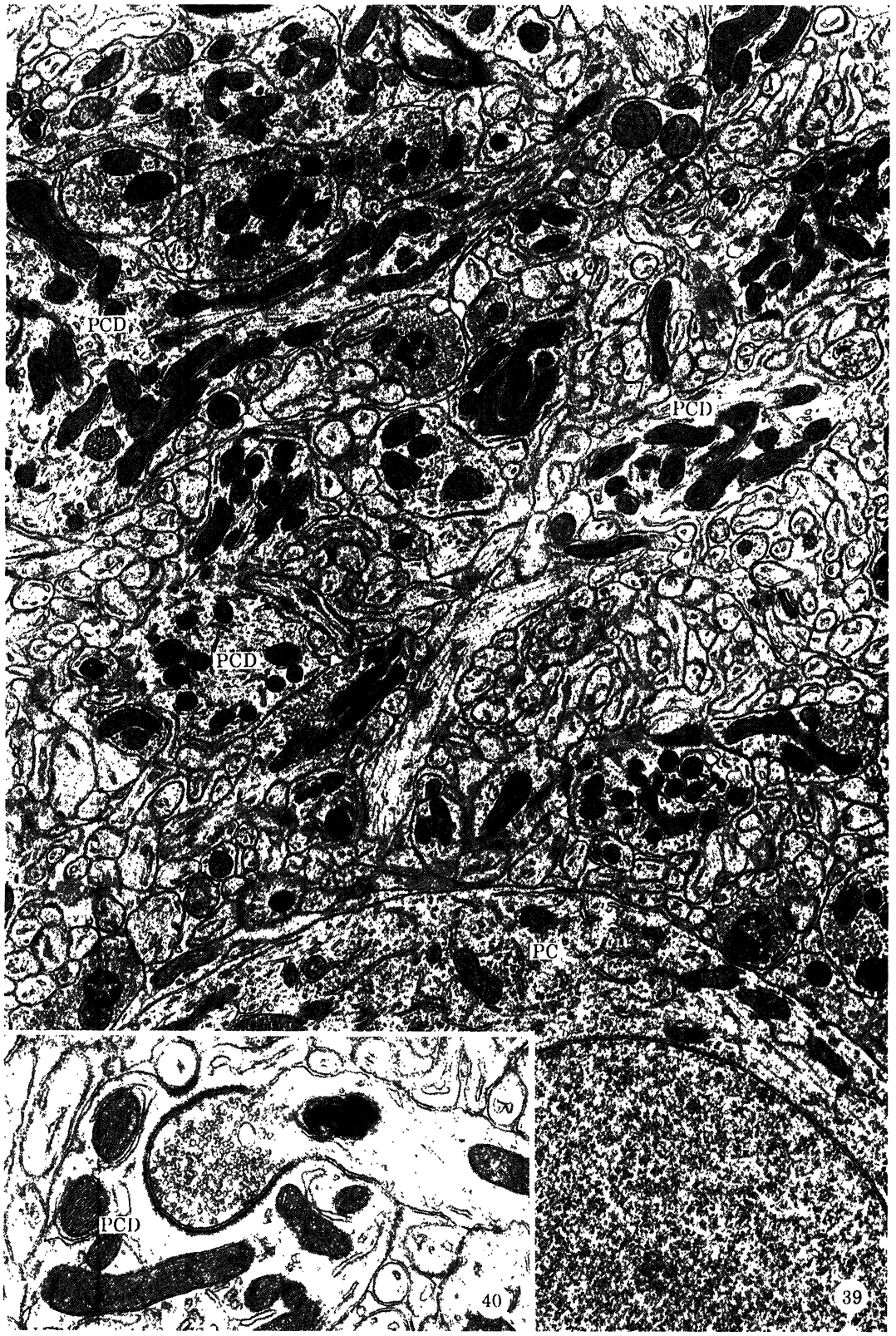
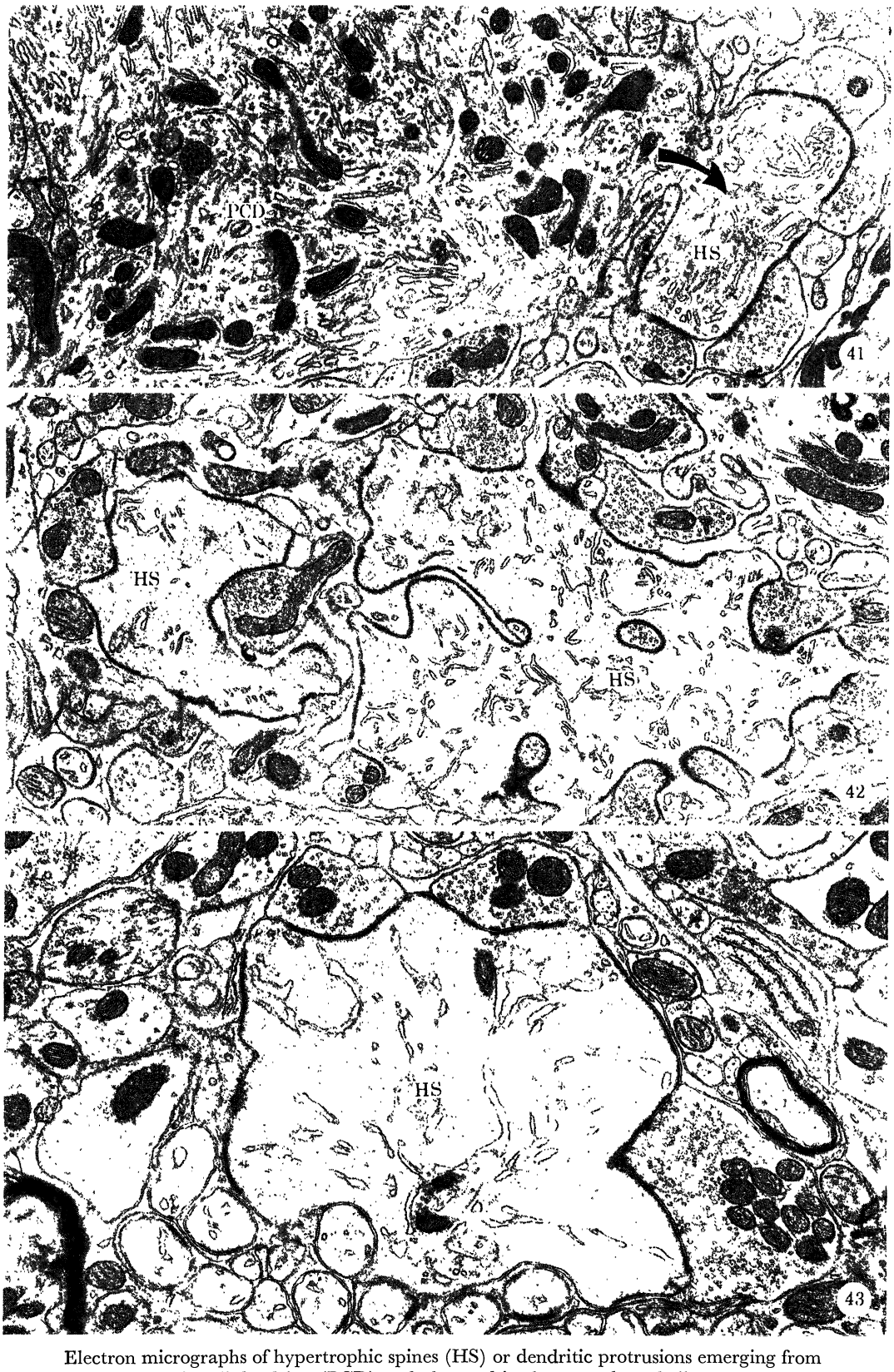


FIGURE 39. Purkinje cell perikaryon (PC) and surrounding neuropil in the central cerebellar mass. This neuropil is almost exclusively formed by Purkinje cell dendritic profiles (PCD) and the innumerables free spines emerging from them. Three climbing fibre varicosities (black circles with transparent stars) synapse on some of these spines. (Magn. × 12000.)

FIGURE 40. Purkinje cell dendrite (PCD) invaginated by an unidentified profile filled with spherical synaptic vesicles. The dendritic membrane invaginated by this profile exhibits a postsynaptic-like differentiation. The unidentified profile does not develop a 'presynaptic vesicular grid'. (Magn. × 23000.)



Electron micrographs of hypertrophic spines (HS) or dendritic protrusions emerging from Purkinje cell dendrites (PCD) and observed in the central cerebellar mass.

FIGURE 41. The neck of the hypertrophic spine is visible (arrow). Almost all the cytoplasmic surface of this profile is undercoated by a postsynaptic-like differentiation, which receives numerous synaptic boutons. (Magn. × 14000.)

FIGURE 42. Note the undercoating of the membrane, the fine granular material of the cytoplasmic matrix and the large amount of smooth cisterns of the endoplasmic reticulum, which characterize dendritic spines. (Magn. × 15000.)

FIGURE 43. In this protrusion, in addition to the organelles present on spines, there are some rare microtubules and mitochondria. (Magn. × 19000.)

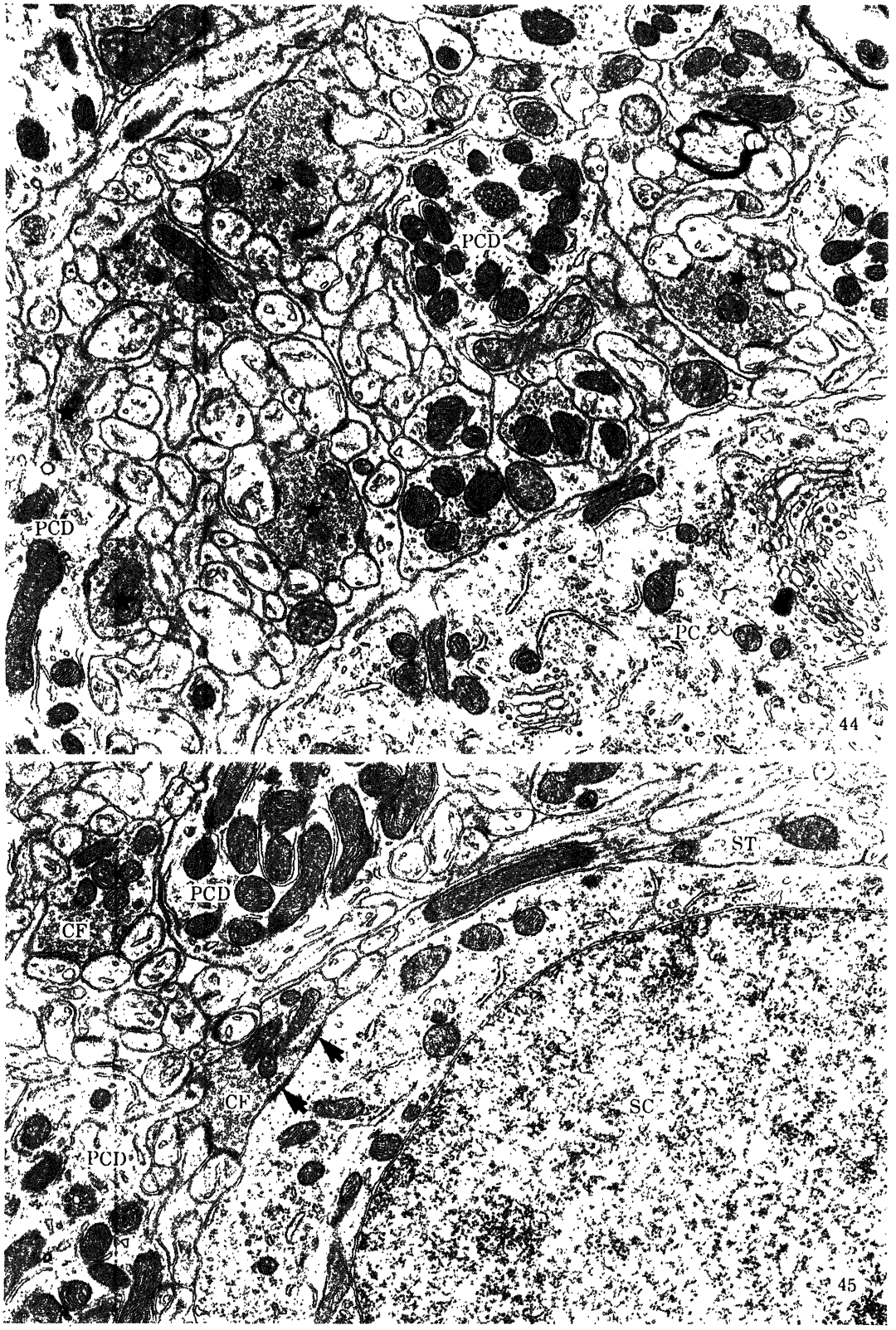
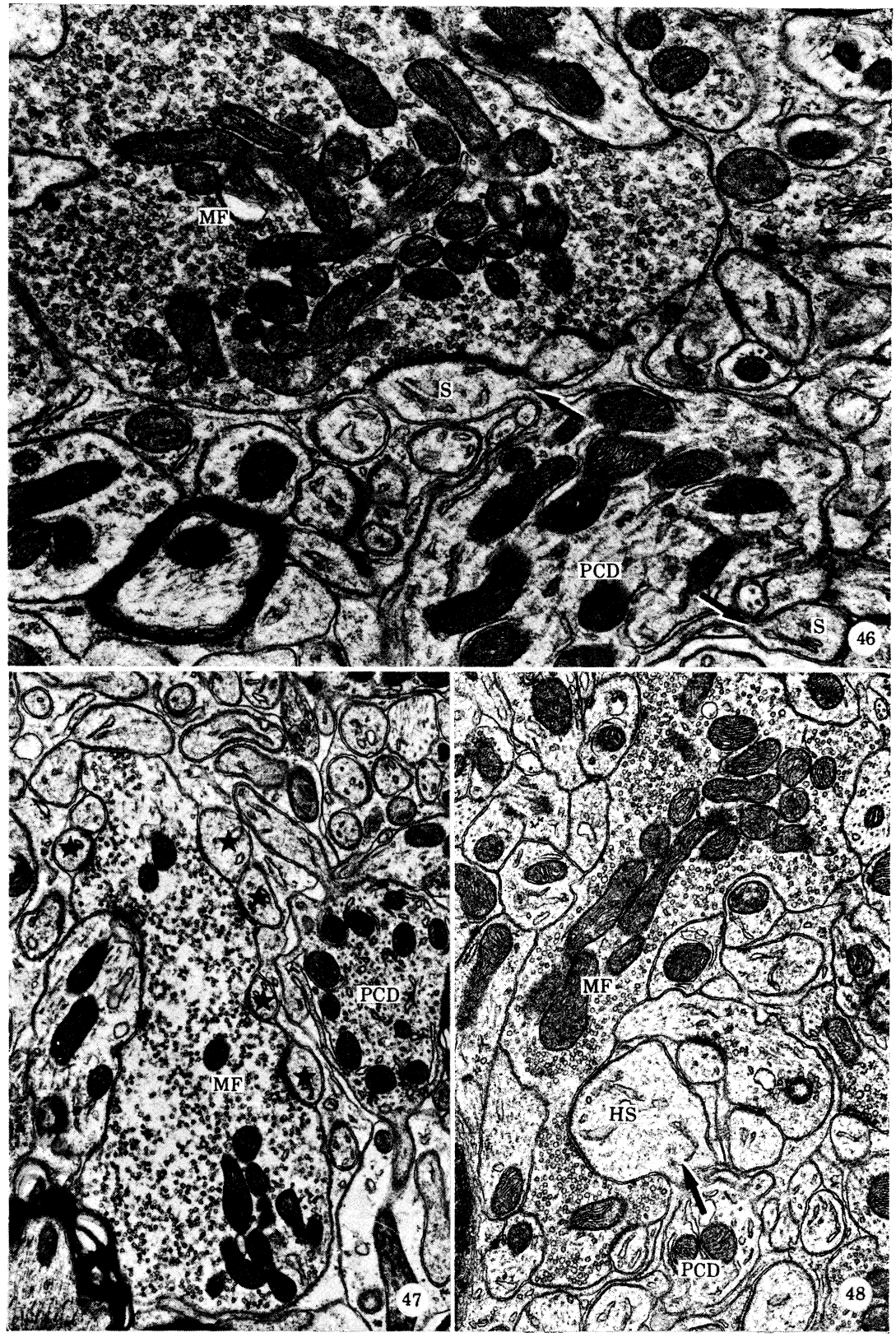


FIGURE 44. Increased density of climbing fibre varicosities (black stars) in the neuropil of the central cerebellar mass. A Purkinje cell perikaryon (PC) with its typical subsurface cistern and associated mitochondrion, occupies the lower right corner of the micrograph. Purkinje cell dendrites (PCD) and numerous free spines characterize this neuropil. (Magn. × 16000.)

FIGURE 45. Central cerebellar mass. A perikaryon of a stellate-like neuron (SC) receives a stellate axon terminal (ST) and a climbing varicosity (CF). The arrows point to the postsynaptic densities of this Scheibel's collateral. Purkinje cell dendrites (PCD) and their free spines form the neighbouring neuropil. (Magn. × 16000.)



Heterologous synapses between mossy fibres (MF) and Purkinje cell dendritic spines in the central cerebellar mass.

FIGURE 46. A Purkinje cell dendrite (PCD) gives rise to two spines (S) within the plane of the section (arrows). One of them is facing a mossy terminal (MF), which has developed a 'presynaptic vesicular grid' at the zone of contact. The other one (right low corner), although facing an axon terminal, is free of innervation. (Magn. × 25000.)

FIGURE 47. A mossy terminal (MF) synapsing on five spines (black stars), probably arising from nearby Purkinje cell dendrites (PCD). (Magn. × 16000.)

FIGURE 48. A mossy terminal (MF) establishes a synaptic contact on a hypertrophic spine (HS) arising from (arrow) a small Purkinje cell dendritic profile (PCD). (Magn. × 20000.)

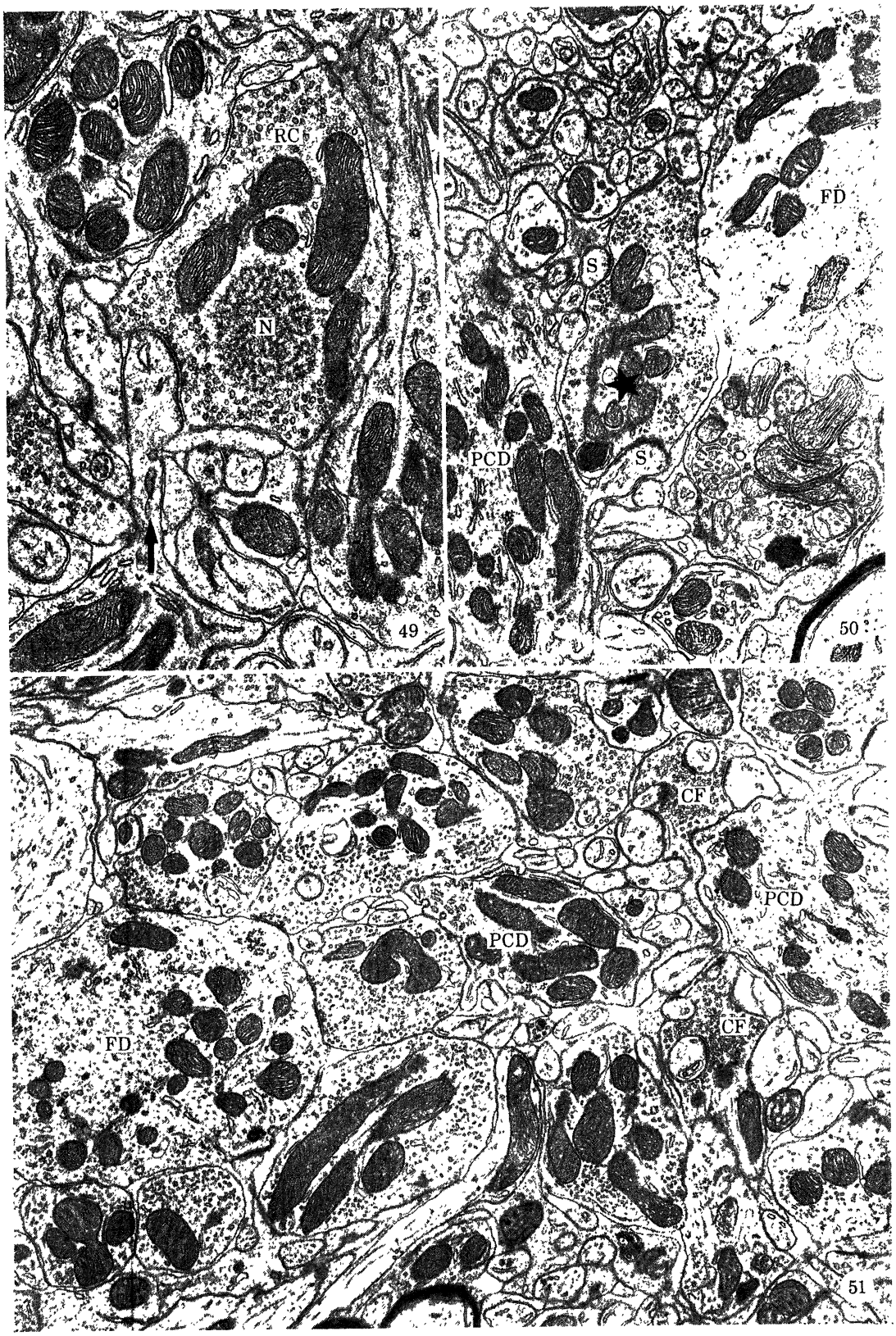


FIGURE 49. Recurrent collateral of a Purkinje cell axon (RC) synapsing on a spine arising from (arrow) a Purkinje cell dendrite. Note the presence of a nematosome (N) in the axoplasm of the terminal. (Magn. × 23 000.)

FIGURE 50. Purkinje cell dendrite (PCD), within the deep cerebellar nuclei, in close relation with a dendrite from a fastigial neuron (FD). An altered axon terminal probably belonging to a Purkinje axon is in synaptic contact with the fastigial neuron. A normal terminal containing pleomorphic synaptic vesicles (black star) is directly apposed to the Purkinje cell dendrite, and two of its spines (S), as well as to the fastigial neuron. (Magn. × 14 000.)

FIGURE 51. Purkinje cell dendrites (PCD) and their normal synaptic investment, climbing fibres (CF) are intermingled with neuronal profiles characterizing the fastigial nucleus. This electron-micrograph illustrates the invasion of disorientated Purkinje cell dendrites through the fastigial nucleus. (Magn. × 13 000.)

Sidman 1973; Sotelo 1975*a*). Indeed, the cellular environment in which these Purkinje neurons develop is as in *weaver* devoid of granular cells. Extremes of the continuum of shapes are illustrated in figures 8 and 12, plate 3. In one instance, a single main dendritic branch emerges from the apical pole; this dendritic segment crosses the white matter and branches *en parapluie* within the granular layer (figure 8, plate 3), keeping the polarity of normal Purkinje cells. On the other hand, some Purkinje cells appear as multipolar neurons giving off 5–6 primary dendritic trunks, which rarely branch (figure 12, plate 3). In these two extremes, as well as in the variety of intermediate shapes, the main characteristics of Purkinje cells in agranular cerebella (see ref. in Sotelo 1975*a*) are observed: spiny branchlets are almost undeveloped but the primary and secondary dendritic branches thicken and exhibit an irregular rough surface due to the presence of numerous spines.

At the ultrastructural level, Purkinje cell perikarya contain all their normal organelles and exhibit a smooth contour (figure 36, plate 11) although occasionally they can give off long, immature-like, spines. The distance separating two neighbouring Purkinje cell perikarya can be as short as 200 A; this is the case when both of them are in direct apposition (figure 36, plate 11). Specialized contacts between the apposed plasma membranes however were not observed.

(*b* 4) *Purkinje cell dendrites penetrating the deep cerebellar nuclei*

A clear-cut border exists between the central regions containing the Purkinje cell perikarya or the cell bodies of the deep cerebellar nuclei. Purkinje cells in the vicinity of the latter sometimes send the whole or a part of their dendritic trees to the nuclear territory. In Golgi-impregnated material, the disorientated Purkinje cell dendrites which invade, for instance, the fastigial nucleus, are similar to those remaining in the central agranular region. They are rather thick dendritic segments studded with spines.

(*c*) *The synaptic investment of the Purkinje cell at its four different positions*

Electron microscopic studies of the Purkinje cells at their various locations in *rl/rl* cerebellum reveal that different synaptic investments follow the observed diversity of shape.

(*c* 1) *Purkinje cells at their normal position in the superficial cortex*

The synaptic investment of Purkinje cells located at the Purkinje cell layer in *rl/rl* cerebellum is similar to that of normal Purkinje cells. Climbing fibre varicosities establish their typical synapses on the stubby spines of the large dendrites (figure 22, plate 6). Ascending collaterals of the basket axons, as well as axon terminals belonging to stellate cells, synapse mainly on the smooth surface of these large dendrites (figures 21, 22, plate 6). Some of the thick dendritic profiles give rise to spines which can be contacted by climbing varicosities and stellate axon terminals, but also by parallel fibres (figure 23, plate 6). These dendrites probably belong to Purkinje cells whose perikarya are located within the granular layer. The large majority of the spines emerging from the spiny branchlets are contacted by parallel fibres (figure 21, plate 6). Spines similar to those normally arising from spiny branchlets and located in the deepermost region of the molecular layer are sometimes postsynaptic to granule cells bodies (figure 19, plate 5). Such 'ectopic' somato-spine synapses are only rarely observed.

In the neurofibrillary silver stained material, each Purkinje cell at the Purkinje cell layer is surrounded by a basket formation (figure 13, plate 4). The ultrastructural study confirms the

existence of basket fibres and reveals that there is an hypertrophy of basket profiles in the neighbourhood of some Purkinje cell perikarya. Thus in some instances, two to three layers of basket fibre profiles enwrap a Purkinje cell perikaryon. The descending collaterals of the basket axons form the pinceau surrounding the initial segment of the Purkinje cells axon (figure 24, plate 7). As in normal mice, the outermost zone of the pinceau is composed of large axonal profiles organized in almost parallel arrays and being directly connected with the slender pericellular basket axons. This palisade of the basket axons forms the boundary of the pinceau region. The inner zone of this complex synapse is formed by coiled thin basket branches and by small finger-like processes originating from the former. Clusters of synaptic vesicles are suspended in the axoplasm of the large majority of these small axonal profiles (figure 24, plate 7). Astroglial processes are intermingled with this broad vortex-like axonal arrangement. Some of these processes can partially enwrap the initial segment of the Purkinje axon.

(*c* 2) *Purkinje cells in the granular layer of the superficial cortex*

A few significant differences have been observed in the synaptic investment of this category of Purkinje cell when compared with the normally positioned Purkinje cells. The granular layer itself shows all its usual elements, granule cells, Golgi cells, mossy fibres, and their common synaptic arrangement, the cerebellar glomeruli. The large majority of the glomeruli correspond to the simple type (figure 27, plate 8), but occasionally, they resemble those described in the *weaver* cerebellum (Rakic & Sidman 1973; Sotelo 1975*a*) because the mossy rosette is only synapsing on Golgi cell profiles (figure 28, plate 8).

These Purkinje cell perikarya exhibit a synaptic investment similar to that of normally located Purkinje cells. It is mainly formed by basket axon terminals (figure 25, plate 7) which belong to basket cells located in the molecular layer. In fact, in reduced silver (figure 13, plate 4) as well as in Golgi preparations (figures 14, 15, plate 4), collaterals of the basket axons descend far down in the granular layer, and end up in atypical basket formations around the Purkinje cell perikarya displaced in this layer (figures 16, 17, plate 4). Electron microscopy discloses bundles of descending basket fibres, which pass across the granular layer within the vertical septa.

The *ascending dendrites of the Purkinje cells* occupy the large majority of the volume which forms the neuropil of the vertical septa. They are large to medium-sized dendritic segments studded with spines, and surrounded by a neuropil containing processes from neurons normally located in the molecular layer (figures 27, 29, plates 8 and 9). Four types of axons accompany these ascending dendrites. (i) Bundles of descending basket fibres from which some axons can reach the smooth surface of the Purkinje cell dendrite and/or some of their spines and establish synaptic connections with them. (ii) Recurrent collaterals of the Purkinje cell axons. These axon terminals can be identified both by their pleomorphic vesicular population and by their emergence from myelinated fibres. Occasionally, they contain unusual organelles, mainly nematosomes (Grillo 1970). These axon terminals can also synapse on the spines. (iii) Boutons *en passant* belonging to axonal varicosities present in the ascending segment of the granule cell axons. Therefore, axon terminals belonging to the same system as the parallel fibres and like those synapsing on spines (figure 27, plate 8). (iv) The most important type is constituted by the climbing fibres. Their synaptic varicosities as well as their thin non-synaptic segments (figure 29, plate 9), share all the ultrastructural features of climbing fibres present in the molecular layer. The only disclosed difference is that some of the varicosities are in synaptic

contact with more numerous postsynaptic spines than in normal cerebellum. An example of a climbing varicosity with an increased number of synaptic contacts is illustrated in figure 30, plate 9). It is also of interest that climbing varicosities and parallel fibres can innervate spines emerging from the same dendritic branch (figure 26, plate 7).

Generally, there is a clear-cut border between the neuropil of the vertical septa and that of the granular layer (figure 29, plate 9). Even at the zones where the branches of the Purkinje cell dendritic tree overlap with elements of the granular layer (figure 27, plate 8) both remain separated. It is remarkable that, in spite of this major disorder, the specificity of the connectivity is almost totally preserved. However, a careful study of these regions disclosed the presence of '*heterologous*' *synapses between mossy rosettes and spines of Purkinje cell dendrites* (figure 31, plate 9). In these instances, the mossy rosette is smaller than in the normal glomeruli, and it is only partially covered by postsynaptic elements, because a large area of its surface is directly apposed to glial processes. Within the plane of the section each of these mossy profiles synapses on 3–4 granule cell dendrites and 1–2 Purkinje cell spines. We consider only here the cases in which, in addition to a direct apposition between the mossy membrane and the spine membrane bearing the postsynaptic dense material, the mossy fibre has developed a presynaptic vesicular grid (figure 31, plate 9) easily recognizable by the presence of the triangular presynaptic dense projections. This way, the non-synaptic appositions between mossy fibres and Purkinje cell spines are disclosed, the latter being more numerous than the real synapses.

The term '*ectopic synapse*' is used here to designate a synapse established between the two normal pre- and postsynaptic partners but at a site on the surface of the neuron different from that found under normal conditions. For example, the 'parallel fibre-omnicellular system' is formed, in normal cerebellum, by the axon terminals of the granule cells axon, the parallel fibres, which establish numerous synapses *en passant* with all the other cellular types. In the granular layer of the *rl/rl* there are somato-spine, somato-dendritic and dendro-spine synapses, in which the presynaptic element can be the perikaryon (figures 32, 34, 35, plate 10) or the dendrite of a granule cell (figure 33, plate 10). The Purkinje cell spines (figures 32, 33, plate 10) generally are the postsynaptic elements of these 'ectopic' synapses. However, small and/or medium sized dendritic profiles belonging to interneurons (Golgi and basket cells) (figures 34, 35, plate 10), can also be postsynaptic to granule cell perikarya. In all these instances rounded synaptic-like vesicles are clustered in the regions in which the perikaryal and/or the dendritic membranes are differentiated to form an 'active-zone'. The morphology of these junctions closely resembles that of a Gray type I synapse (figures 32–35, plate 10).

(*c* 3) *Purkinje cells intermingled with the white matter*

As already mentioned these cells are in a rather 'abnormal' milieu which, in many respects, resemble that of the agranular 'weaver' cerebellum.

There exists a great variability in the amount of axon terminals investing different Purkinje cell perikarya, but the complex synaptic arrangement characteristic of the pinceau formation around the initial segment does not exist (figures 37, 38, plate 11). The synaptic investment of Purkinje cell perikarya is formed by boutons originated from recurrent collaterals of the Purkinje cell axon, and from axon terminals which must arise from the interneurons (stellate-like cells) present at these areas. Occasionally one of these axon terminals can diverge and establish contacts on two neighbouring Purkinje cells (figure 36, plate 11). No typical contact

with basket axon was ever found which is not unexpected since basket cells are missing from the central mass.

Similarly to what happens in the *weaver* cerebellum, the neuropil here is mainly formed by the profiles of randomly oriented Purkinje cell dendrites (figure 39, plate 12). The presence of innumerable spines devoid of innervation also characterizes these regions. *Postsynaptic-like differentiations* are frequently observed at these free spines (figures 39, 44, 45, 46, plates 12, 14, 15). Most of them resemble those arising in normal Purkinje cells from the spiny branchlets; however, the atypical forms described in *weaver* (Sotelo 1975*a*), branching and hypertrophic spines, are also present. In addition, a new type of gigantic spine or dendritic protrusion has been observed. It consists of irregular profiles, measuring up to 6 μm in diameter, which contain an electron-lucent fine granular matrix, where numerous cisterns of the smooth endoplasmic reticulum and some occasional mitochondria are suspended (figures 41, 42, 43, plate 13). Their inner surfaces are undercoated by an almost continuous band of postsynaptic-like material, and on their outer surfaces numerous axon terminals establish synaptic contacts (figures, 41 42, 43, plate 13). We have considered those structures as dendritic spines or protrusions because: (i) they originate from large Purkinje cell dendritic trunks, appearing as excrescences sometimes connected to the parent dendrite by a narrow neck (figure 41, plate 13); (ii) they contain the organelles found in normal spines; and (iii) they are postsynaptic structures.

As in *weaver* the Purkinje cell dendrites exhibit postsynaptic-like differentiations undercoating segments of variable length at the dendritic shafts. Coated vesicles, formed by a pit of the dendritic membrane bearing the postsynaptic differentiation are also frequently observed. They are the morphological counterparts of a process of membrane sequestration (Sotelo 1975*a*). To achieve this parallelism between *wv/wv* Purkinje cell dendrites and the dendrites of the *rl/rl* Purkinje cells located in the deep cerebellar mass, it is sufficient that in the latter there are also neuronal unidentified profiles which deeply invaginate the Purkinje cell dendrite at the level of its postsynaptic undercoating. The cytoplasm of the profile invaginated on the dendrite is filled with spherical synaptic-like vesicles, but a 'presynaptic vesicular grid' formation has not been observed (figure 40, plate 12).

On the dendrites of the Purkinje cells straddling the cerebellar central mass, several types of axon terminals have been identified. The most frequent ones are the *climbing fibres*. The synaptic varicosities of these fibres are widespread all over the central cerebellar mass, where they establish synapses on the Purkinje cell spines (figures 39, 44, plates 12, 14); although occasionally they can develop 'active zones' on the shaft of the dendrites and even on the perikarya of the interneurons present at this central location (figure 45, plate 14). Owing to these synaptic relations, the latter can be identified with the Scheibels' collaterals of normal cerebellum. In this agranular cerebellar region, the relative density of climbing varicosities per surface area is largely increased (figures 39, 44, plates 12, 14) compared to the density of these fibres in the granular or the molecular layers. In fact, this increase in the density of the climbing fibre varicosities seems to be one of the characteristic features of agranular cerebella (Hamori 1969; Sotelo 1975*a*) (compare figure 44, plate 14, of this paper with figure 14 in Sotelo 1975*c*).

Recurrent collaterals of the Purkinje cell axon are also frequent in this region. As they were described in the section devoted to Purkinje cells in the granular layer, these axon terminals are easily identified because, among the synaptic boutons which originate from a myelinated

fibre, they are the only ones to contain a pleomorphic vesicular population. In addition, they can exhibit different kinds of abnormal inclusions, mainly stacks of tubular membranes and/or nematosomes (figure 49, plate 16). These axon terminals can establish synaptic contacts with dendritic shafts as well as with dendritic spines (figure 49, plate 16).

Axon terminals belonging to stellate-like neurons are present in the cerebellar central mass and beside synapsing on perikarya (figure 36, plate 11) can also contact the spines and dendritic shafts of the Purkinje cells. These terminals share the ultrastructural features of stellate axon terminals of control animals. They contain pleomorphic vesicles, but their axoplasmic matrix is generally electron-lighter than that of the recurrent collaterals of Purkinje cell axons. Since neither in the Golgi-impregnated material nor in the electron microscope has it been possible, till now, to identify these cells as stellates or Golgi II cells, they are reported here as stellate-like in the general sense given by Ramōn y Cajal (1911).

The *density of mossy fibres* in this agranular region is very low, since most of the fibres belonging to this system pass through the narrow band of pure white matter up to the granular layer, where they are numerous. However, some of the mossy fibres reach the central cerebellar mass. They can be easily identified by the following criteria: (i) they constitute the largest terminal profiles observed at this location; (ii) they contain a pure population of rounded vesicles; (iii) in all instances, their axoplasm is electron-lighter than those of the climbing varicosities. Axon terminals exhibiting all these features, have been observed in synaptic contact with Purkinje cell spines (figures 46, 47, 48, plate 15). These 'heterologous' synapses are rare but unequivocally present. In figure 46, plate 15, the large mossy rosette synapses on two Purkinjee cell spines; a 'presynaptic vesicular grid' is evident on the membrane facing the spine which is in continuity with its parent dendrite. Occasionally, the mossy rosette can be synapsing on numerous Purkinje cell spines, as illustrated in figure 47, plate 15, or it can even synapse on the surface of hypertrophic spines described above (figure 48, plate 15).

(*c* 4) *Purkinje cell dendrites penetrating the deep cerebellar nuclei*

In this totally abnormal environment, dendritic profiles of Purkinje cells and dendritic profiles of cerebellar nuclear neurons can be observed, side by side, with the electron microscope (figures 50, 51, plate 16). The latter have a rich synaptic investment as it occurs in normal cerebellum (figure 51, plate 16) (Angaut & Sotelo 1973). The spines of the Purkinje cell dendrites can be postsynaptic to their own normal inputs, mainly climbing varicosities (figure 51, plate 16) or apposed to the axon terminals present in the cerebellar nuclei (figure 50, plate 16).

Results II: Electrophysiological studies of afferent pathways to Purkinje cells

The various responses of Purkinje cells were recorded extra- and intracellularly and an attempt was made to correlate these responses with the localization of Purkinje cells within the mutant cerebellum. In this first study, the Purkinje cells have been ranged in only two main classes: the superficial ones corresponding to cell normally located or intermingled with the granular cells of the cortical region, and the deep ones recorded from the central mass of Purkinje cells.

(*a*) *Extracellular recordings from Purkinje cells*

The electrical stimulation of the cerebellar white matter in the region of the fastigial nucleus (JF stimulation) was used to excite both the several afferents of the Purkinje cells (orthodromic activation) and the axon of the Purkinje cell (antidromic invasion). This fast antidromic response was considered as a criterion for the identification of Purkinje cells (see Eccles *et al.* 1966*c*).

Figures 52A and 52B illustrate the extracellular unitary responses recorded after JF stimulation, from superficially located Purkinje cells. The response typically consisted of an antidromic spike potential with a short and fixed latency of less than one ms (figure 52A) which followed repetitive stimulations up to 300/s. Two distinct orthodromic responses usually followed the antidromic spike potential.

The first one (figure 52A_1) consisted of a simple spike with a latency range of 4 to 7 ms at threshold and considered as due to the activation of the Purkinje cell via the mossy fibre-granular cell pathway (Eccles *et al.* 1966*b*). The second one (figure 52A_2, 52B_1, 52B_2) consisted of typical direct and reflex climbing fibre response (CFR) (Eccles *et al.* 1966*a*) whose latency ranges were respectively 2–3 ms and 6–8 ms at threshold. The all-or-none character

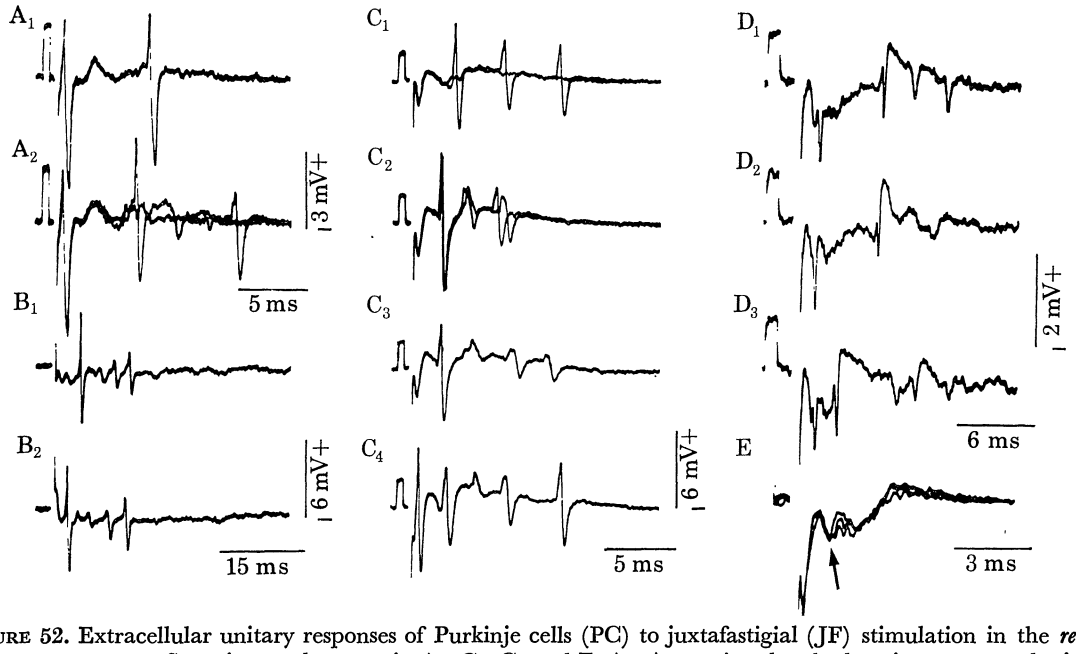


FIGURE 52. Extracellular unitary responses of Purkinje cells (PC) to juxtafastigial (JF) stimulation in the *reeler* mutant mouse. Superimposed sweeps in A_2, C_1, C_2 and E. A_1, A_2: anti and orthodromic responses obtained from a superficially located PC. In A_1 the orthodromic response consisted of one single spike. In A_2 a typical all-or-none climbing fibre response (CFR) was evoked. B_1–B_2: CFR recorded from another superficial PC. The intensity of the JF stimulus was increased between B_1 and B_2, and the latency of the CFR decreased abruptly without any change in the morphology of the response. C_1–C_4: responses of a PC located within the central cerebellar mass. In C_1 a threshold stimulus evoked an all-or-none burst of three single spikes. The stimulation strength slightly increased from C_1 to C_4, revealing the graded character of the orthodromic response (full explanation in text); in C_4 an antidromic spike was also evoked by the JF stimulus. D_1–D_3: responses of another deeply located PC. In D_1 a slightly suprathreshold stimulus evoked a short latency (1.65 ms) single spike followed by the reflex CFR. With higher stimuli (D_2 and D_3) the inactivation of partial spikes within the reflex CFR increased (D_2) and the direct CFR appeared (D_3). E: field potentials recorded at the same depth than D_1–D_3. The antidromic field is followed by a short latency negative field (arrow) timed with the single spike response.

of the direct and reflex CFR suggested that these Purkinje cells were innervated by only one climbing fibre as it normally occurs in adult mammals (Ramōn y Cajal 1911; Eccles *et al.* 1966*a*).

Neurons located in the central cerebellar mass as revealed by methyl blue deposit (at a depth from 300 to 1100 μm below the pial surface) were identified as Purkinje cells by their antidromic response (figure 52C_4). They exhibited orthodromic responses generally different from those elicited in superficial Purkinje cells. A typical example is illustrated in figure 52C: a JF stimulus at threshold evoked a high frequency burst of 3 simple spikes (figure 52C_1); when the intensity of stimulation became slightly greater, the interspike interval within the response markedly decreased and the number and the inactivation of the successive spikes of the burst increased (figure 52C_2, 52C_3); with the highest stimulation strength used the orthodromic response exhibited the typical waveform of a climbing fibre response (figure 52C_3, 52C_4). These activities resemble those of Purkinje cells recorded extracellularly in adult X-irradiated rats and interpreted as caused by a synaptic activation of Purkinje cells via several independent climbing fibre endings (Woodward *et al.* 1974; Crepel *et al.* 1976*a*). For other deeply located Purkinje cells the orthodromic responses to a JF stimulus were graded from only one simple spike at threshold to 3 or 4 spikes for the maximum response (not illustrated). This latter response also resembled a typical climbing fibre response. For these cells a contribution of the mossy fibre-granular cell pathway could not be precluded from these electro-physiological data; however, this contribution seems very unlikely on the basis of the morphological organization of the central mass of Purkinje cells where granule cells are very few or even absent (see morphological section of this paper).

Finally a small number of the deep Purkinje cells tested (4 out of a total of 26) exhibited two distinct orthodromic responses (figure 52D): a simple spike response with a short latency of 1.6–1.8 ms at threshold, followed by the direct and reflex CFR previously described. This early orthodromic response was timed with a short latency negative field potential following the antidromic one (figure 52D_4). The short latency simple spike response looked like responses attributed to a direct impingement of mossy fibre onto Purkinje cells in agranular cerebella of virally infected ferrets (Llinas *et al.* 1973) or X-irradiated rats (Crepel *et al.* 1976*a*).

(*b*) *Intracellular recordings from Purkinje cells*

Eighty-one Purkinje cells were impaled and their responses to the climbing fibres by stimulation of the inferior olivary nucleus were analysed. In some of the Purkinje cells tested a full spike (Eccles *et al.* 1966*a*) typical of the climbing fibre response was recorded before deterioration of the cell (figure 53A). In most of the Purkinje cells tested, the degradation of the cell led rapidly to a suppression of the spike potentials and only the underlying excitatory postsynaptic potentials (e.p.s.ps) caused by activation by the climbing fibres were recorded during several minutes. Their shape was similar to that obtained in other mammals under the same experimental conditions (Eccles *et al.* 1966*a*; Martinez *et al.* 1971). Furthermore they occurred at exactly the same latency as the climbing fibre response recorded before deterioration of the cell.

For 29 Purkinje cells among the 81 studied these postsynaptic potentials were typically all-or-none in character either when evoked by stimulation of the inferior olivary nucleus (figure 53B) or when they occurred spontaneously (figure 53C), thus suggesting that the Purkinje cells were innervated by only one climbing fibre; 21 of the 29 Purkinje cells were recorded

70–200 μm below the pia, i.e. at a depth corresponding to Purkinje cell somas normally located or scattered in the granular layer. The 8 other all-or-none excitatory postsynaptic potentials were recorded from Purkinje cells clearly located in the central mass.

For the 52 remaining Purkinje cells out of the 81, the size of the otherwise typical excitatory postsynaptic potentials mediated via climbing fibres (figure 53E) was graded by steps when smoothly increasing the intensity of stimulation in the inferior olive (2–4 steps depending on cells). From the same Purkinje cells spontaneous excitatory postsynaptic potentials mediated via climbing fibres were also recorded (figure 53E, 53F) and their size fluctuated in the same stepwise manner. These results strongly suggest that these 52 Purkinje cells are innervated by several climbing fibres like an important proportion of Purkinje cells in the young rat (Crepel *et al.* 1976*b*) and in the agranular cerebellum of X-irradiated rats (Woodward *et al.* 1974; Crepel *et al.* 1976*a*) and of weaver mutant mice (Crepel & Mariani 1976).

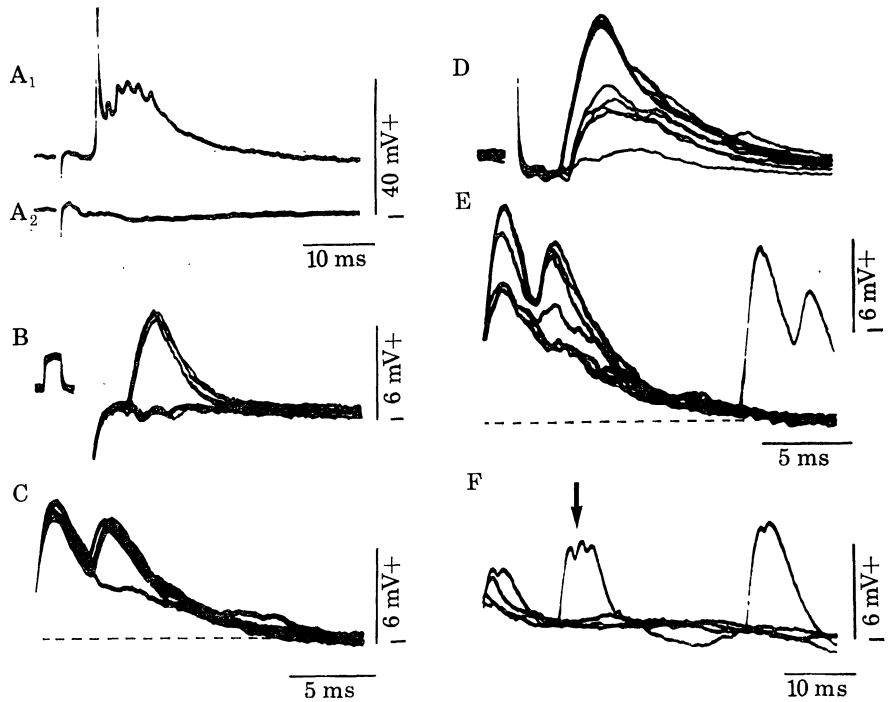


FIGURE 53. Intracellular responses of Purkinje cells (PC) to climbing fibres (CF) inputs in the *reeler* mouse. Superimposed sweeps in all traces except A_1. A_1: climbing fibre response (CFR) evoked by inferior olive (IO) stimulation and recorded before deterioration of the cell. A_2: extracellular field after withdrawal of the microelectrode. B: typical all-or-none e.p.s.p. mediated via CF and evoked by JF stimulation in a superficial PC. C: spontaneous all-or-none e.p.s.ps due to CF input in another superficial PC. D: e.p.s.p. evoked via CF in a deeply located PC. It was graded in a stepwise fashion by smoothly increasing the IO stimulation. E, F: spontaneous e.p.s.ps mediated via CF in two different PCs, located within the central cerebellar mass. The amplitude of the e.p.s.ps fluctuated in a stepwise manner. In F recorded at a lower sweep speed, the successive e.p.s.ps exhibited at least three different sizes, but one of these e.p.s.p. (arrow) just followed a previous one. In C, E, F, each sweep was triggered by the rising edge of the response.

Among these 52 Purkinje cells, 48 were located in the central mass and only 4 were located within the granular layer (two at 200 μm and two at 150 μm). This distribution with depth is summarized in table 1. It can be noticed that no differences appeared between the two strains of mice used in the present study.

In conclusion, the most significant difference noticed between the two categories of Purkinje cell lies in the climbing fibre response. The response of the superficial ones, like in the normal cerebellum, is typical of a one-to-one relation between climbing fibre and Purkinje cell; while in the deep ones the observed response shows signs of a multiple innervation by several climbing fibres. Our data also suggest that the heterologous synapses between mossy fibres endings and Purkinje cells disclosed in the central mass (see Results I) are functional like those formed in typical agranular cerebella (Llinas, Hillman & Precht 1973; Crepel *et al.* 1976*a*).

TABLE 1. REPARTITION OF THE EXCITATORY POSTSYNAPTIC POTENTIALS (e.p.s.p.) MEDIATED VIA CLIMBING FIBRES (CF) AND RECORDED FROM SUPERFICIALLY AND DEEPLY LOCATED PURKINJE CELLS IN THE TWO STRAINS OF HOMOZYGOUS *REELER* (*rl/rl*) MICE

		70–200 μm		300–1100 μm	
depth below the pia		C57Bl/6	Balb/C	C57Bl/6	Balb/C
e.p.s.ps mediated via CF	all-or-none	8	13	3	5
	graded by steps	1	3	19	29

RESULTS III: QUANTITATIVE CHANGES IN THE CELL POPULATION REVEALED BY BIOCHEMICAL STUDIES

No attempt was made to develop biochemical studies on single neurons or even on dissected cerebellum layers despite the fact that this last technique has yielded useful information with the normal cerebellum (Mikoshiba & Changeux, in preparation). The global measurements made on the whole cerebellum may, however, monitor quantitative changes in the population of cells and thereby complement the morphological and electrophysiological approach. Thanks to gel electrophoresis in sodium dodecyl sulphate, variations in the content of proteins characteristic of a given cell type (for instance the P_{400} protein (Mallet *et al.* 1976)) may even be revealed and assigned to a modification of a particular category of cell.

(*a*) *DNA, RNA and protein content of* rl/rl *cerebellum*

All determinations were carried out on homozygous *reeler* mice from both C57Bl and Balb/C genetic background and little, if any, difference was noticed between normal individuals from these two inbred strains. Table 2 shows that in the *rl/rl* cerebella the total wet mass and protein content decrease to almost the same value: approx. $\frac{1}{3}$–$\frac{1}{4}$ that of the normal cerebellum. In *rl/rl* cerebellum the deficit in the total content of DNA is even more dramatic: it falls by a factor of 7–8, the effect being slightly more pronounced on the C57Bl/6J than on the Balb/C genetic background. As a consequence, and in agreement with previous findings on the *weaver* and *staggerer* cerebella (Mallet *et al.* 1976), the DNA to protein ratio varies as a consequence of the *reeler* mutation. It ranges from 27–31 μg/mg in 20-day old *rl/rl* to 47–48 μg/mg in 26-day old ones while in the wild type the corresponding values are 65–73 and 61–64 μg/mg respectively. Such a low DNA:protein ratio cannot be accounted for by an immaturity of the mutant cerebellum since in the new born it is higher than in the adult.

The variation in the total content of RNA does not follow that of DNA: in the homozygous *reeler* of 20–26 days it is about 3 times smaller than in the wild type, i.e. about the same as the decrease in the total protein content. As a consequence, the RNA to protein or the RNA to wet weight ratio remains about the same as in the wild type.

TABLE 2. WET MASSES, PROTEIN, DNA AND RNA CONTENT OF THE CEREBELLA FROM HOMOZYGOUS *REELER* MUTANT AND WILD TYPE MICE FROM DIFFERENT STRAINS: C57Bl AND Balb/C

(Assays and fractionation methods for DNA, RNA and protein are given in Methods.)

	strain			wet mass (mg)	protein (mg/ cere-bellum)	DNA (μg/ cere-bellum)	DNA/ wet mass (μg/mg)	DNA/ protein (μg/mg)	RNA (μg/ cere-bellum)	RNA/ wet mass (μg/mg)	RNA/ protein (μg/mg)	DNA/ RNA (μg/mg)
1	C57Bl/C	(*rl/rl*)	20 days	14.75	1.29	37.91	2.57	29.39	81.13	5.50	62.89	0.467
2		(*rl/rl*)	20	12.70	1.16	31.31	2.47	26.99	52.96	4.17	45.66	0.592
3		(*rl/rl*)	20	12.70	1.21	37.02	2.92	30.60	85.47	6.73	70.64	0.433
1	C57Bl/C	(+/+)	20 days	37.50	3.80	276.38	7.37	72.73	204.38	5.45	53.78	1.351
2		(+/+)	20	41.65	4.28	311.96	7.49	72.89	185.34	4.45	43.30	1.695
3		(+/+)	20	35.70	3.78	262.40	7.35	69.42	175.29	4.91	46.37	1.493
4		(+/+)	20	41.55	4.24	273.81	6.59	64.58	192.79	4.64	45.47	1.429
1	Balb/C	(*rl/rl*)	22 days	21.15	1.98	64.30	3.04	32.47	79.31	3.75	40.06	0.813
2	Balb/C	(+/+)	22	53.30	5.29	316.34	6.03	59.80	258.51	4.85	48.87	1.220
1	Balb/C	(*rl/rl*)	26 days	18.10	1.65	78.74	4.35	47.72	93.43	5.05	56.62	0.840
2		(*rl/rl*)	26	14.05	1.28	59.71	4.25	46.65	60.21	4.24	47.04	0.990
1	Balb/C	(+/+)	26 days	52.65	5.29	321.96	6.59	60.86	231.66	4.40	43.79	1.389
2		(+/+)		47.00	4.41	274.95	5.85	62.35	205.39	4.37	46.57	1.333
3		(+/+)		46.10	4.62	297.35	6.45	64.36	199.15	4.32	43.11	1.493

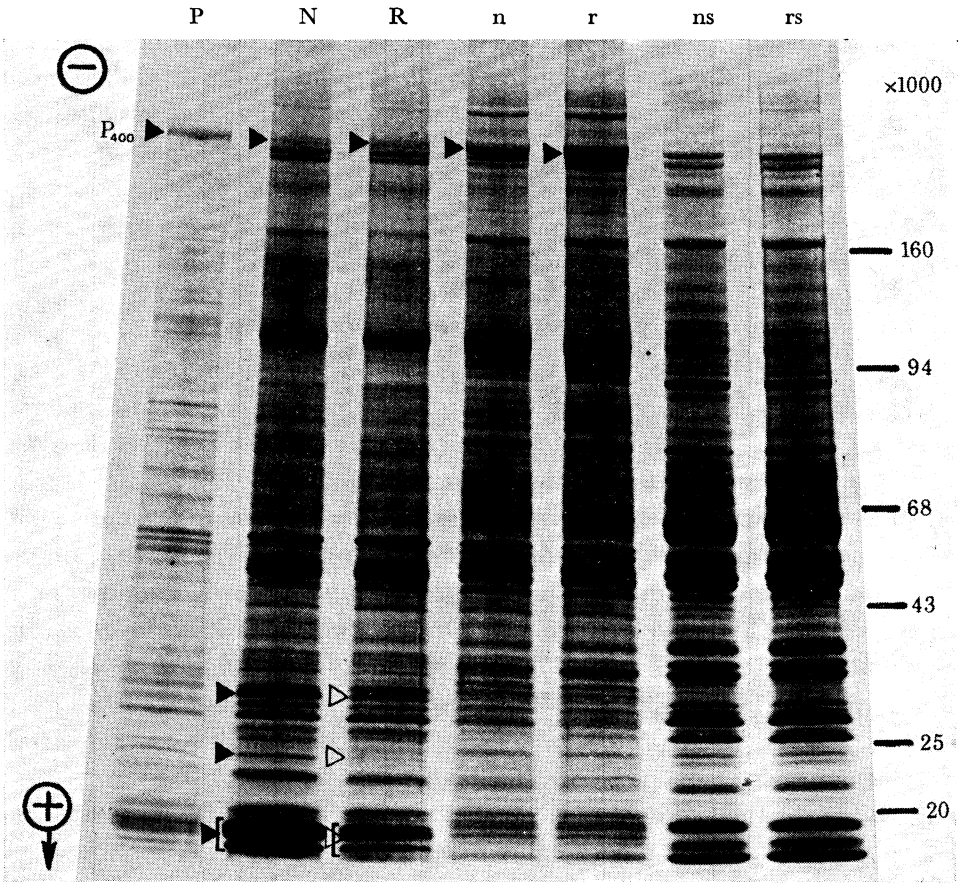


FIGURE 54. Protein patterns obtained by polyacrylamide gel electrophoresis in sodium dodecyl sulphate of fractions from cerebella of homozygous *reeler* mutant and wild type mice. P: Purkinje cells isolated from normal rat according to the method of Sellinger, Legrand, Clos & Ohlsson (1974). R: l.s.p. fractions from homozygous *reeler* mutant; N: l.s.p. fractions from heterozygous wild mice; r: h.s.p. fractions from homozygous *reeler* mutant; n: l.s.p. fractions from heterozygous wild mice; rs: supernatant of the cerebella of homozygous *reeler* mutant; ns: supernatant of the cerebella of heterozygous mice. 'Molecular mass of protein' markers are given on the right of the figure. They are phosphorylase a (molec. mass 94000), phosphorylase b (molec. mass 94000), bovine serum albumin (molec. mass 68000), ovalbumin (molec. mass 43000), chymotrypsinogen (molec. mass 25000), Kunitz trypsin inhibitor (molec. mass 20100).

(*b*) *Protein pattern of the* rl/rl *cerebellum*

The same fractions as those used in a previous study on *weaver* and *staggerer* mutant mice (Mallet *et al.* 1976) were submitted to electrophoresis in a linear 5–10 % polyacrylamide gel in the presence of sodium dodecyl sulphate. The 'low speed pellet' (l.s.p.) contains mostly nuclei, mitochondria and various cell debris; the 'high speed pellet' (h.s.p.) is rich in microsomes and in the final supernatant (s) remain the soluble cytoplasmic proteins (see Methods). In figures 54–57 we compare the patterns given by these fractions in homozygous *reeler* and wild type cerebella. No difference was observed with the supernatants which give qualitatively and quantitatively identical electrophoregrams. On the other hand, in the l.s.p. the four bands of low apparent molec. mass (from 30000 to lower values), identified in a previous work (Mallet *et al.* 1976) as the histone bands, are relatively reduced. Such a reduction which confirms previous findings was expected from the decrease of the DNA to protein ratio found in the mutant cerebellum. Another still unidentified band in the 20000 molec. mass range is also missing in the *rl/rl* cerebellum. On the other hand, the P_{400} protein, characteristic of the Purkinje cells (Mallet *et al.* 1976), is abundant in both the low and high speed pellets. A close comparison of the densitometric scans recorded with normal and mutant l.s.p. even show that, after normalization of the majority of the protein bands to identical values in the two scans, a slight enrichment of the P_{400} protein exists in the *reeler* extract. A similar observation was also previously reported with the *weaver* cerebellum (Mallet *et al.* 1975).

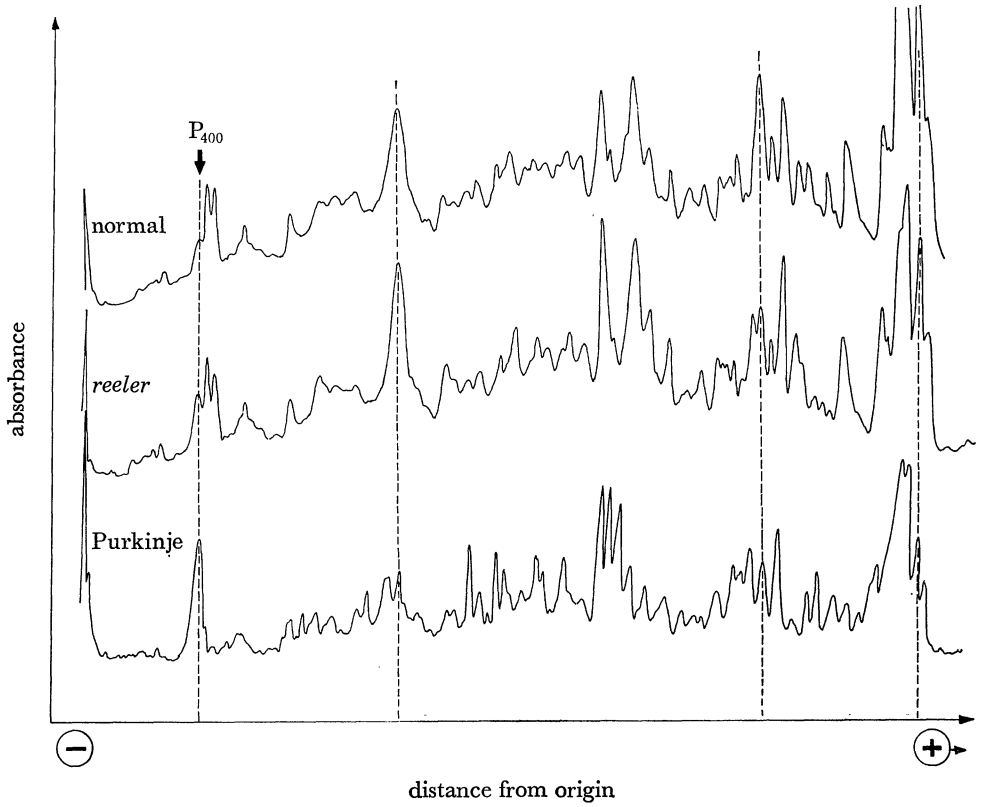


FIGURE 55. Densitometric scans of the electrophoretograms of the low speed pellets (l.s.p.) from cerebella of homozygous *reeler* mutant and wild type mice (same gel as in figure 54). The method from preparation of low speed pellets is given in Methods.

In conclusion, the gross biochemical alterations noticed with *reeler* cerebellum: marked deficit in total DNA content, decrease of the DNA to protein ratio, relative enrichment in P_{400} protein, are in many respects similar to those found with the agranular *weaver* cerebellum. They may equally reflect a significant loss in the total number of granular cells.

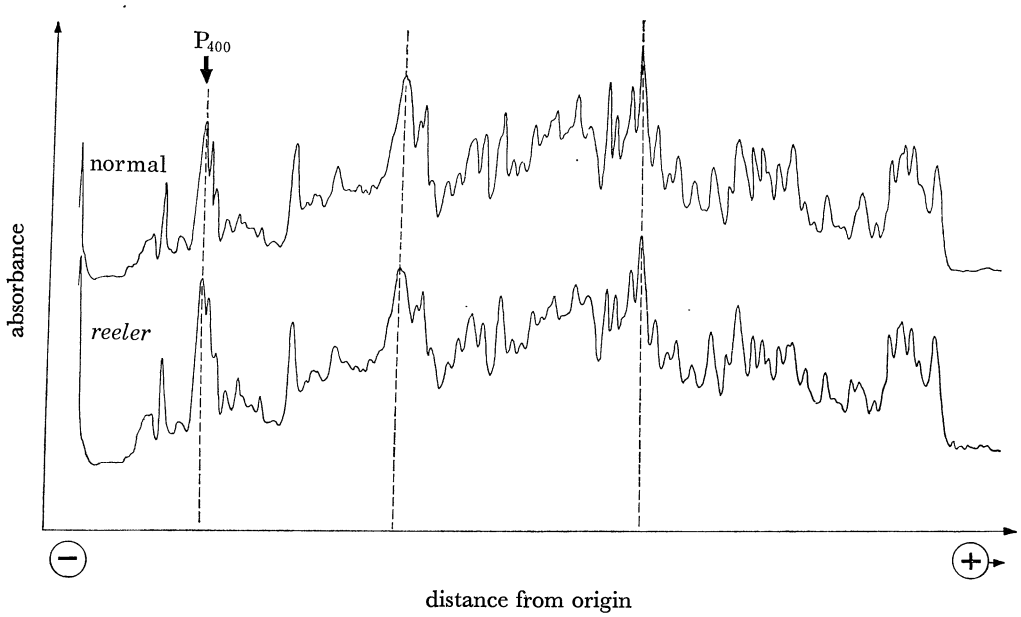


FIGURE 56. Densitometric scans of the electrophoretograms of the high speed pellets (h.s.p.) from cerebella of homozygous *reeler* mutant and wild type mice (same gel as in figure 54). The method for preparation of high speed pellets is given in Methods.

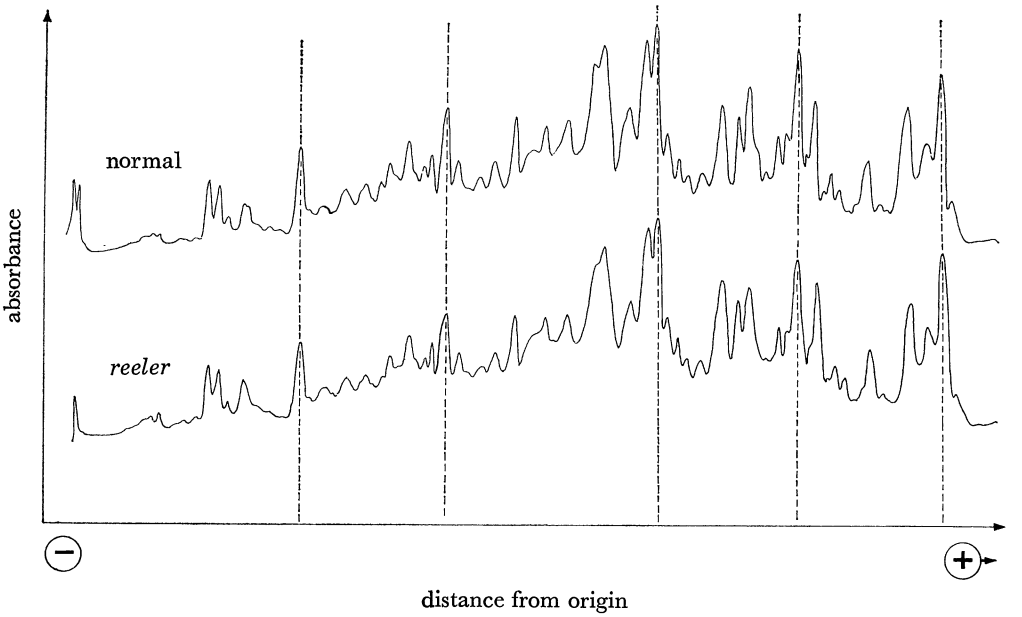


FIGURE 57. Densitometric scans of the electrophoretograms of supernatants (s) from cerebella of homozygous *reeler* mutant and wild type mice (same gel as in figure 54). The supernatant was obtained by centrifugation for 75 min at 30000 rev min^{-1} in a RP65 Beckman Rotor (see Methods).

Discussion

The *reeler* mouse is of particular interest since, as recognized by Sidman (references in Sidman 1974), its cerebellar and cerebral cortex exhibit major defects in cell alignment and orientation but nevertheless, preserve all the major classes of synapses found in normal animals. The present findings bring additional support to this conclusion and further reveal that the shape and synaptic investment of a given category of neuron may significantly vary with position and orientation within the cerebellum; also it is shown that special kinds of synaptic contacts, which are absent in the normal adult are found in the mutant mouse. The analysis of the '*reeler*' cerebellum, therefore, brings an insight to the mechanisms by which the cellular environment of a neuron may affect its development and that of its afferent synapses.

One of the most obvious effects of the *reeler* mutation in homozygous animals is a marked decrease in size of the cerebellum accompanied by a reduction of the number of fissures. The dry mass is 3–4 times smaller than in normal animals and the content in DNA falls by a factor of 7 or 8. A dramatic decrease in cell number accompanies this reduction of size. In order to investigate if the observed cellular hypoplasia affects equally well the diverse categories of cerebellar neurons we took advantages of former biochemical studies done with other cerebellar mutants (Mallet *et al.* 1974, 1975, 1976) and with the *reeler* mutant mouse (Beckingham Smith 1976). It was shown in particular that a protein of *apparent* molecular mass 400000, the P_{400} protein, is abundant in isolated Purkinje cell perikarya but markedly reduced or absent in the cerebella from two mutants, *staggerer* and *nervous*, which exhibit a marked alteration or a deficit in the Purkinje neurons. On the other hand, the almost completely agranular cerebellum of the homozygous *weaver* mouse contains relatively higher levels of a P_{400} protein than normal cerebellum. Interestingly, the protein pattern given by homozygous *reeler* cerebella resembles those observed with the homozygous *weaver* mouse. In other words the marked deficit in cell number noticed in *rl/rl* cerebellum primarily affects the granular rather than the Purkinje cell. A qualitative inspection of the histology of *reeler* cerebellum indeed confirms that a marked hypoplasia of the granular neurons take place.

Still the main disturbance affecting the *rl/rl* cerebellum is a severe disalignment of the Purkinje cells (Sidman 1972; Rakic 1976). The distribution of those neurons, however, does not look random but rather reveals an *emboîtement* of a deep mass of Purkinje cells by a quasi-normal cerebellar cortex. In addition, Purkinje cells are seen embedded within the granular layer, or intermingled within the white matter, as if they were stopped in their migration towards the superficial cortex. The primary cellular defect caused by the *reeler* mutation is not known. At least two, equally plausible, possibilities may have to be considered. Following Yoon's (1976) suggestion in the case of the *staggerer* mouse, the *reeler* mutation has a pleiotropic effect altering simultaneously and independently both the proliferation of the granular cells and the position of the Purkinje cells. An alternative hypothesis is that the *reeler* mutation only affects the position of the Purkinje cells the hypoplasia of granular cells being secondary to the malposition of the Purkinje cell-type and, as in *staggerer*, resulting from a transynaptic degeneration (Sotelo & Changeux 1974; Sotelo 1975*b*). (As proposed by Mallet *et al.* 1976, the proliferation of granular cell might be regulated by a factor emitted by the Purkinje cell dendrites, which would be present in smaller concentration in the peripheral cortex of *reeler* cerebellum.) In any case, it is clear that the *reeler* mutation affects the position of the Purkinje cells (Sidman 1968; Rakic 1976; Yoon 1976). Their abnormalities in shape and synaptic

investment, which are also found in entirely different mutant cerebella (e.g. *weaver*) and their phenocopies (e.g. X-irradiated rats), have therefore to be related to the cellular environment in which they develop rather than to a direct effect of the mutation. The *reeler* cerebellum offers a 'natural' experimental situation to analyse to what extent local interactions are required to achieve the three-dimensional unique arrangement of the Purkinje cell and its synaptic investment.

Hypothesis have been proposed to explain the orderly growth of the dendritic tree of the Purkinje cell (Herndon & Oster-Granite 1975; Woodward *et al.* 1975; Altman 1976; Berry & Bradley 1976). It seems clear, however, that an intrinsic mechanism allows the Purkinje cell dendrites to grow autonomously. Indeed, even *in vitro*, almost totally deafferented Purkinje cells (Privat & Drian 1974) grow dendrites although these are randomly oriented. It is also clear that the presence of all the normal inputs to the Purkinje cell is necessary for the achievement of the planar disposition and the normal branching pattern of their dendrites (Altman 1976; Bradley & Berry 1976; Sotelo & Arsenio-Nunes 1976).

Cajal in 1929 already suggested that parallel fibres are the main organizing element for the planar distribution of the Purkinje cell dendrites. Support for this interpretation has recently been given by Altman (1973) with rat cerebella submitted to early postnatal irradiation. In these cerebella the regeneration of the external granular layer is followed by a misorientation of some bundles of parallel fibres and the dendritic tree of the Purkinje cells is always oriented perpendicular to the parallel fibres. Such orthogonal orientation allows the formation of a maximum of synaptic contacts between parallel fibres and Purkinje cell dendrites (Herndon & Oster-Granite 1975). Altman (1976) advanced the hypothesis that 'the oriented perpendicular growth of a single stem dendrite depends on the presence of basket cell axons, the outgrowth of smooth branches on the presence of stellate cell axons and the proliferation of spiny branchlets on the interaction with parallel fibres'. He does not consider, however, that the remaining input to the Purkinje cell dendrites, the climbing fibres, may also be responsible for some of the features of the normal dendritic pattern. On the other hand, Kawaguchi, Yamamoto, Mizumo & Iwahori (1975), Bradley & Berry (1976) and Sotelo & Arsenio-Nunes (1976) have shown that Purkinje cell dendrites developed in the absence of climbing fibres, have a branching pattern different from that of Purkinje cells developed under normal conditions.

The present analysis of the *reeler* cerebellum supports the view that parallel fibres are necessary for the planar disposition of the Purkinje cell dendrites and for the development of the spiny branchlets. The variation in shape of the Purkinje cells located at the central cerebellar agranular region would then mimic that described in the *weaver* cerebellum (Rakic & Sidman 1973; Sotelo 1975*a*), in which these cells have randomly oriented dendrites, devoid of spiny branchlets.

More difficult to explain is the existence of three different kinds (see Results) of Purkinje cells among those located within the granular layer. The cellular environment is essentially identical for these three morphological types and all these Purkinje cells receive the same synaptic inputs. The fact that the first class of Purkinje cell has a single main dendrite, which ascends without branching to the molecular layer, would support Altman's hypothesis that basket fibres are involved in the moulding of the Purkinje cell dendritic tree. However, the second and the third classes of Purkinje cell have also a significant basket investment and nevertheless their main dendritic stems, instead of being unbranched, give off numerous medium-sized dendritic

segments randomly distributed. Similar observations have been made with *weaver* cerebellum. In this instance, and in spite of the presence of numerous basket and stellate cells, the Purkinje cells have totally disoriented dendritic trees (Rakic & Sidman 1973; Sotelo 1975*a*).

The electrophysiological results offer clues to the identity of the various categories of functional synapses received by Purkinje cells in their different positions. In the normal adult cerebellum, the all-or-none response to climbing fibre stimulation indicates that the Purkinje cell receives only one climbing fibre. Such an all-or-none response was found with the superficial Purkinje cells located in the peripheral cortex of the *reeler* cerebellum. These cells can also be activated antidromically and via the mossy-fibre-granular cell pathway as with normal cells. It must be emphasized that no electrophysiological difference was noticed between normally located Purkinje cells and those whose somata were intermingled with granule cells. On the other hand, the Purkinje cells located in the deep cerebellar mass exhibit responses, during both extra- and intracellular recordings, which were graded by steps as a function of stimulus intensity. In this respect they resemble the Purkinje cells found in the agranular cerebella of X-irradiated rats or homozygous *weaver* mice (Woodward *et al.* 1974; Crepel *et al.* 1976*a*; Crepel & Mariani 1976). The interpretation proposed here is that these Purkinje cells receive several climbing fibres instead of only one. In agreement with this interpretation is the anatomical observation that the climbing fibre varicosities found on the deep Purkinje cells increase in density, as also occurs in the *weaver* cerebellum (Sotelo 1975*a*).

The presence in the adult state of supernumerary axon terminals at sites which, under normal conditions, receive only one of them, may be interpreted in terms of the recent theoretical suggestion that processes of selective stabilization take place during the development of neuronal networks (Changeux, Courrège & Danchin 1973; Changeux & Danchin 1974, 1976; Changeux 1972). At critical stages a limited 'redundancy' and 'fluctuation' of the connectivity would exist and the early activity of the circuits by reducing this transient redundancy would bring additional order to the system. Some of the multiple contacts made would become selectively stabilized while, in a concomitant manner, the other would regress. The development of the innervation of the Purkinje cell in rodent cerebellum may, in this respect, present analogies with that of the neuromuscular junction where the establishment of the one-to-one relation between muscle fibres and motor axons results from the regression of transient supernumerary collaterals (Redfern 1970; Bennett & Pettigrew 1974; Bagust, Lewis & Westerman 1973, Benoit & Changeux 1975; Jansen, Van Essen & Brown, 1976; Brown, Jansen & Van Essen 1976). It has been recently shown by unitary recordings that in 8–9-day old rats the response of the Purkinje cell to climbing fibre stimulation is graded by steps indicating that more than 50% of them would be innervated by at least two distinct climbing fibres (Crepel *et al.* 1976*b*). In other words, in the 'deep' Purkinje cells of *reeler* cerebellum or in those of the *weaver* mouse, as well as in those of the X-irradiated rat, the transient redundancy of climbing fibre innervation would persist even in a 226-day old adult. In all these cases, a common situation exists where the local environment of the Purkinje cell presents a marked deficit in granular cell axons. A tempting assumption would be that the transition from multiple to single innervation is due to the establishment of synaptic contacts between parallel fibres and Purkinje cell dendrites at a critical stage of development. Alternative hypothesis may have, then, to be considered: (1) the regression of the multiple innervation is due to a direct competition by 'steric hindrance' for a limited postsynaptic space (Prestige & Willshaw 1975) and therefore does not depend on the state of activity of the system; or (2) on the contrary, it is caused by a

modification of the state of activity of the Purkinje cell due to the absence of the parallel-fibre input. As a consequence the internal regulatory mechanisms (see Changeux & Danchin 1976), which may account for the selective stabilization of its afferent climbing fibres, would be perturbed. It is at present difficult to distinguish between these alternatives.

Another feature of these 'deep' Purkinje cells is that they develop numerous free spines and even long postsynaptic-like differentiations undercoating part of their dendritic shafts. They exhibit the same features as the Purkinje cells in *weaver* mouse (Sotelo 1975*a*), indicating again, that since the same picture is obtained with two non-allelic genes, these features are related to 'local' factors governing the growth and development of the Purkinje cell rather than to a direct effect of the mutation. Again, as already discussed in the case of the climbing fibres innervation, the abundant production in the adult of postsynaptic elements might be related to the lack of either an efficient occupation of space, or to the perturbation of an internal regulation of the Purkinje cell which accounts for selective stabilization of adequate post-synaptic element.

As already mentioned, the development of a synaptic connection is a rather complex process which may involve at least two major steps: an early 'recognition' between cell surfaces and the subsequent active 'stabilization' of the pre-formed labile contact (for a discussion see Changeux 1974; Changeux & Danchin 1976). The *reeler* cerebellum offers an opportunity to investigate the 'specificity' of the recognition step because in this cerebellum morphologically identified synaptic contacts may be formed either between the correct partners but at an abnormal location (ectopic synapse), or between cells which, under normal conditions, never form synaptic contacts ('heterologous' synapses). Ectopic synapses between the soma and/or the dendrites of the granule cells and the spines of Purkinje cell dendrites are found in the granular layer or lower molecular layer of the *rl/rl* cerebellum. Their occurrence may simply be accounted for by an impoverishment of granular cells since they have been already found in such a situation either in tissue cultures (Kim 1974) or, in the hemispheres of the *weaver* cerebellum (Sotelo 1975*a*). They have also been observed in ectopic granule cells located in the molecular layer of rabbit cerebellum (Spacek, Parizek & Lieberman 1973). The ectopic synapses between the dendrites of interneurons and the soma of granular cells reported here, may also be relevant to a similar interpretation.

In normal conditions, the climbing fibres occupy the spines arising from the thick dendritic segments, whereas the distal spiny branchlets synapse with the parallel fibres. Previous studies (Sotelo, Hillman, Zamora & Llinas 1975; Sotelo & Arsenio-Nunes 1976) have shown that under climbing fibre deafferentiation in adult or in immature animals, there is an induction of spine formation mainly at the thick dendrites of the Purkinje cells. Therefore it was concluded that climbing fibres exert a suppressible action on the thick dendrites. In the *reeler*, on the ectopic Purkinje cells located in the granular layer, thick dendritic trunks, which ascend towards the molecular layer and sometimes spread in this layer, can bear neighbouring spines contacted, one by a climbing varicosity, the other by a parallel fibre. This result makes less general the conclusion reached on cerebella devoid of climbing fibres. How this situation, which does not exist in normal cerebellum, is obtained in *reeler*, is still an unanswered question.

Although the specificity of most of the synaptic contacts are preserved in *reeler* cerebellum, heterologous synapses may form despite a major disorder in Purkinje cell distribution and shape. For instance, as in the case of *weaver* cerebellum (Sotelo 1975*a*), mossy fibres reach some

of the unoccupied Purkinje cell spines and there establish typical synaptic contacts. Furthermore, the electrophysiological results suggest that at least some of these 'heterologous' mossy fibre–Purkinje cell synapses are functional, as in viral infected ferrets and in X-irradiated rats (Llinas *et al.* 1973; Crepel *et al.* 1976*a*). This last observation, which confirms previous studies done with the homozygous *weaver* mouse, indicate that the 'recognition' step between cell surfaces may not require a complementarity of structure as exquisite as that generally assumed to account for the specificity of synapse formation (Sperry 1963 and references in Gaze 1970). It clearly means that the acquisition of synaptic specificity is a progressive and sequential process which in its late stages involves a process of stabilization regulated by its cellular environment and possibly the state of activity of the developing neuronal network.

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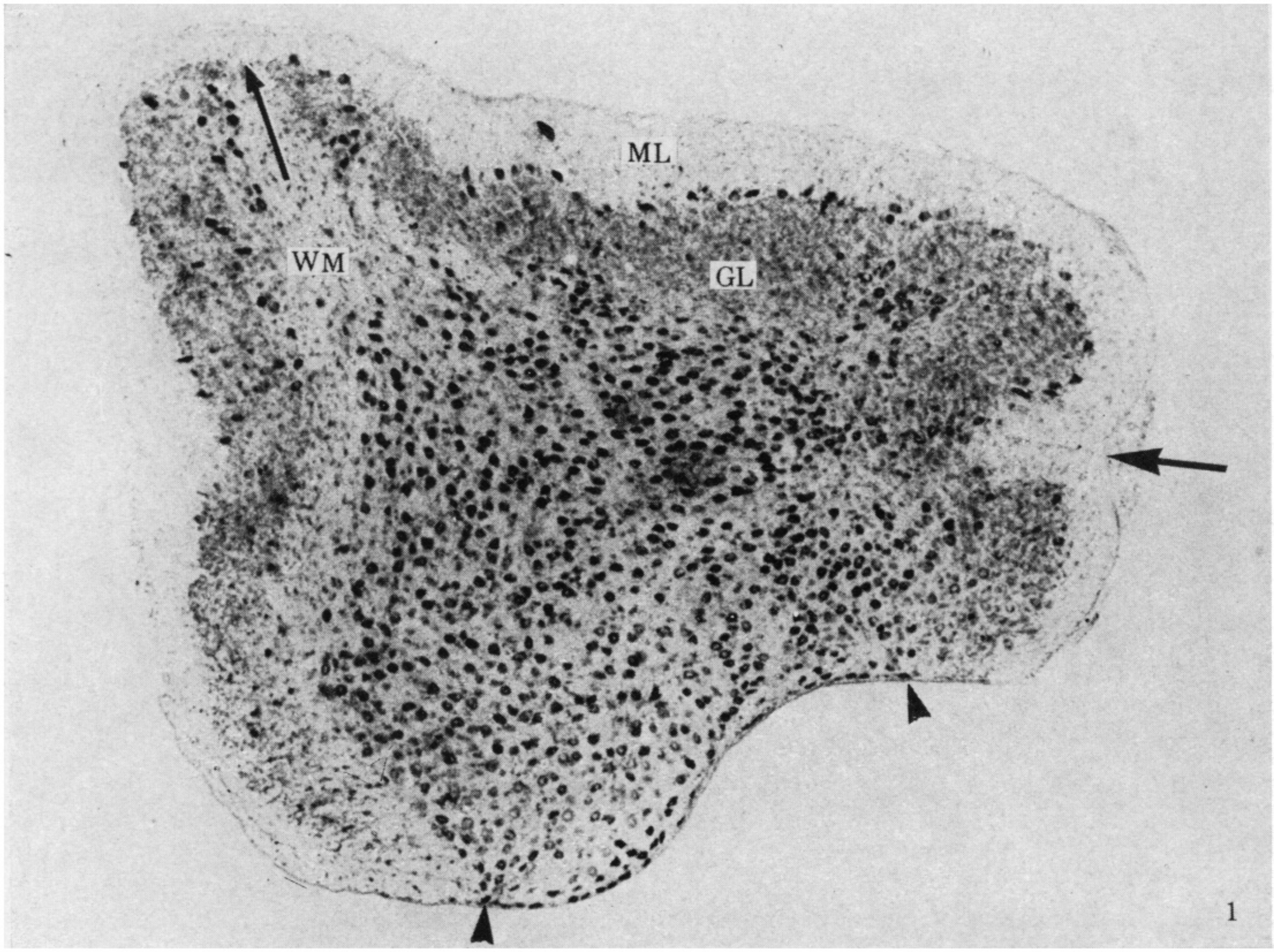

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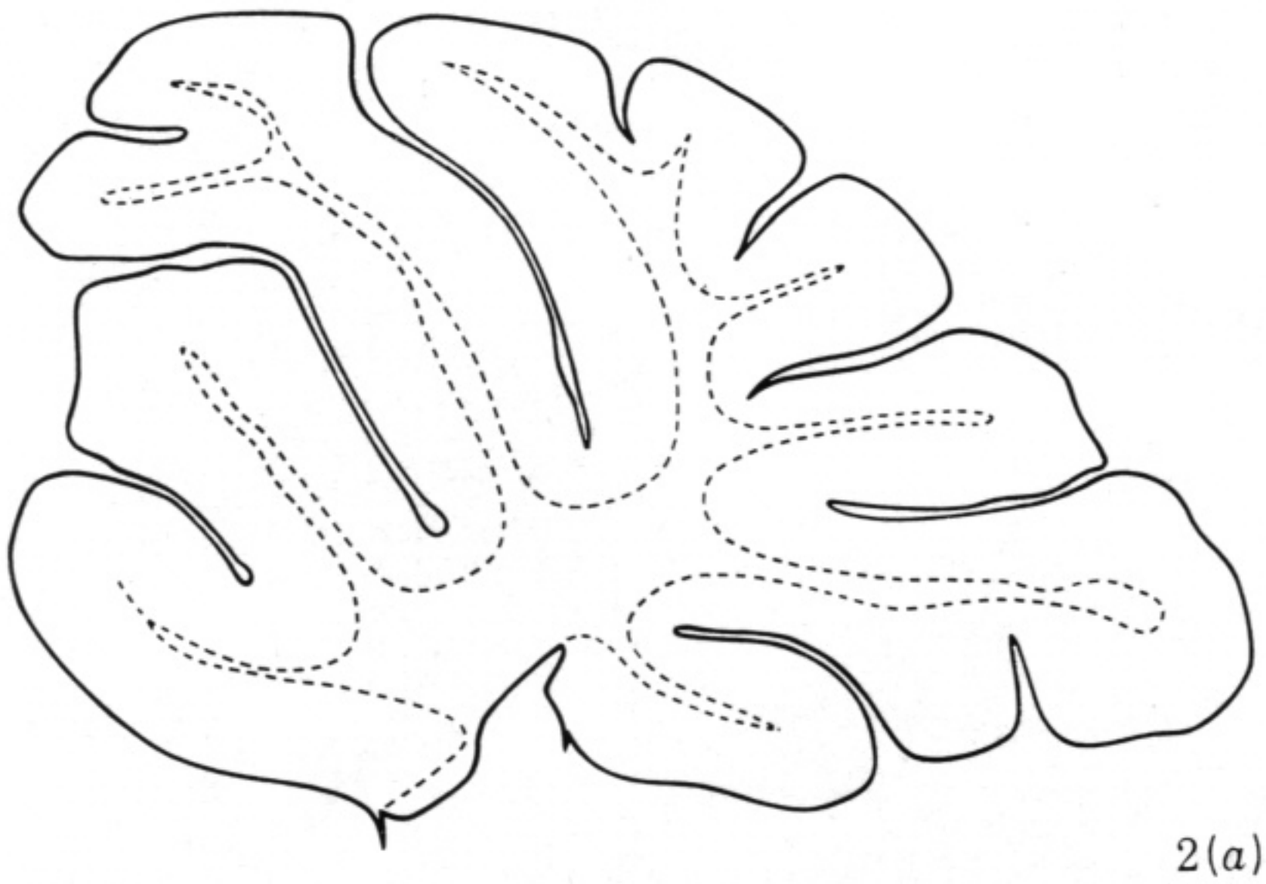

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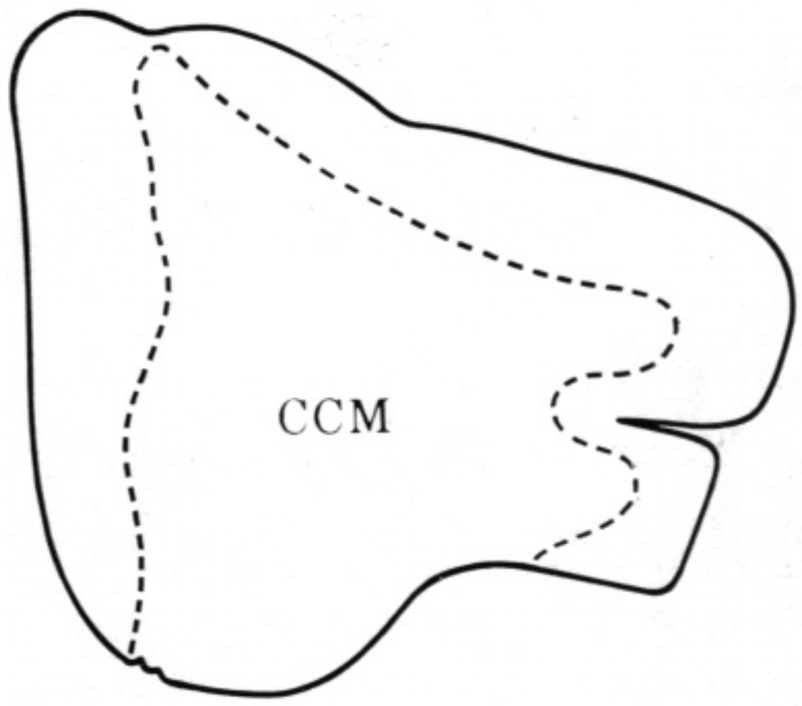
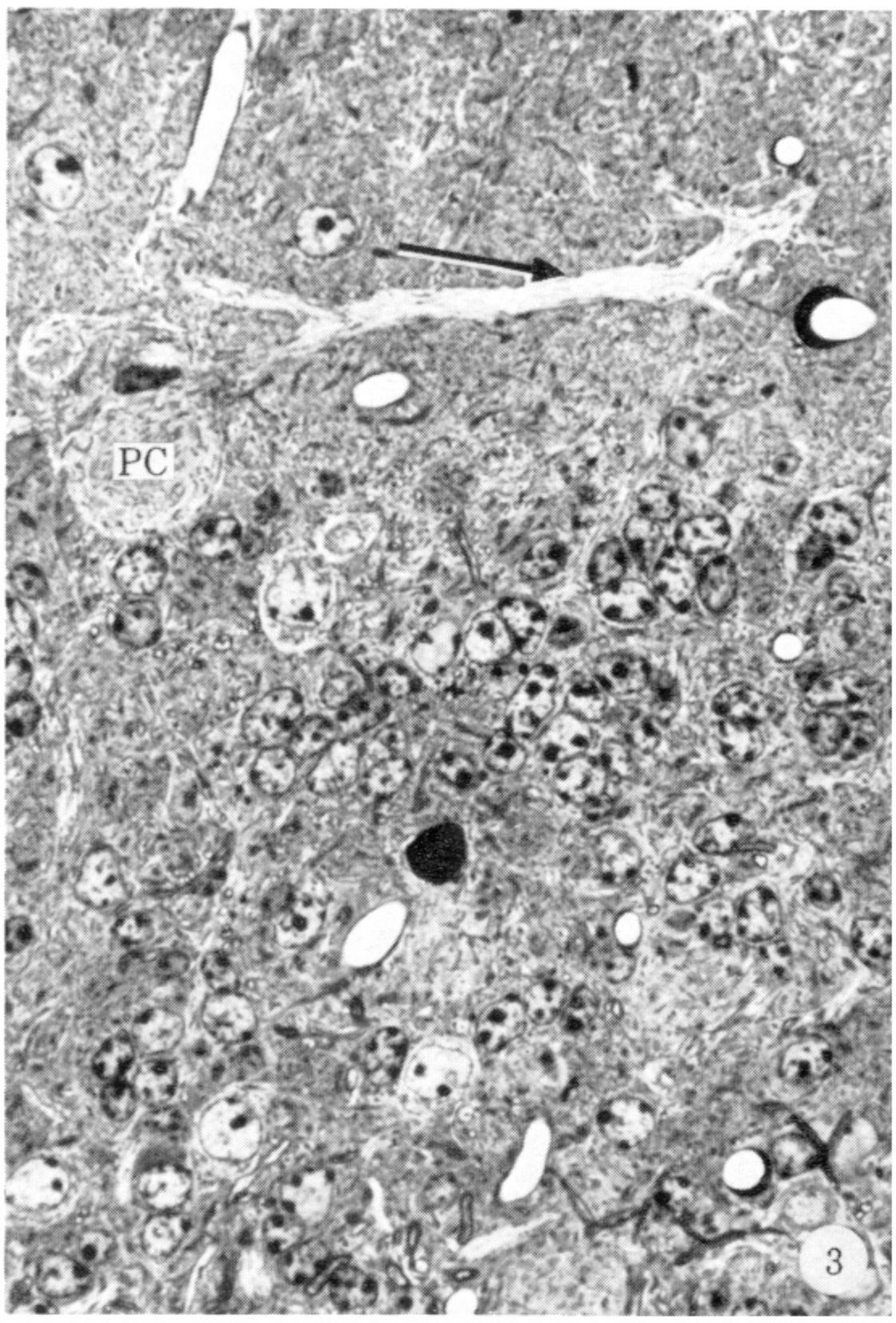
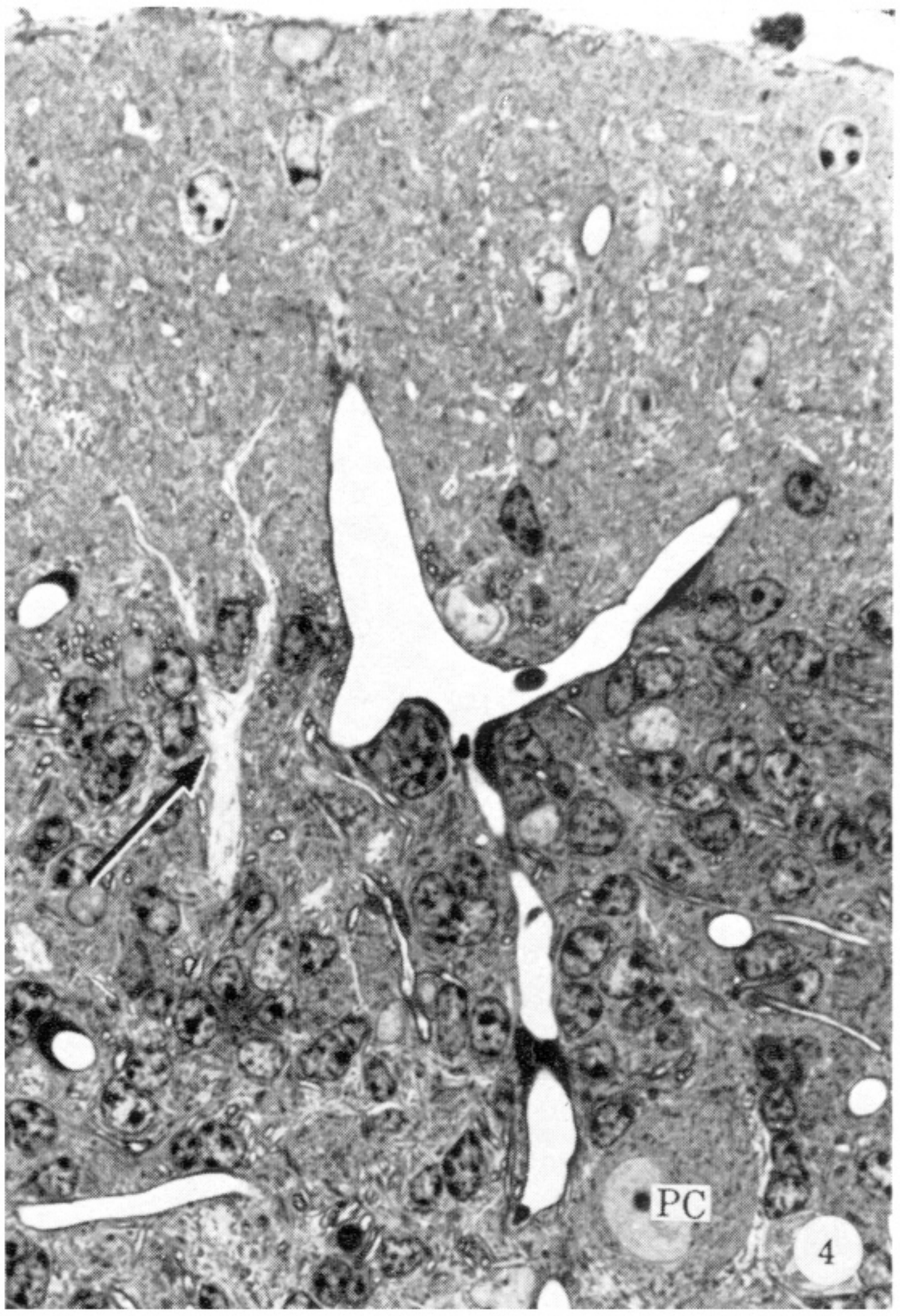


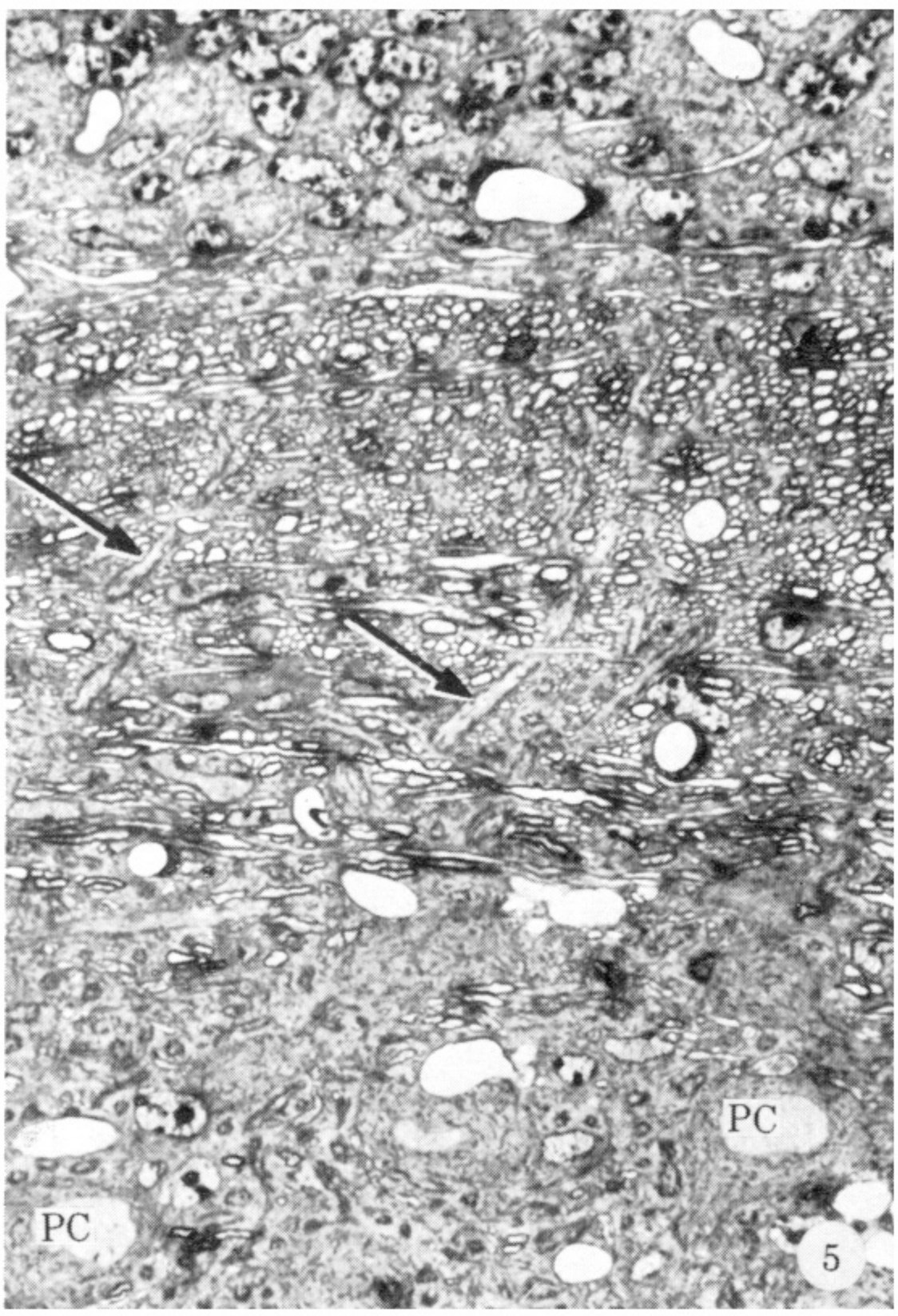
(*b*)

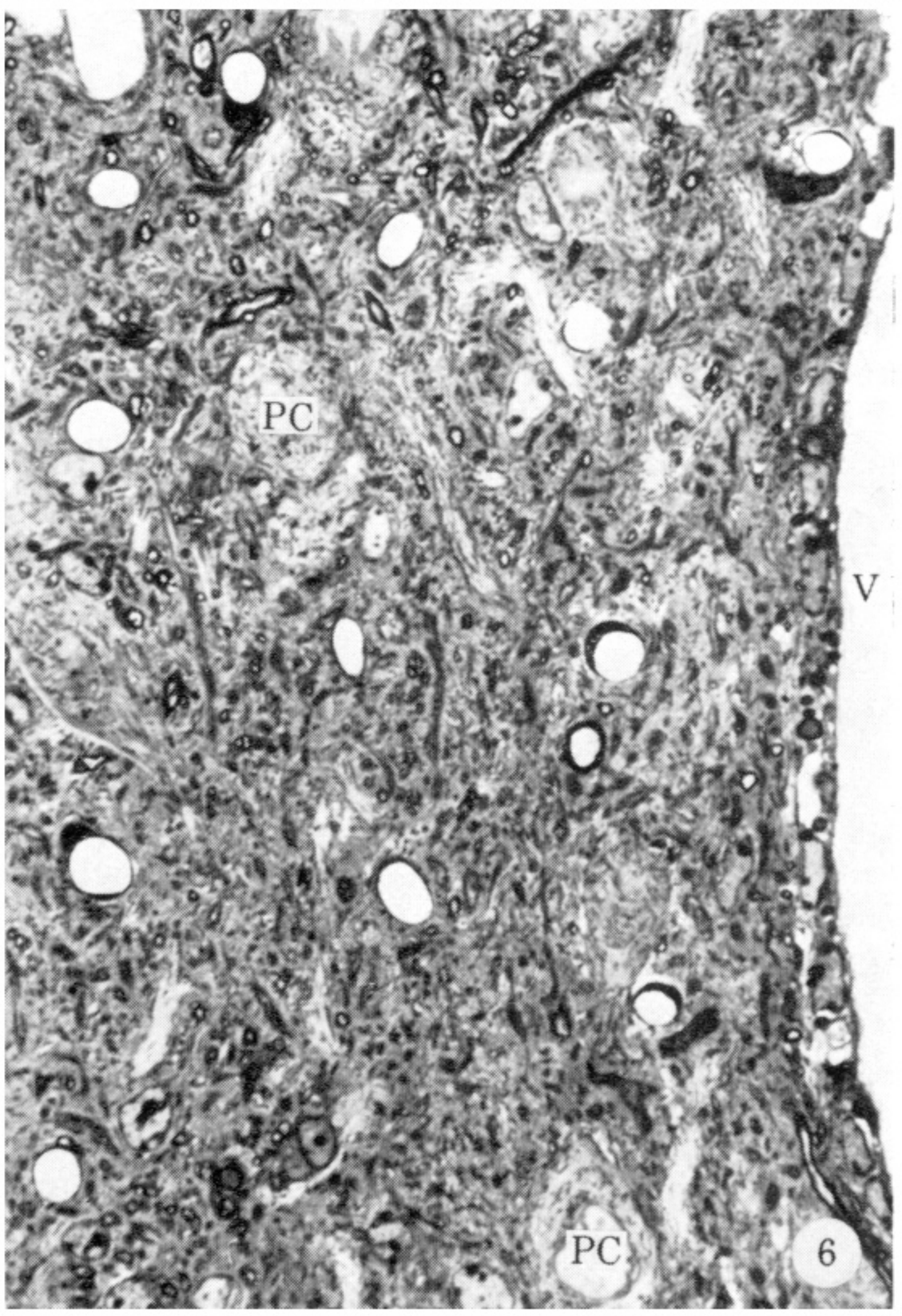
FIGURE 1. Midsagittal section of the vermis of a 226-day old *rl/rl* mouse. The periphery is occupied by a quasi-normal cerebellar cortex, composed by a molecular layer (ML), a Purkinje cell layer and a granular layer (GL), underlayed by a white matter (WM) of variable thickness. This peripheral cortex covers a central cellular mass, almost entirely formed by mispositioned Purkinje cells. The large arrow points to the *fissura secunda*. The small arrow indicates the site of interruption of the granular layer by the white matter, which probably marks the position of a vestigial *fissura prima*. The two arrow heads delimits the ventricular surface. (Magn. × 70.)

FIGURE 2. Schematic outline of a 1 μm thick plastic sagittal section passing through the vermis of a 226-day old *rl/rl* mouse (2*b*) and the vermis of its normal littermate (2*a*). Most of the *reeler* cerebellum is occupied by a central cerebellar mass (CCM), where is located the bulk of the Purkinje cell population. (Magn. × 27.)









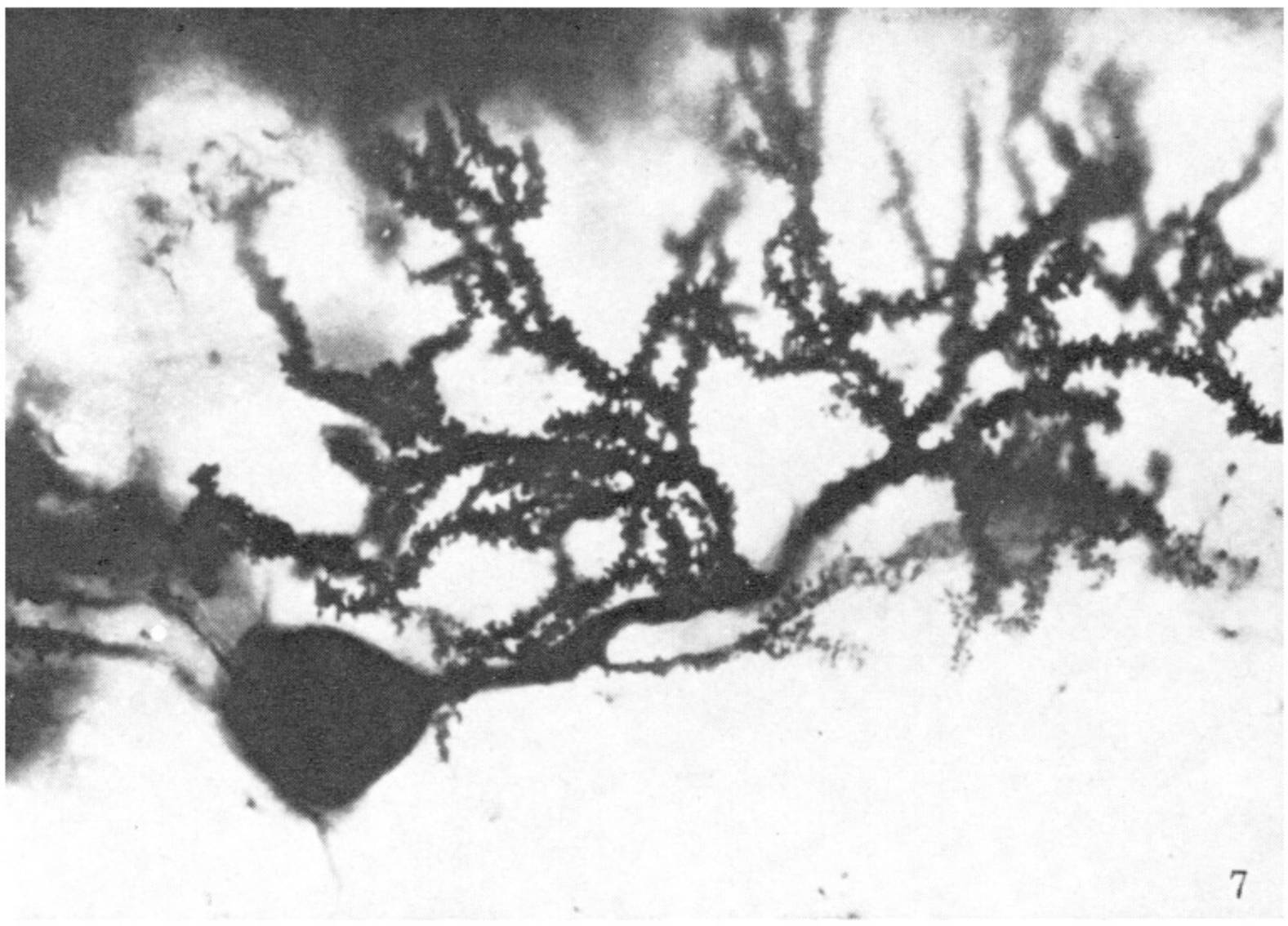
1 μm thick plastic sections stained with toluidine blue, illustrating Purkinje cells at different positions within the cerebellum.

FIGURE 3. Purkinje cell (PC) located at its normal position between the molecular and the granular layers. The arrow points to the secondary dendrite spreading in the molecular layer in an almost parallel direction to the pial surface. (Magn. × 640.)

FIGURE 4. Purkinje cell (PC) within the granular layer. The arrow points to a thick dendritic trunk, ascending in a vertical direction through the granular layer and branching at the molecular layer level. (Magn. × 640.)

FIGURE 5. Purkinje cells (PC) at the periphery of the central cerebellar mass. These cell bodies are just beneath the white matter of the superficial cerebellar cortex. Some of the thick dendritic branches of these Purkinje cells cross the white matter (arrows) and spread within the granular layer. (Magn. × 640.)

FIGURE 6. Purkinje cells (PC) at the central cerebellar mass, close to the ventricular surface (V). (Magn. × 640.)



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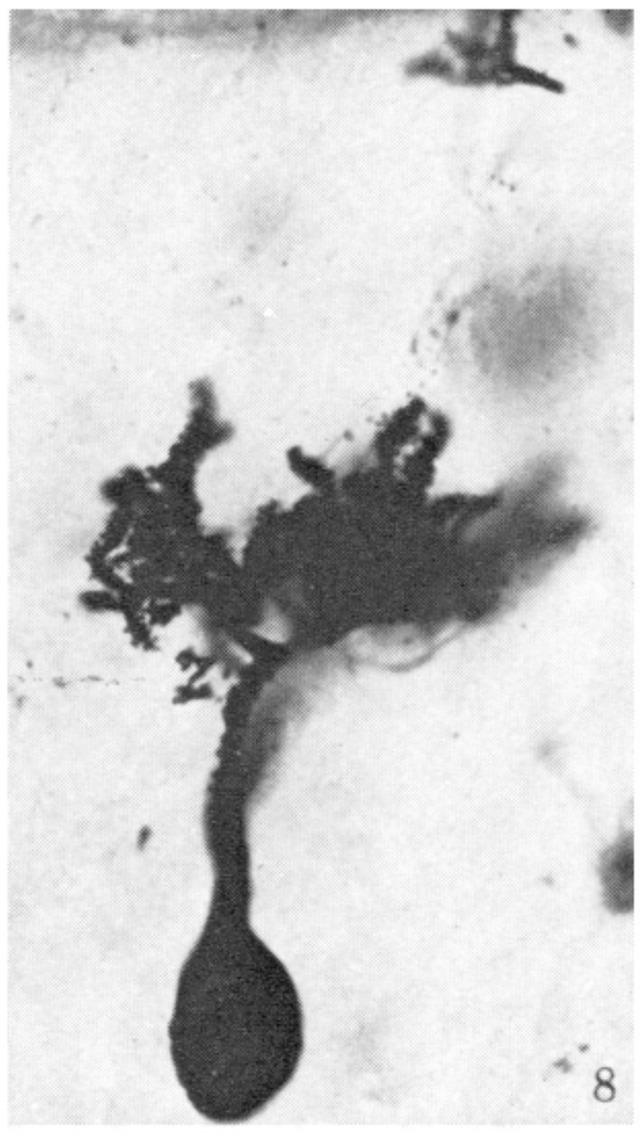

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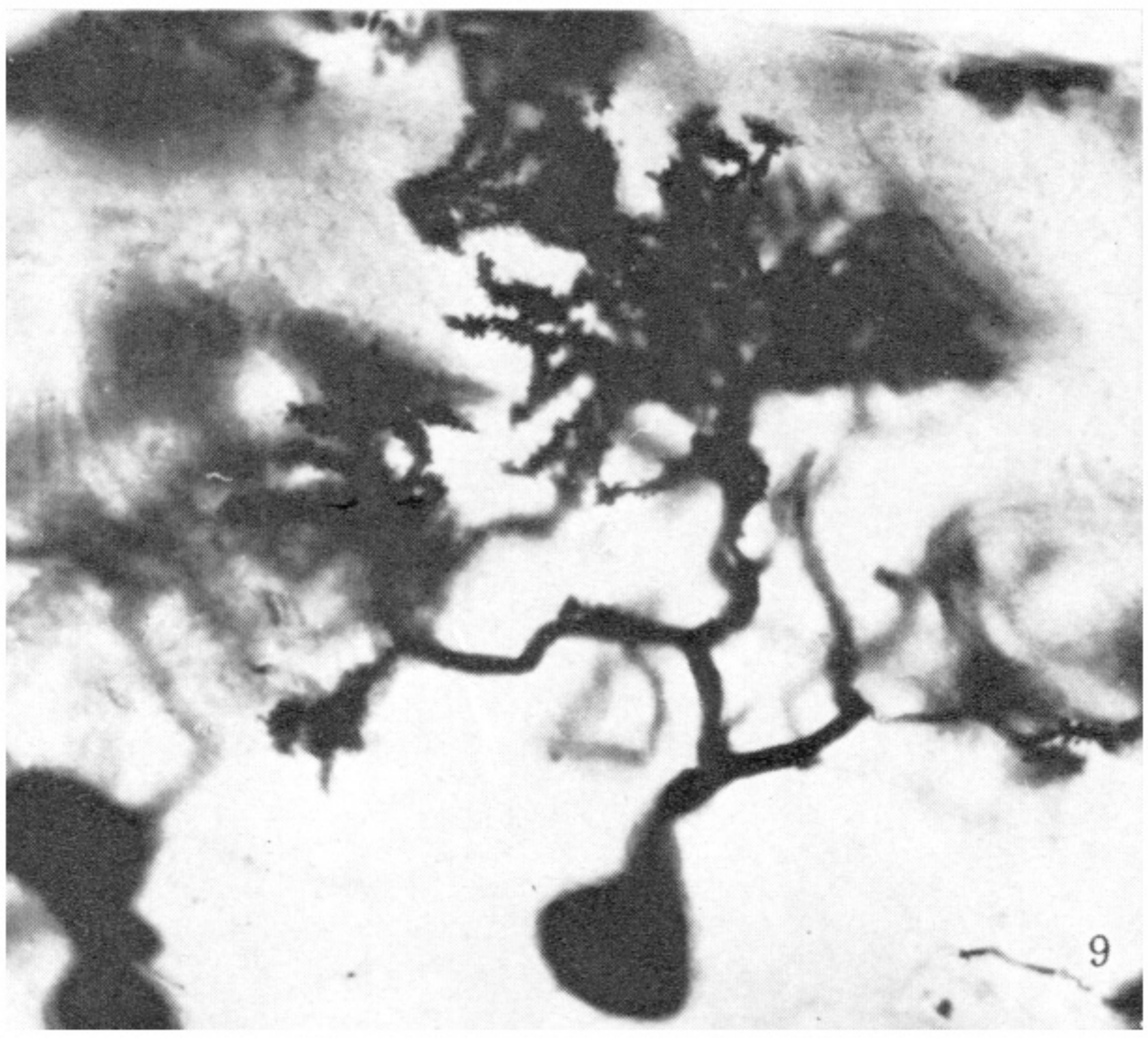

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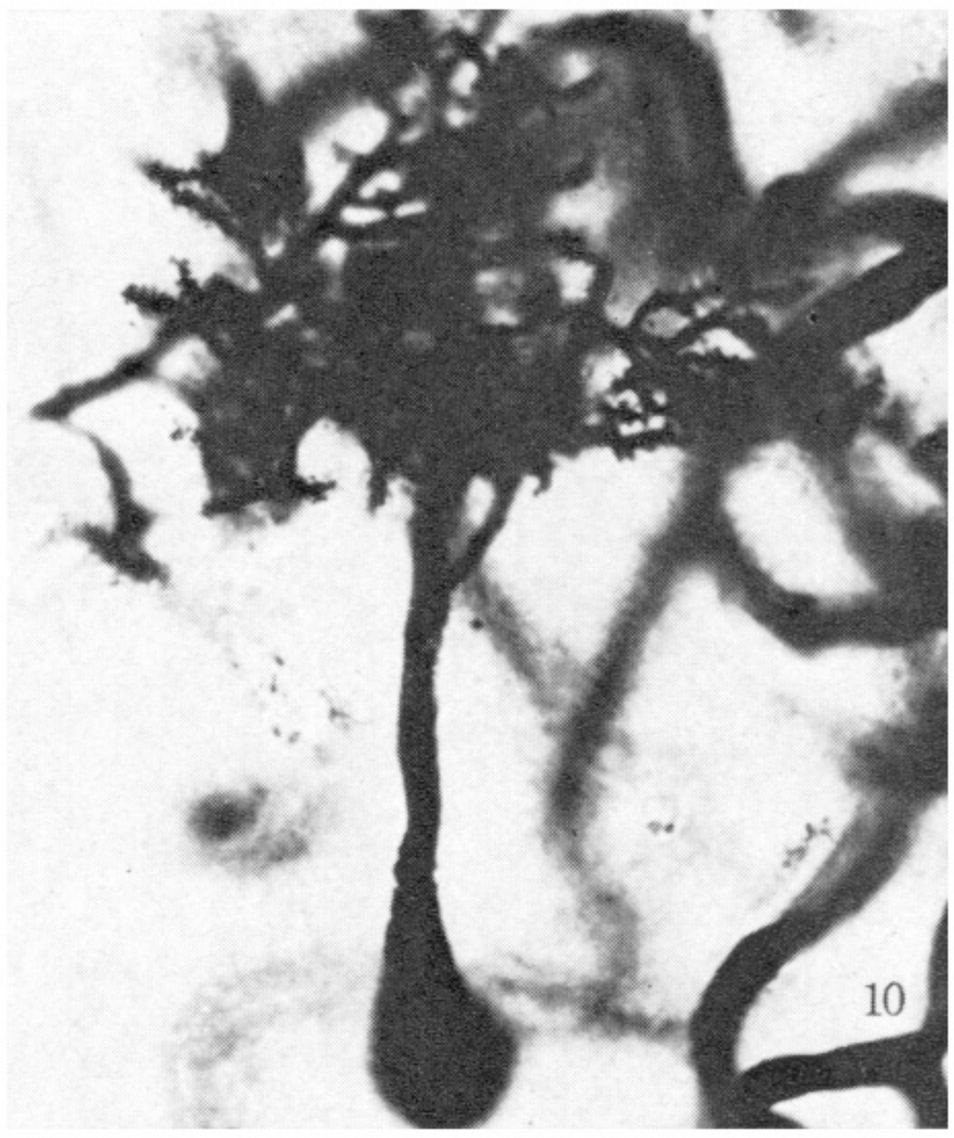

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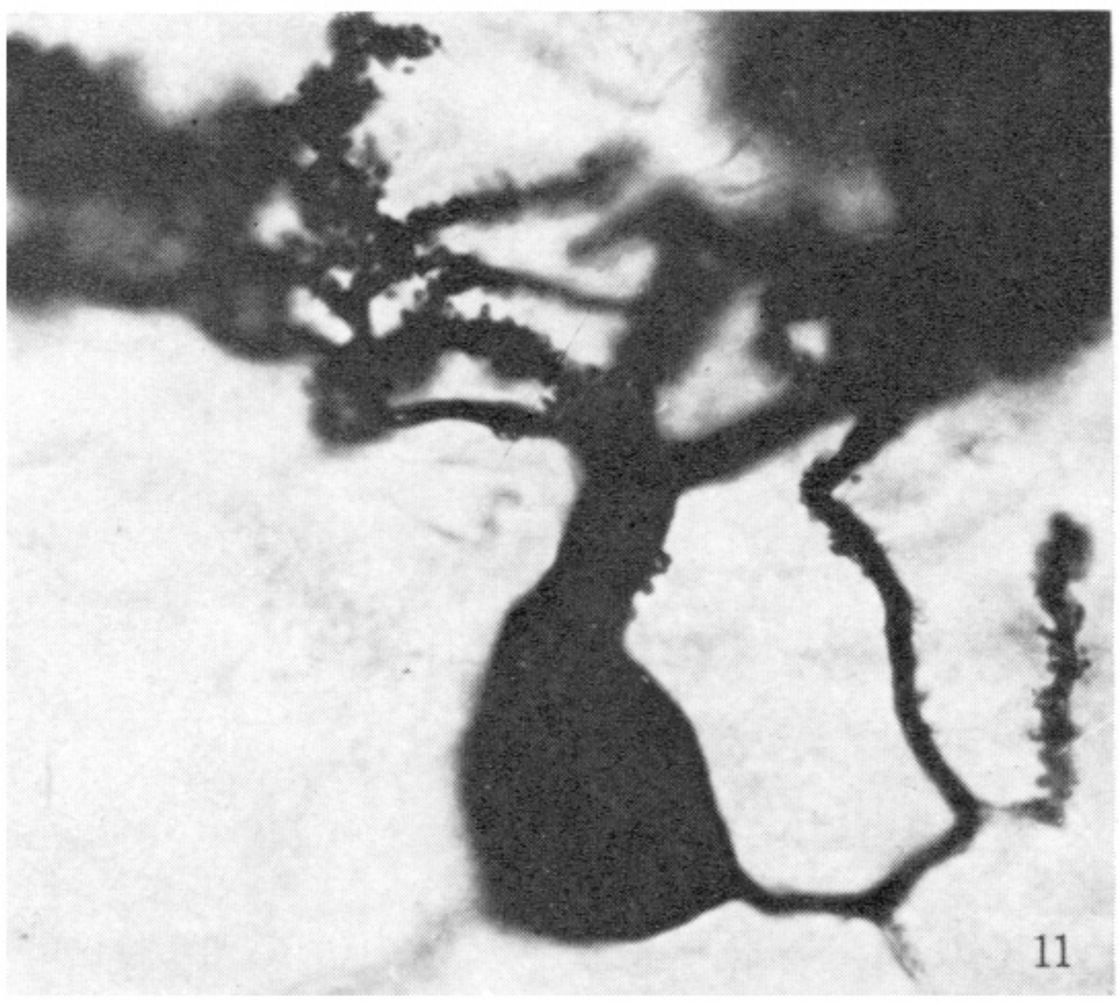

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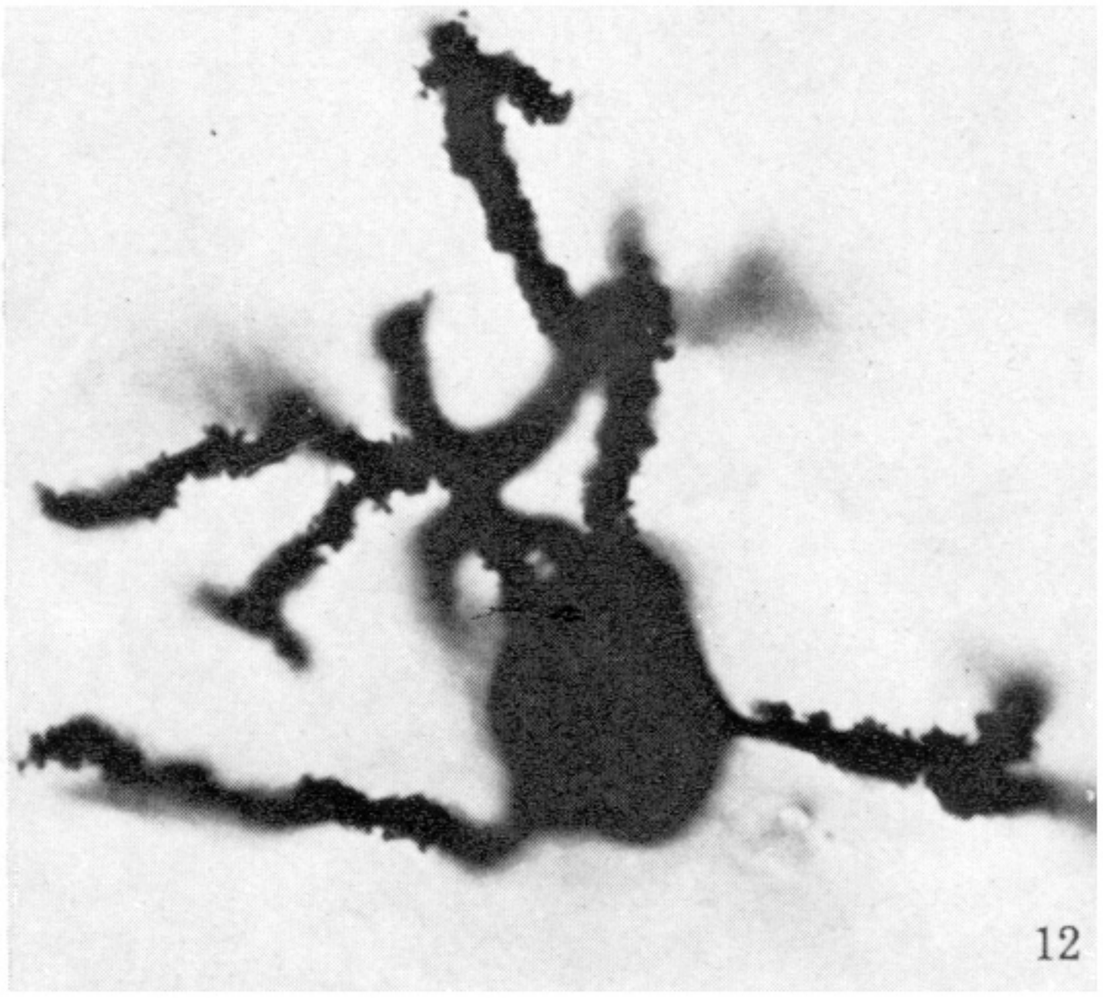

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Golgi impregnation of Purkinje cells located at different levels of the *rl/rl* cerebellum.

FIGURE 7. Purkinje cell which succeeded to reach its normal position. Its dendritic tree spreads within the molecular layer keeping its normal planar orientation. (Magn. × 640.)

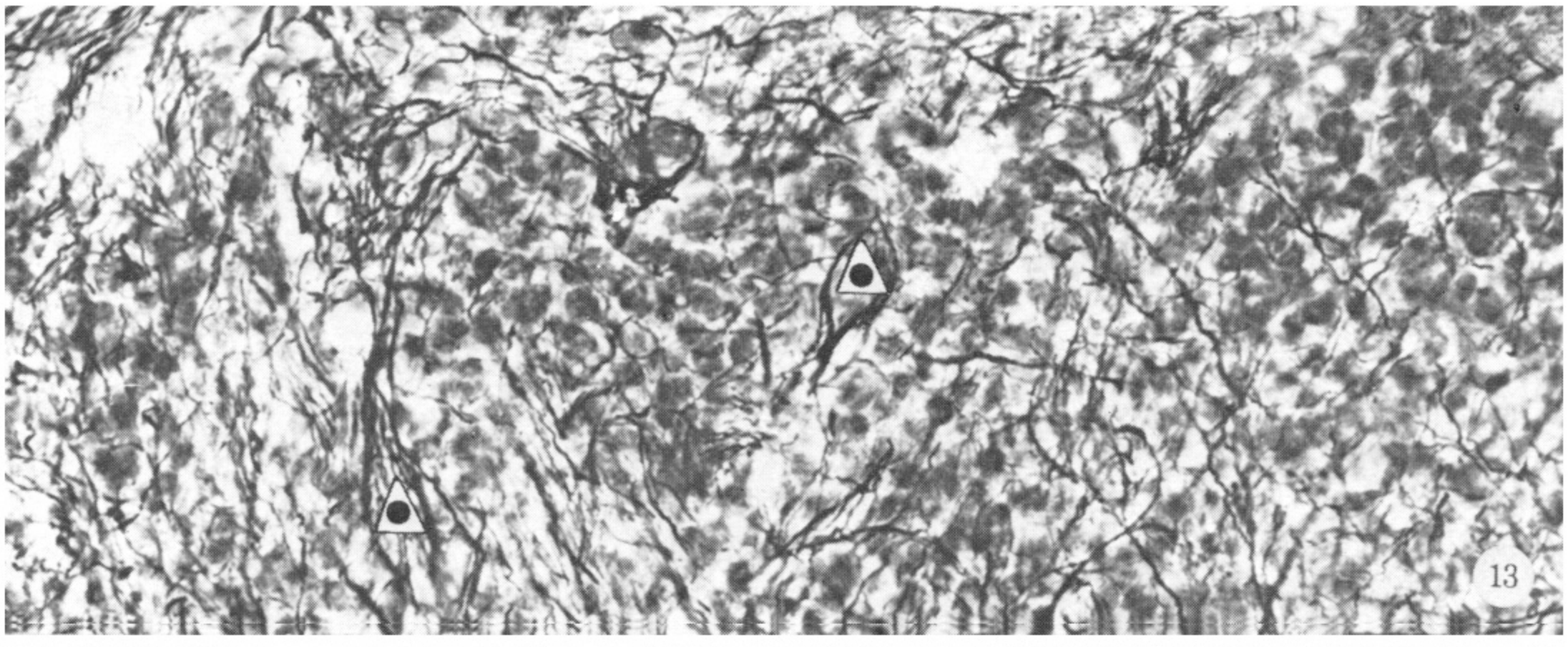
FIGURE 8. Purkinje cell at the periphery of the central cerebellar mass. Its main dendrite, studded with spines, crosses the white matter and branches within the granular layer. (Magn. × 540.)

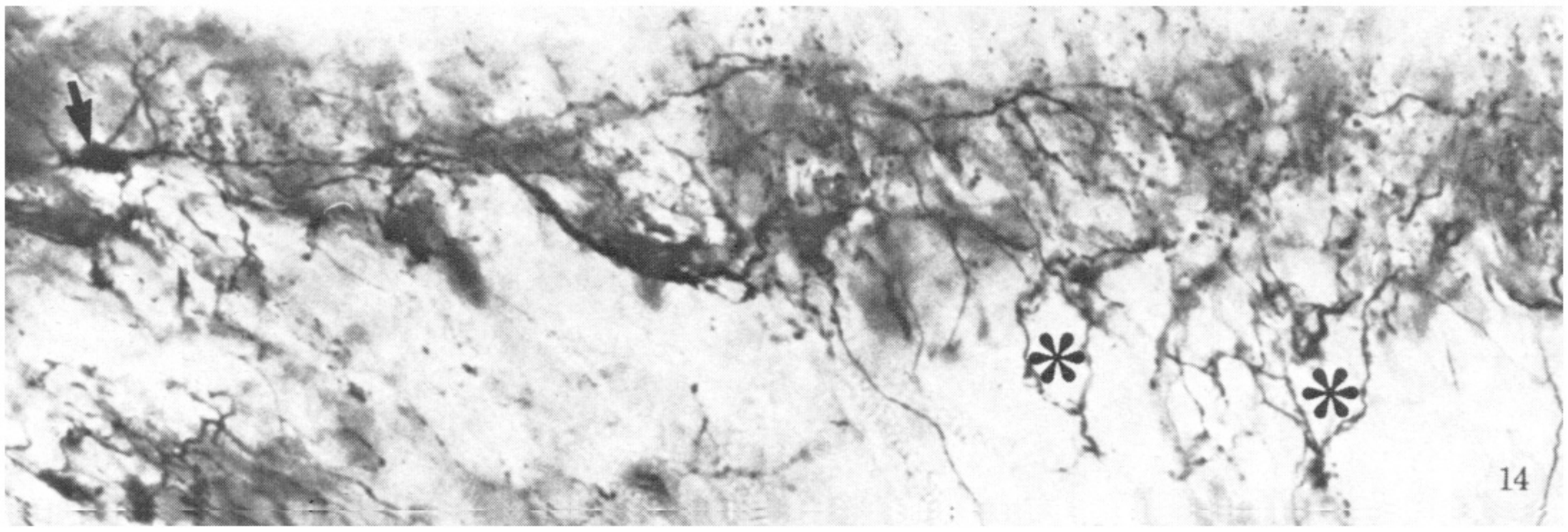
FIGURE 9. Purkinje cell within the granular layer. Its main dendrite branches close to the perikaryon, given rise to large dendritic trunks which spread within the granular layer. One of these trunks ascends towards the molecular layer, where it branches profusely. It therefore corresponds to the mixed type. (Magn. × 590.)

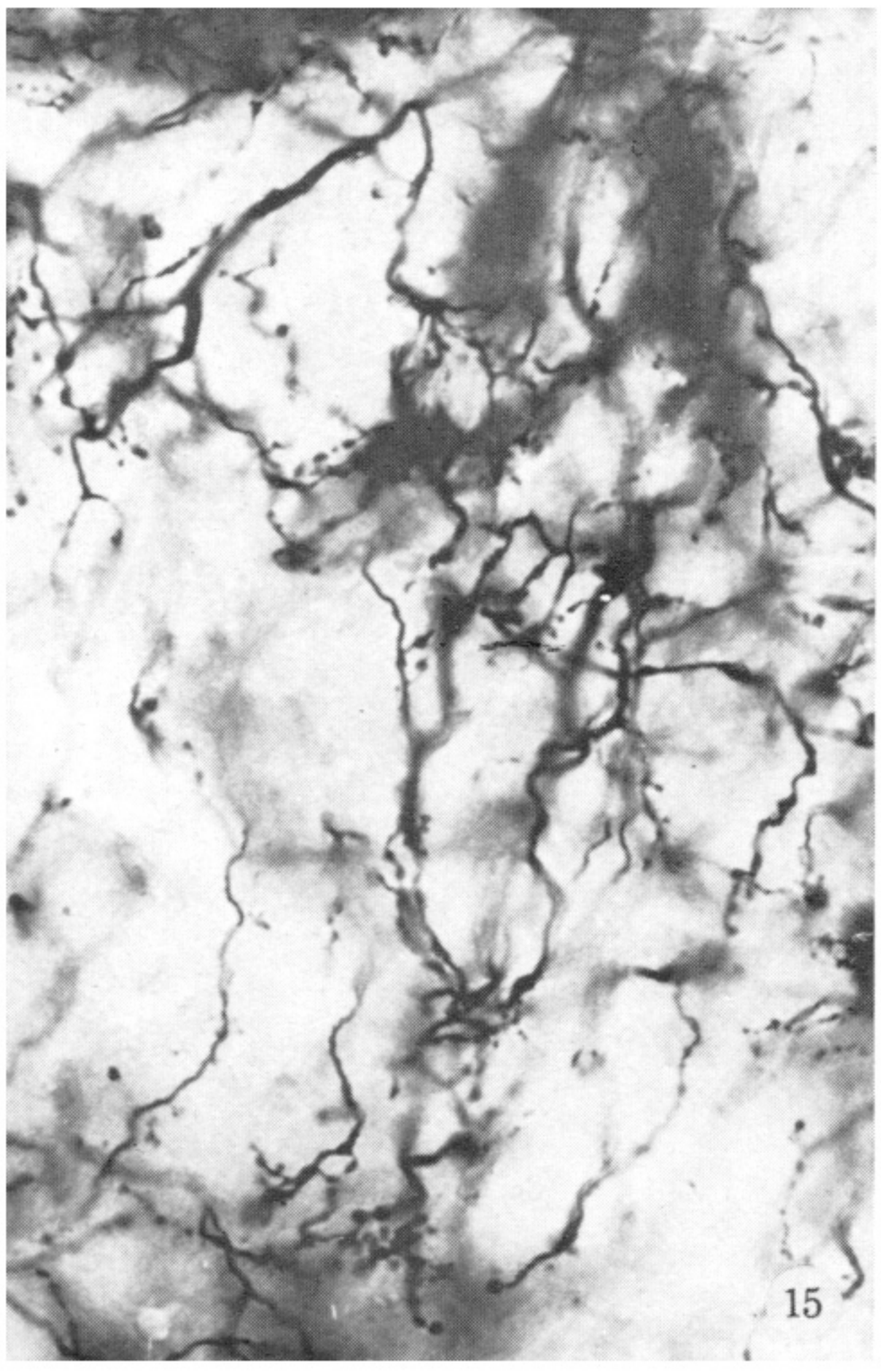
FIGURE 10. Purkinje cell within the granular layer, illustrating the *en parapluie* type. (Magn. × 570.)

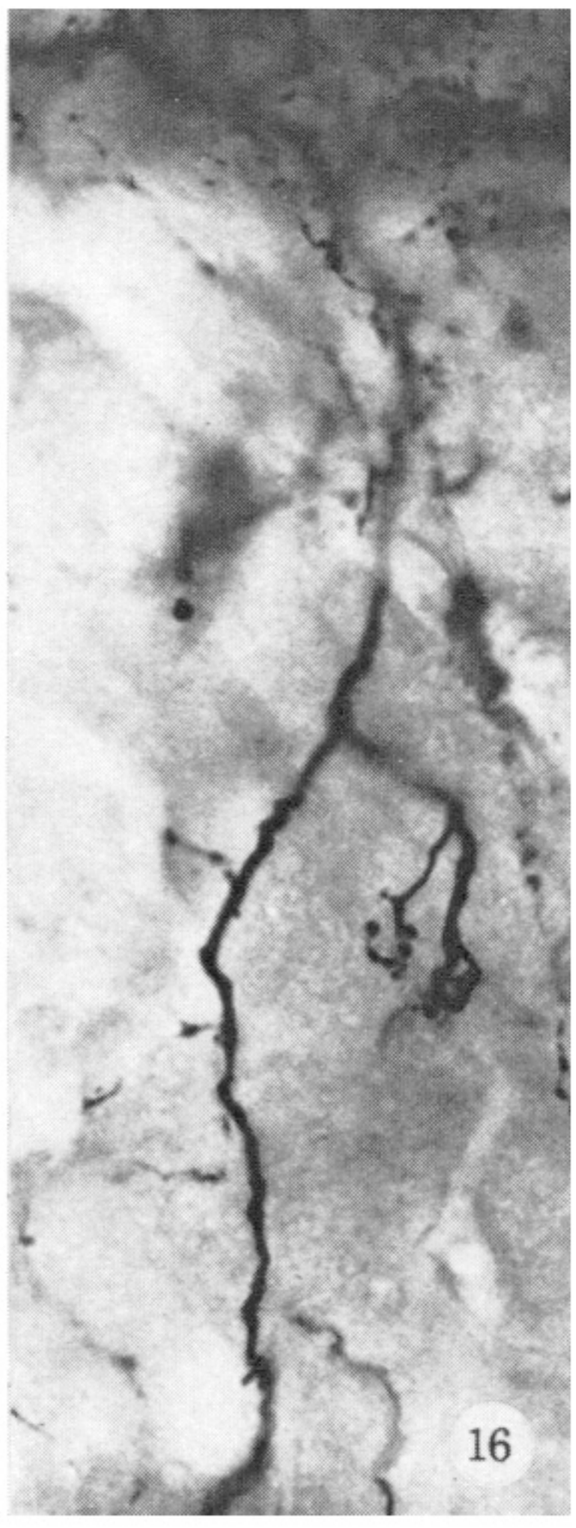
FIGURE 11. 'Multipolar' Purkinje cell, which perikaryon is located within the granular layer. (Magn. × 880.)

FIGURE 12. 'Multipolar' Purkinje cell located at the central cerebellar mass. This cell has randomly oriented dendrites, which are studded with spines. (Magn. × 800.)









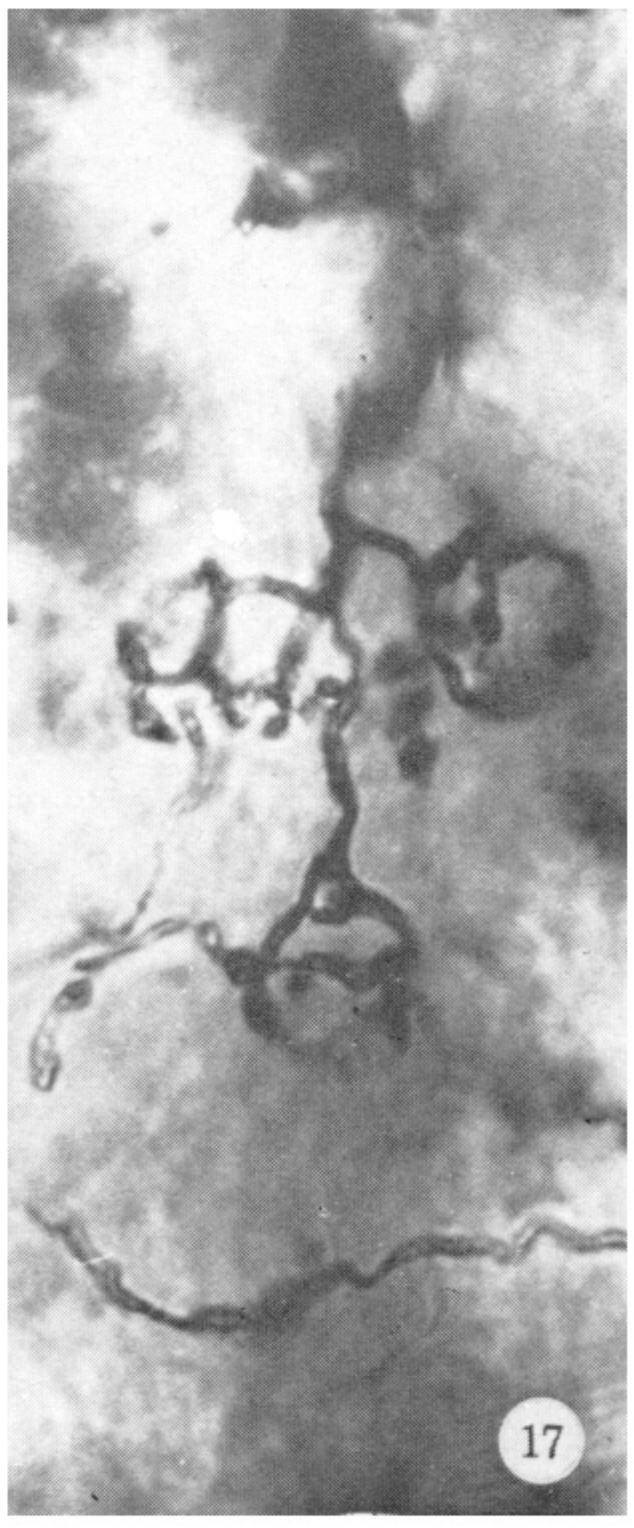


FIGURE 13. Reduced silver impregnation of basket fibres around two Purkinje cell perikarya (white triangle with a black spot) dispersed within the granular layer. (Magn. × 500.)

FIGURES 14–17. Golgi impregnation of a basket cell and numerous basket fibre terminals.

FIGURE 14. The arrow points to a basket cell perikaryon located at the deep region of the superficial molecular layer. Its axon gives rise to a prominent plexus which invades the granular layer to invest some of the malpositioned Purkinje cell perikarya (asterisks). (Magn. × 330.)

FIGURE 15. Pericellular plexus formed by basket fibres investing Purkinje cell perikarya within the granular layer. (Magn. × 600.)

FIGURES 16 and 17. Terminal axonic arborizations of individual basket fibres embracing Purkinje cell perikarya in the granular layer. (Figure 16: Magn. × 600; figure 17: Magn. × 750.)

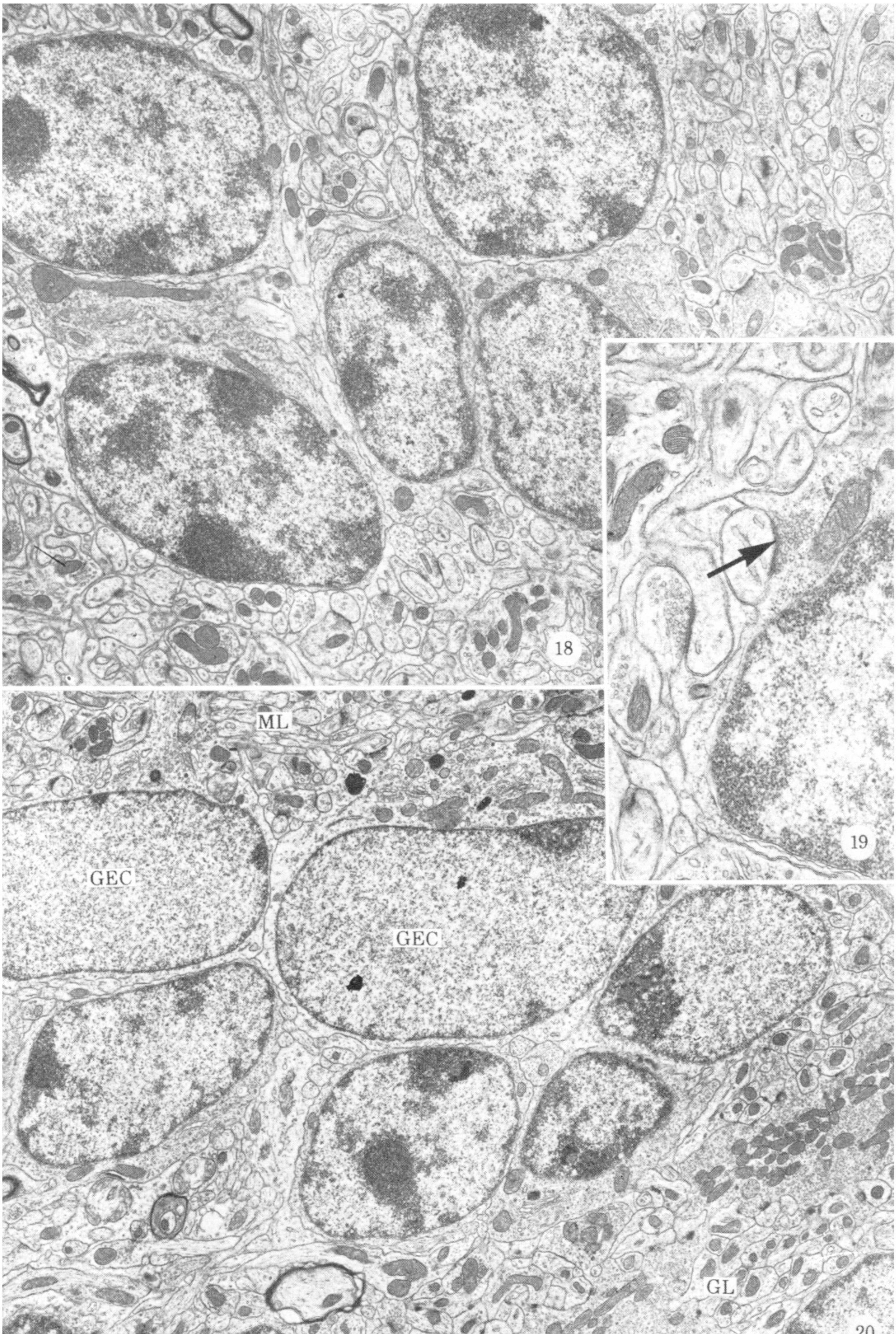


FIGURE 18. Ectopic granule cells in the molecular layer. A cluster of five granule cells is surrounded by a neuropil characteristic of the molecular layer. (Magn. × 8000.)

FIGURE 19. Somato-dendritic synapse (arrow) between an ectopic granule cell and a spine of a Purkinj cell dendrite. (Magn. × 18000.)

FIGURE 20. Border region between the molecular layer (ML) and the granular layer (GL). At this region there are not Purkinje cell perikarya, but Golgi epithelial cells (GEC) separate the molecular from the granular layer. (Magn. × 7000.)

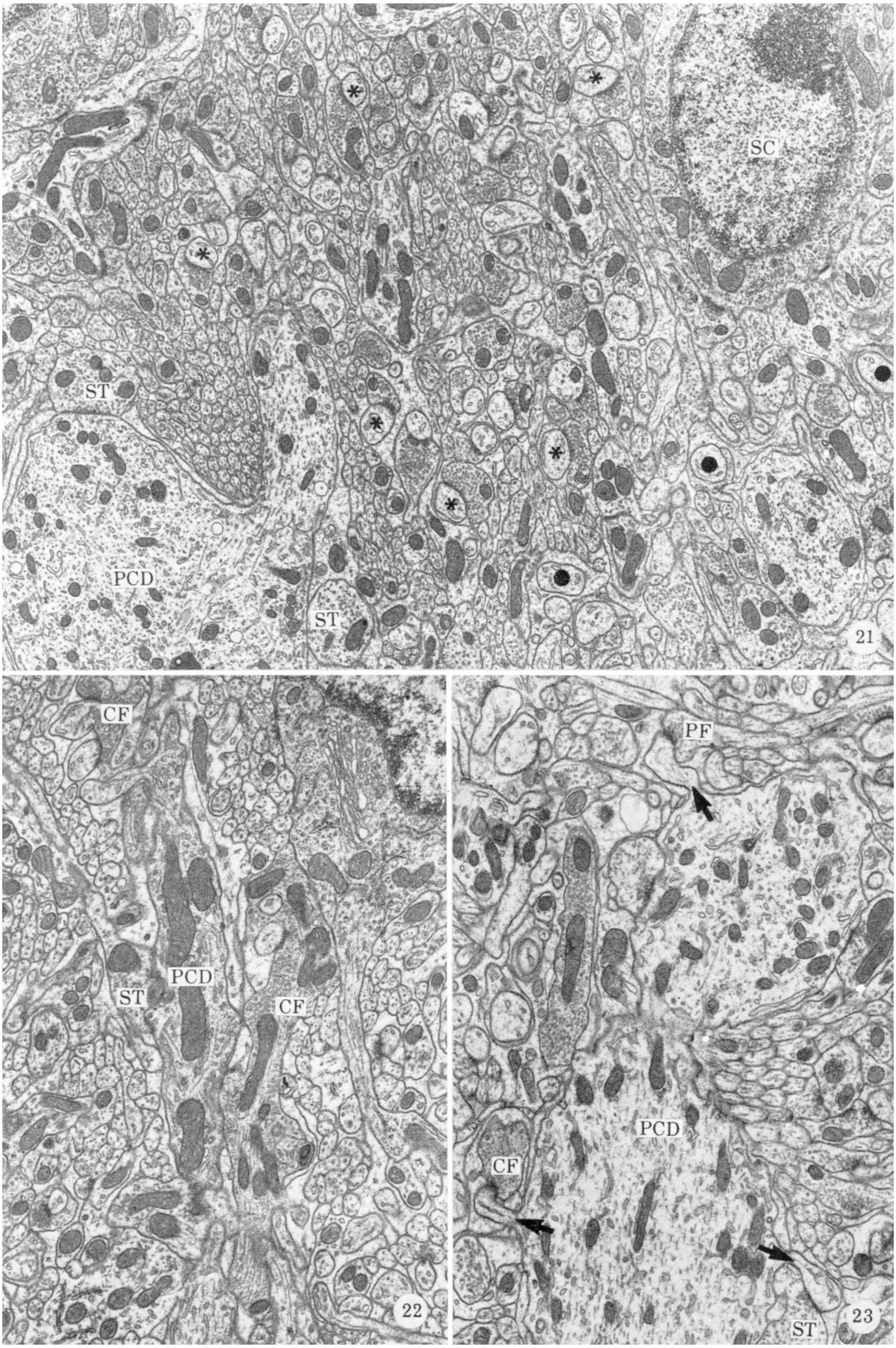


FIGURE 21. Molecular layer. The appearance of this layer is normal. At the right high corner of the micrograph there is a stellate cell perikaryon (SC). A large profile of a Purkinje cell dendrite (PCD) receives stellate axon terminals (ST) on its surface. Many Purkinje cell spines (asterisks) are postsynaptic to parallel fibres; whereas, some of them (black spots) are free of innervation, but do not exhibit postsynaptic-like differentiations. (Magn. × 10000.)

FIGURE 22. Medium-sized Purkinje cell dendritic profile (PCD) contacted on its spines by climbing varicosities (CF) and on its shaft by a stellate axon terminal (ST). (Magn. × 11000.)

FIGURE 23. Three spines (arrows) emerge from a large Purkinje cell dendritic profile (PCD) in the molecular layer. One of them is contacted by a parallel fibre (PF), another by a climbing varicosity (CF) and the third one by a stellate axon terminal (ST). (Magn. × 12000.)

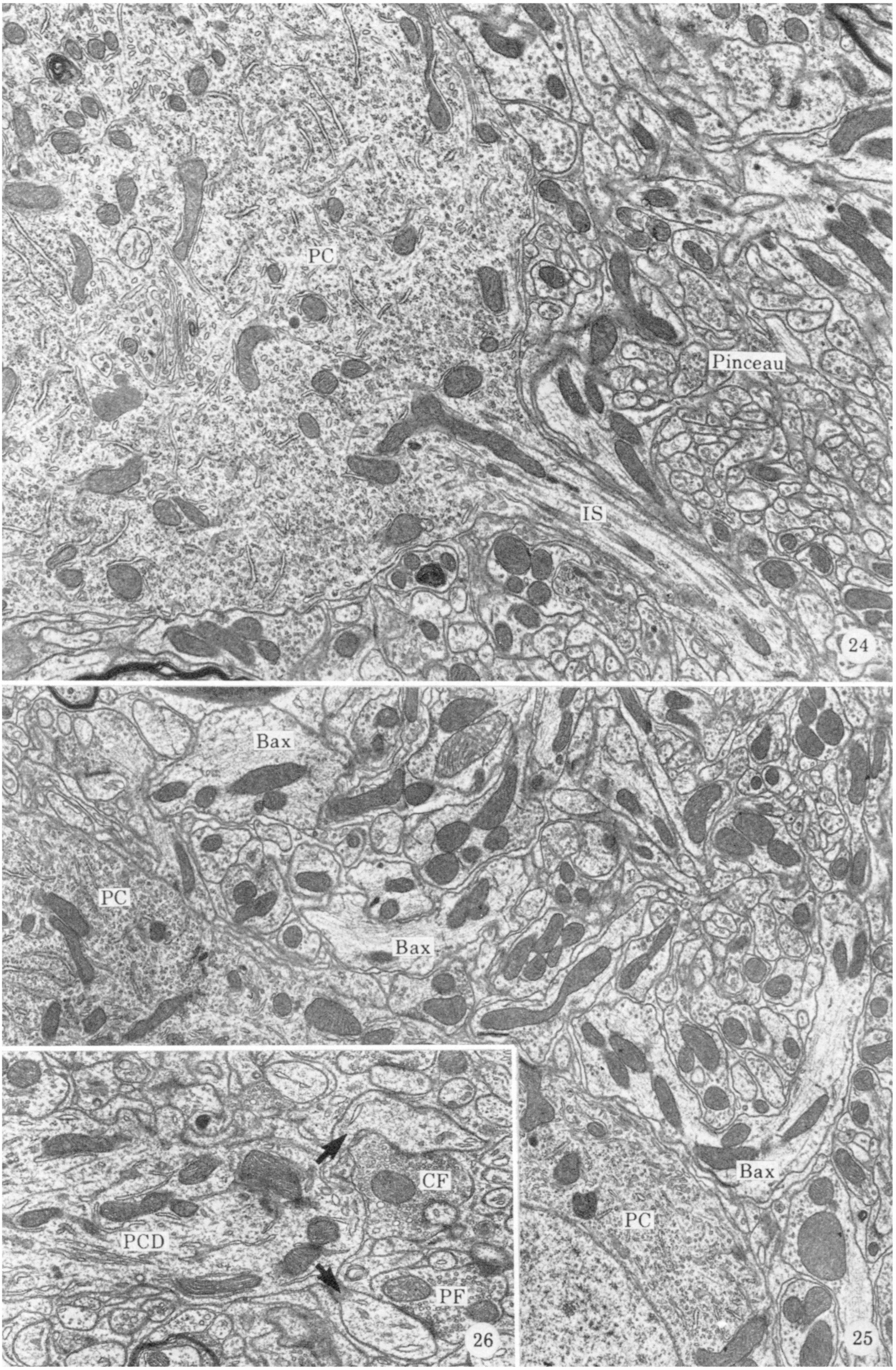
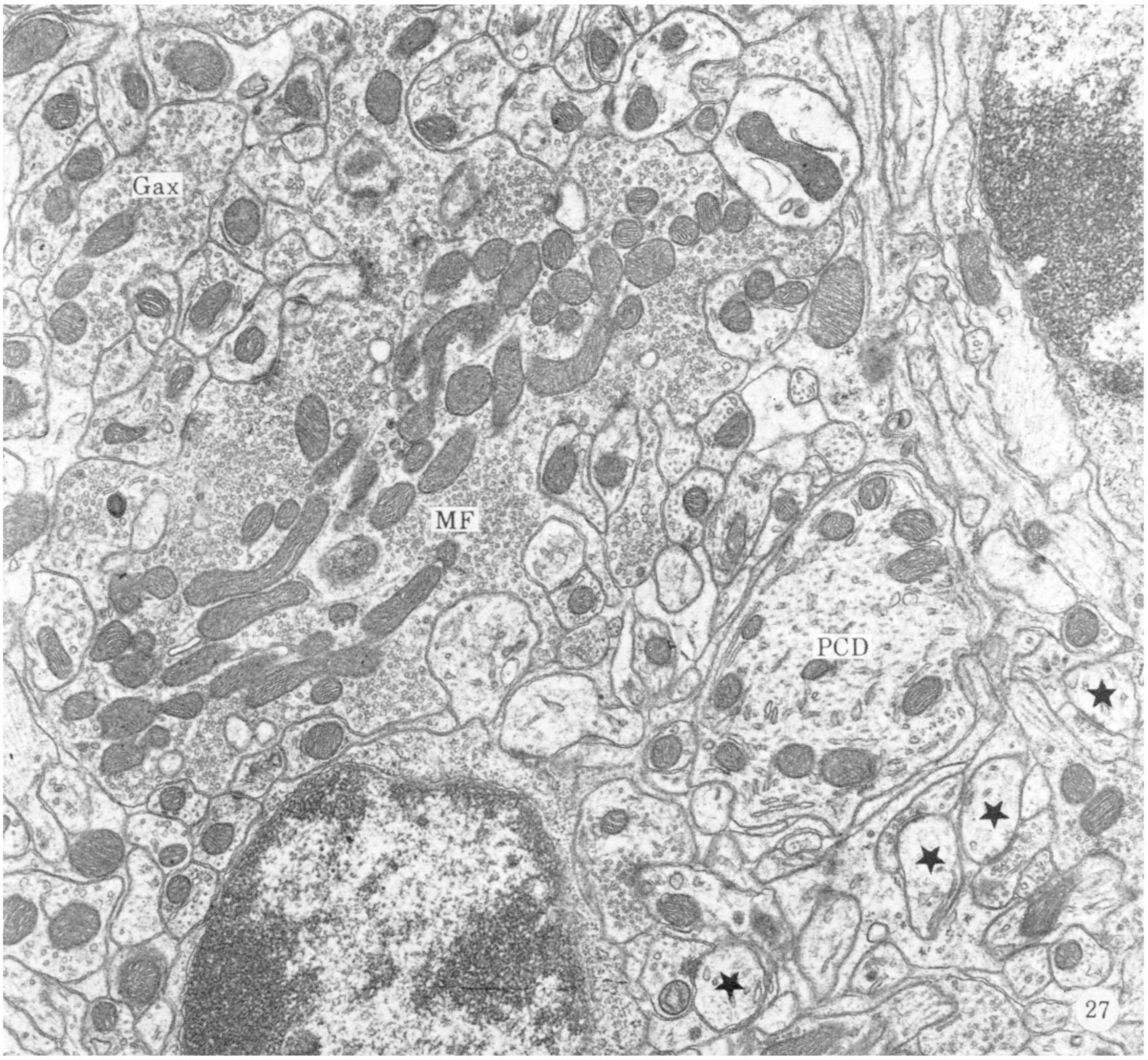


FIGURE 24. A Purkinje cell perikaryon (PC) at its usual location. Note the fine basket processes forming the pinceau around the initial segment (IS) of the Purkinje cell axon. (Magn. ×14000.)

FIGURE 25. Basket fibre (Bax) investment of a Purkinje cell perikaryon (PC) located within the granular layer. (Magn. ×13000.)

FIGURE 26. Electron micrograph of an ascending Purkinje cell dendrite (PCD) within the granular layer. Two spines (arrows) emerge from this dendritic profile. One is contacted by a climbing fibre varicosity (CF) and the other by a parallel fibre (PF). (Magn. ×14000.)



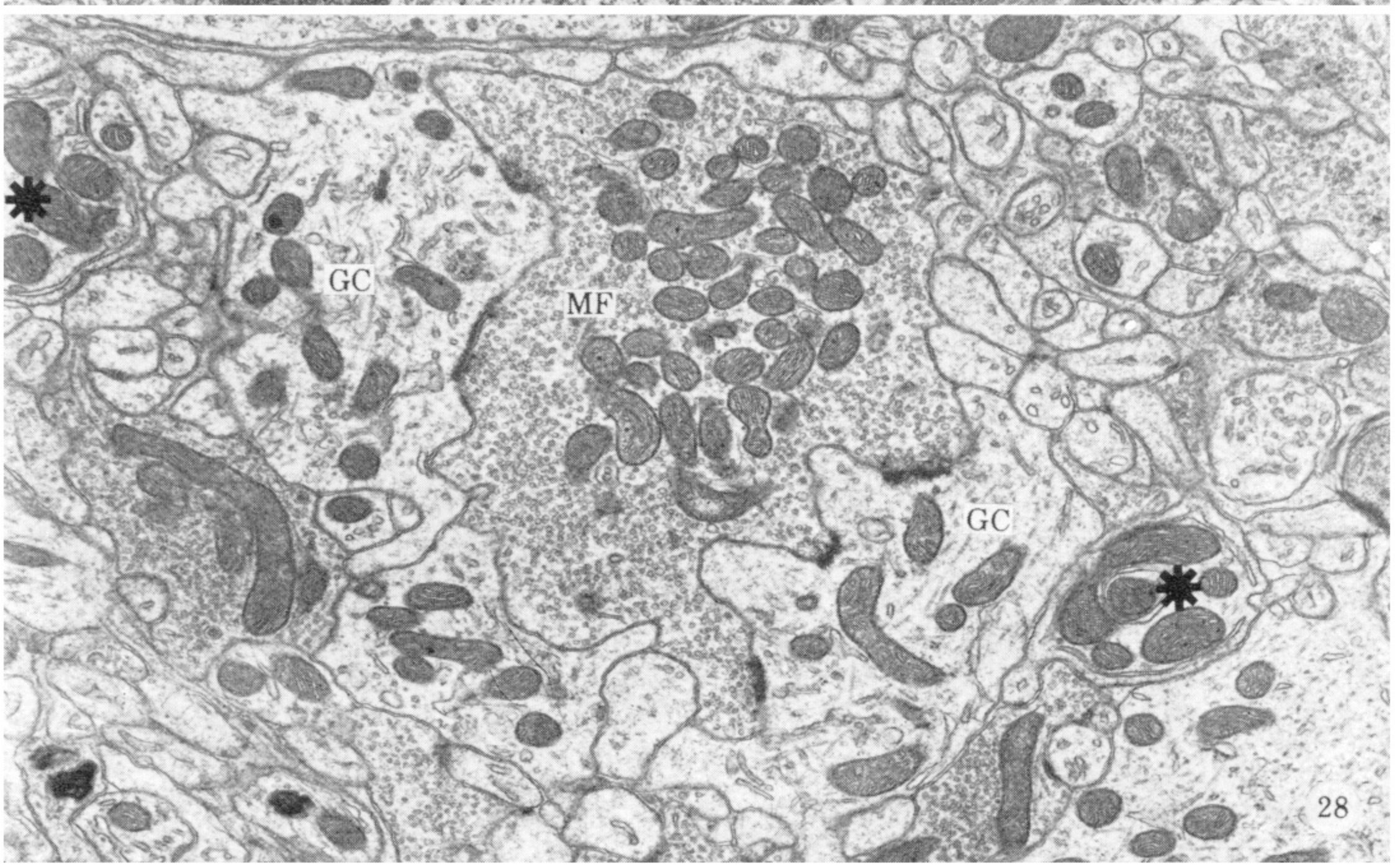
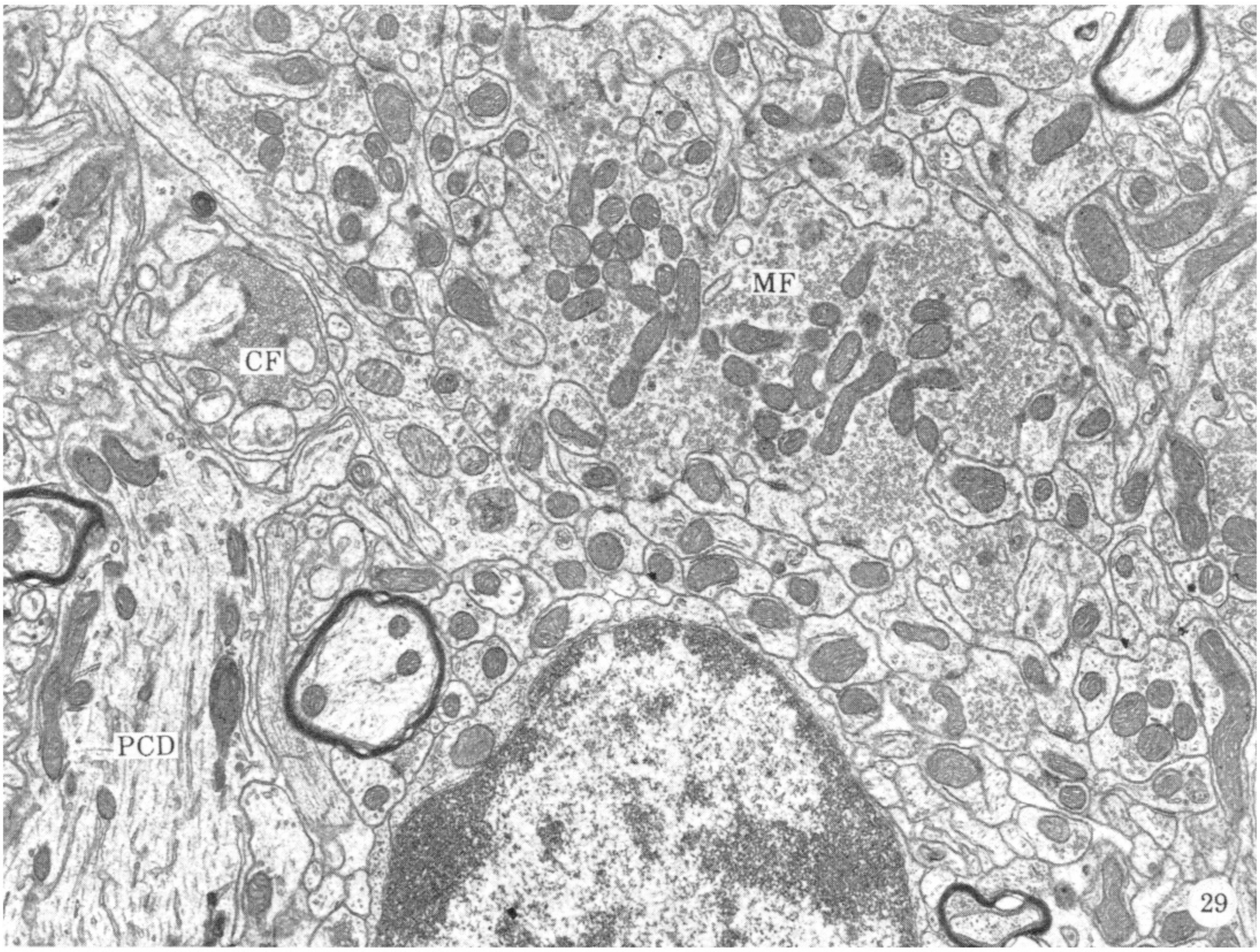
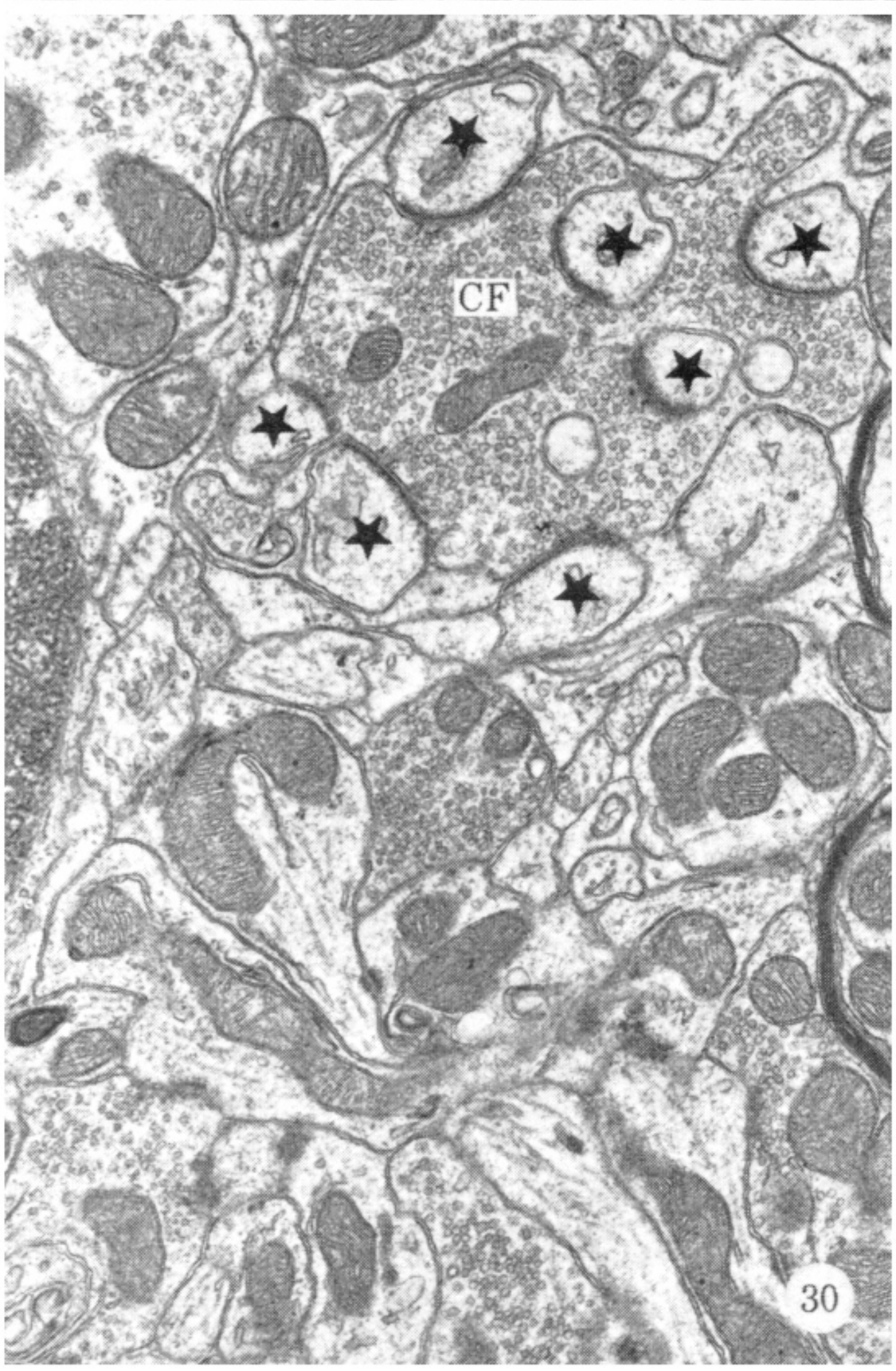


FIGURE 27. Typical appearance of the granular layer in *reeler*. A cerebellar glomerulus composed by a central mossy rosette (MF) and peripheral Golgi axons (Gax) is nearby a Purkinje cell dendritic profile (PCD). The spines (asterisks) of this dendrite are contacted by parallel fibres. (Magn. × 14000.)

FIGURE 28. Atypical glomerulus formed by mossy fibre (MF) synapsing on Golgi cell dendrites (GC). Small Purkinje cell dendritic profiles (large asterisks) are nearby the glomerulus. (Magn. × 15000.)





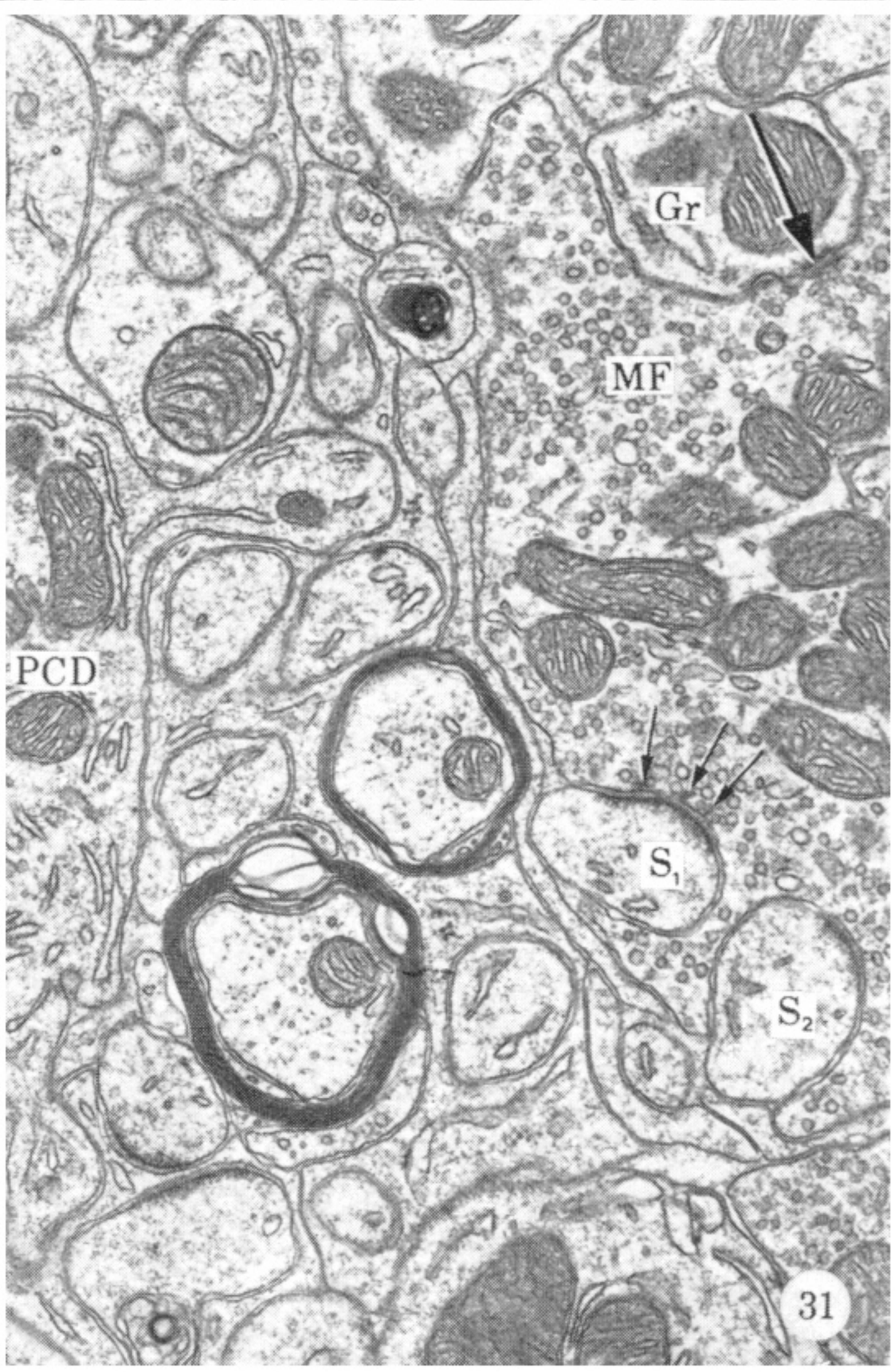
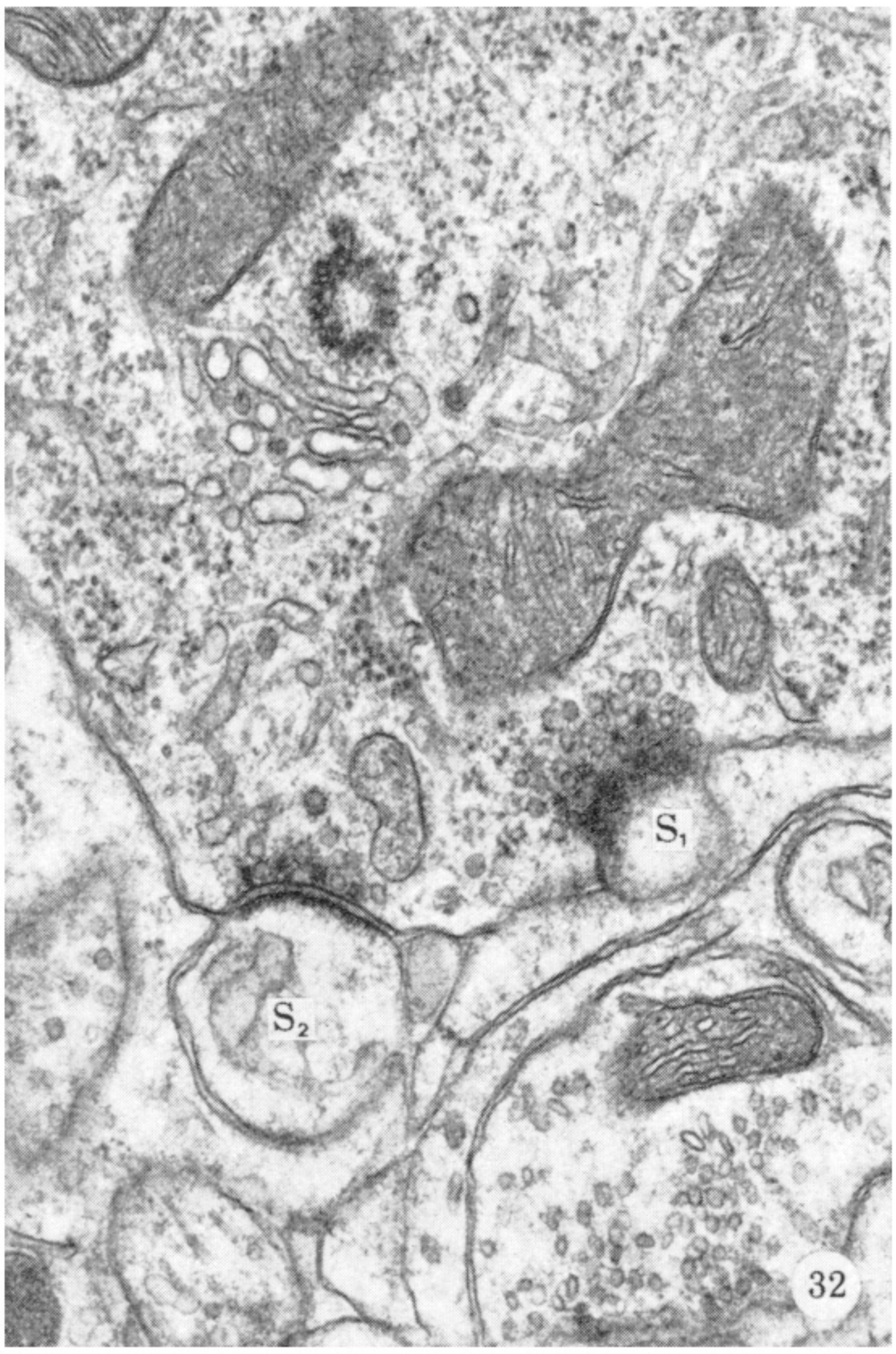
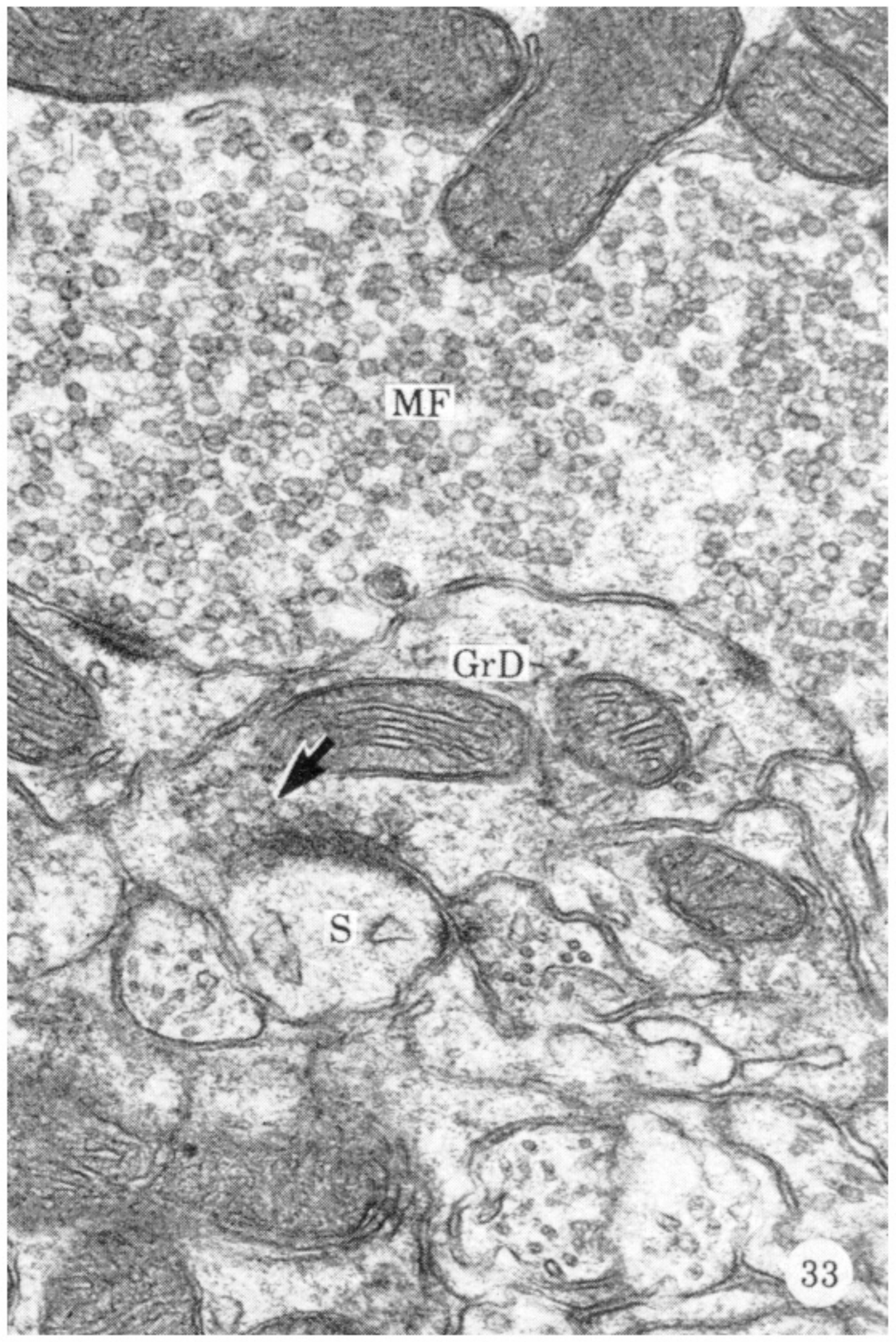


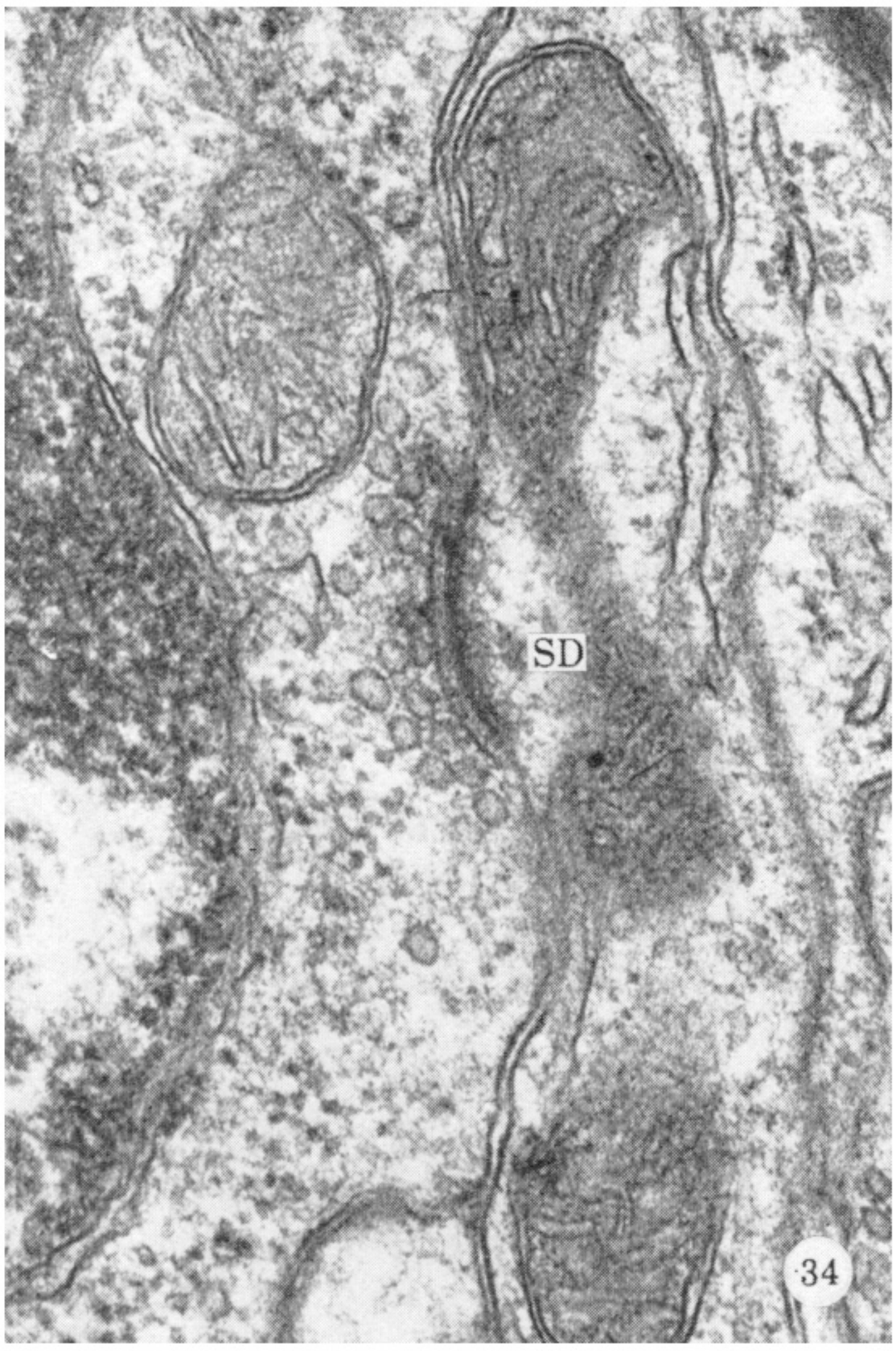
FIGURE 29. The left area of the micrograph is occupied by an ascending Purkinje cell dendrite (PCD) which receives a climbing varicosity (CF). The rest of the micrograph is occupied by neuronal elements characteristic of a granular layer. The two afferent systems, climbing fibres (CF) and mossy fibres (MF) are very close, but each one preserves its synaptic specificity. (Magn. × 12000.)

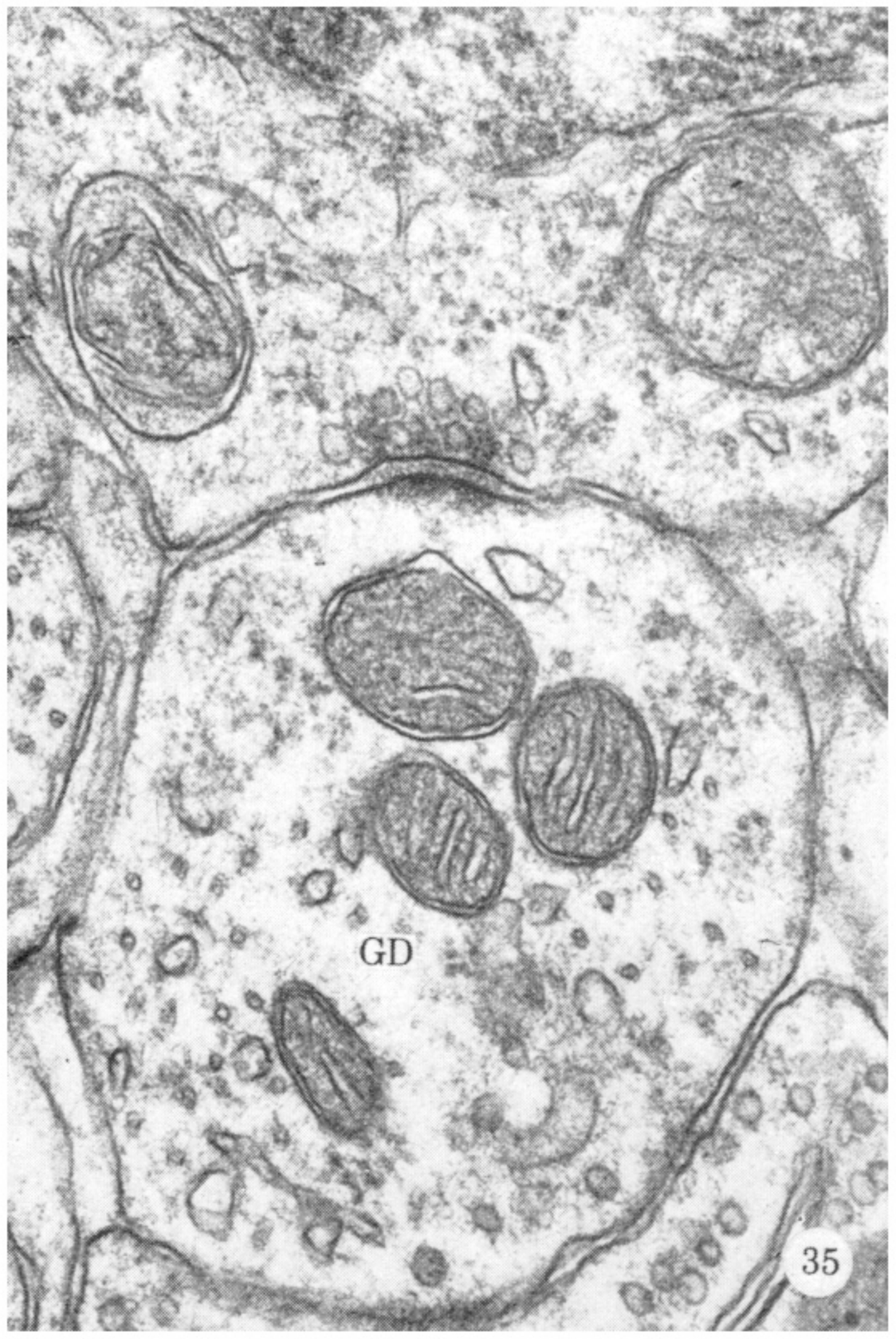
FIGURE 30. Climbing fibre varicosity (CF), within the granular layer, synapsing on numerous Purkinje spines (asterisks). (Magn. × 16000.)

FIGURE 31. Mossy fibre rosette (MF) synapsing on a granule cell (Gr) dendrite (large arrow). Two Purkinje cell dendritic spines (S_1 and S_2) are directly apposed to the mossy fibre. The mossy fibre has only developed a presynaptic vesicular grid (small arrows), morphological counterpart of a synaptic contact, facing one of the spines (S_1). (Magn. × 21000.)









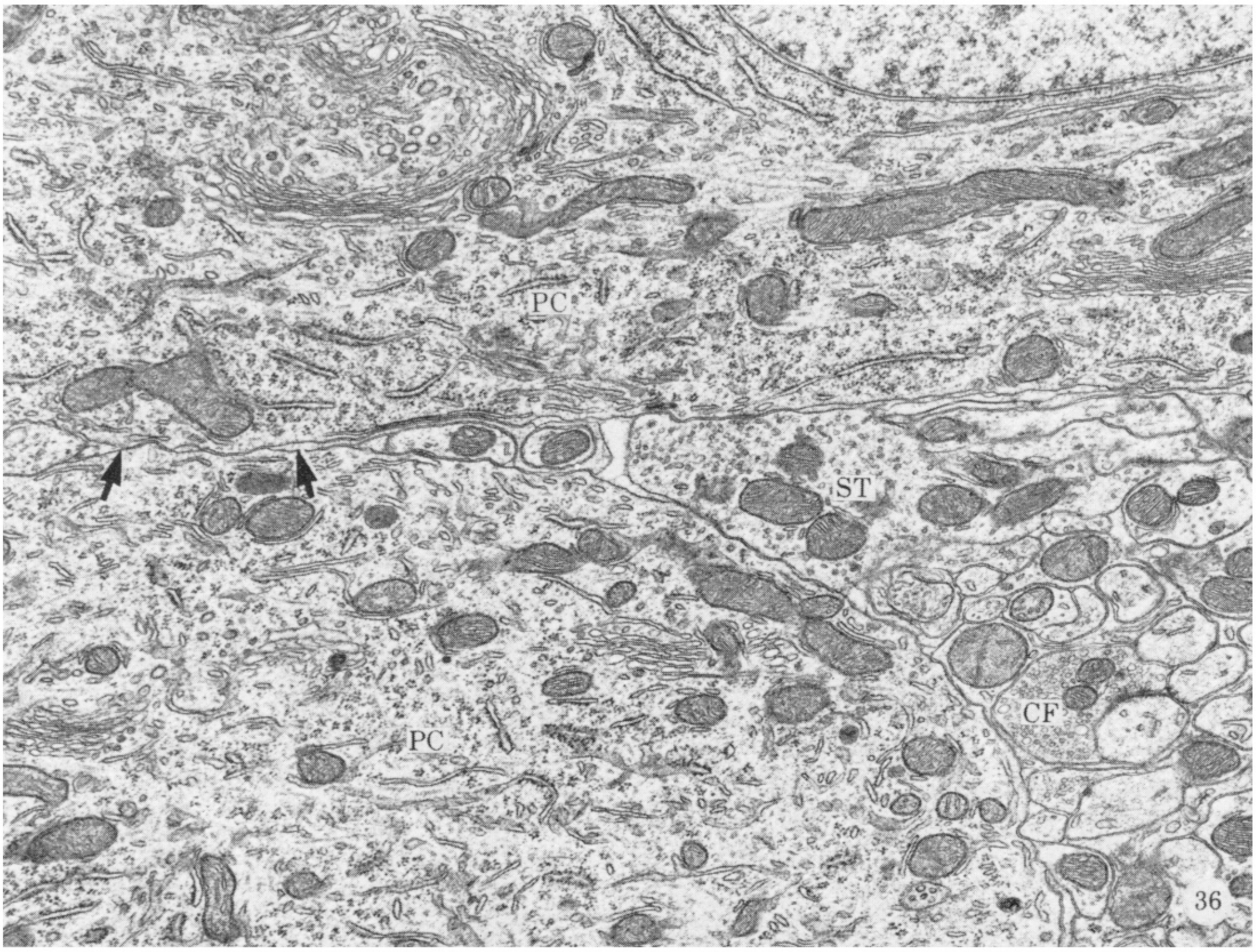
Somato-dendritic and dendro-dendritic ectopic synapses in the granular layer.

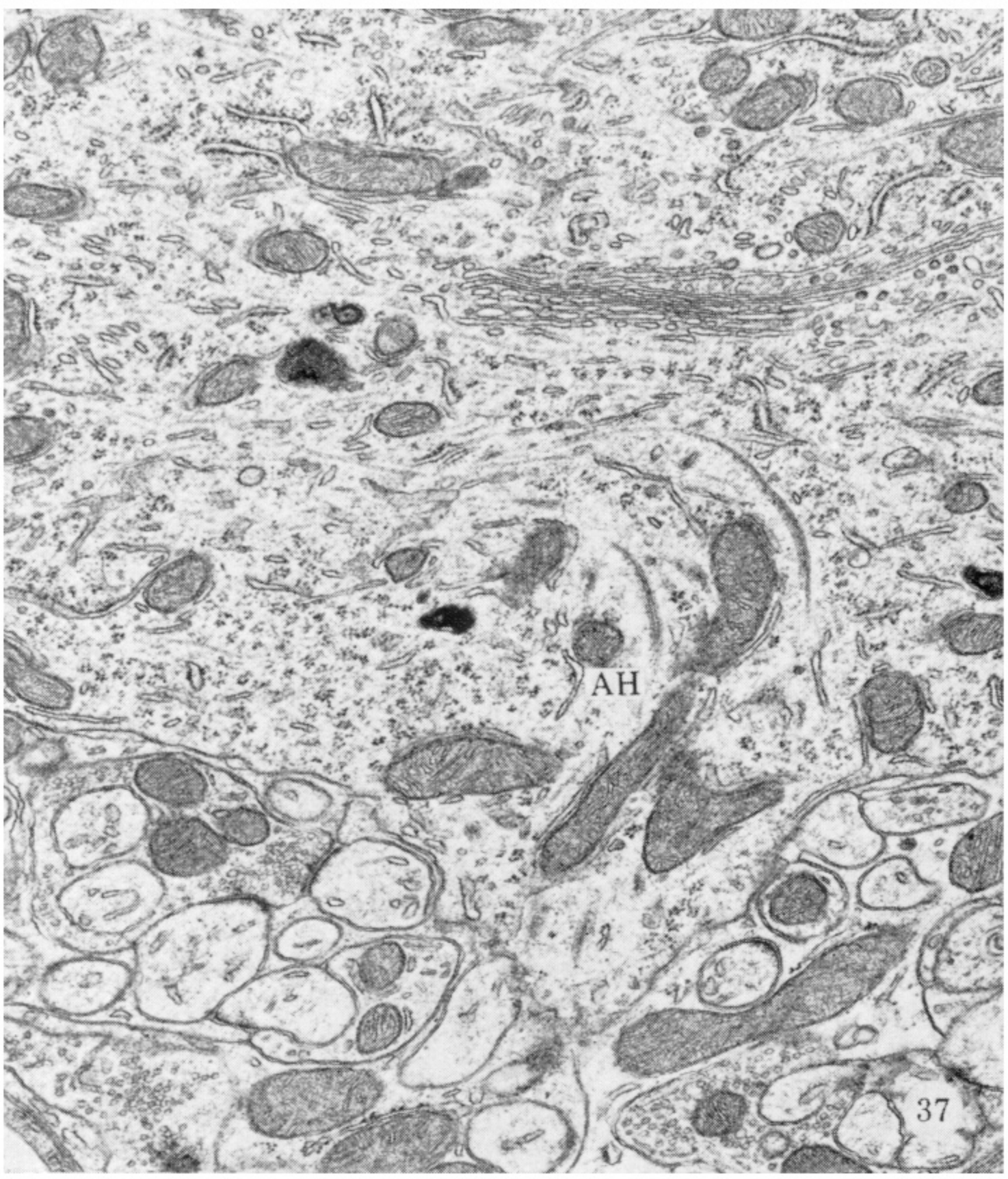
FIGURE 32. Two Purkinje cell dendritic spines (S_1, S_2) are postsynaptic to a granule cell perikaryon. (Magn. × 36 000.)

FIGURE 33. Cerebellar glomerulus. The mossy fibre (MF) is in synaptic contact with a granule cell dendrite (Gr D). The cytoplasm of this dendritic profile contains synaptic vesicles (arrow) forming an 'active' zone with a Purkinje cell dendritic spine (S). (Magn. × 39 000.)

FIGURE 34. Granule cell perikaryon forming an 'active' zone of the Gray type I on the surface of a small dendritic profile (SD), identified as belonging to an interneuron. (Magn. × 60 000.)

FIGURE 35. Granule cell perikaryon synapsing on a large dendritic profile probably belonging to a Golgi cell (GD). (Magn. × 54 000.)





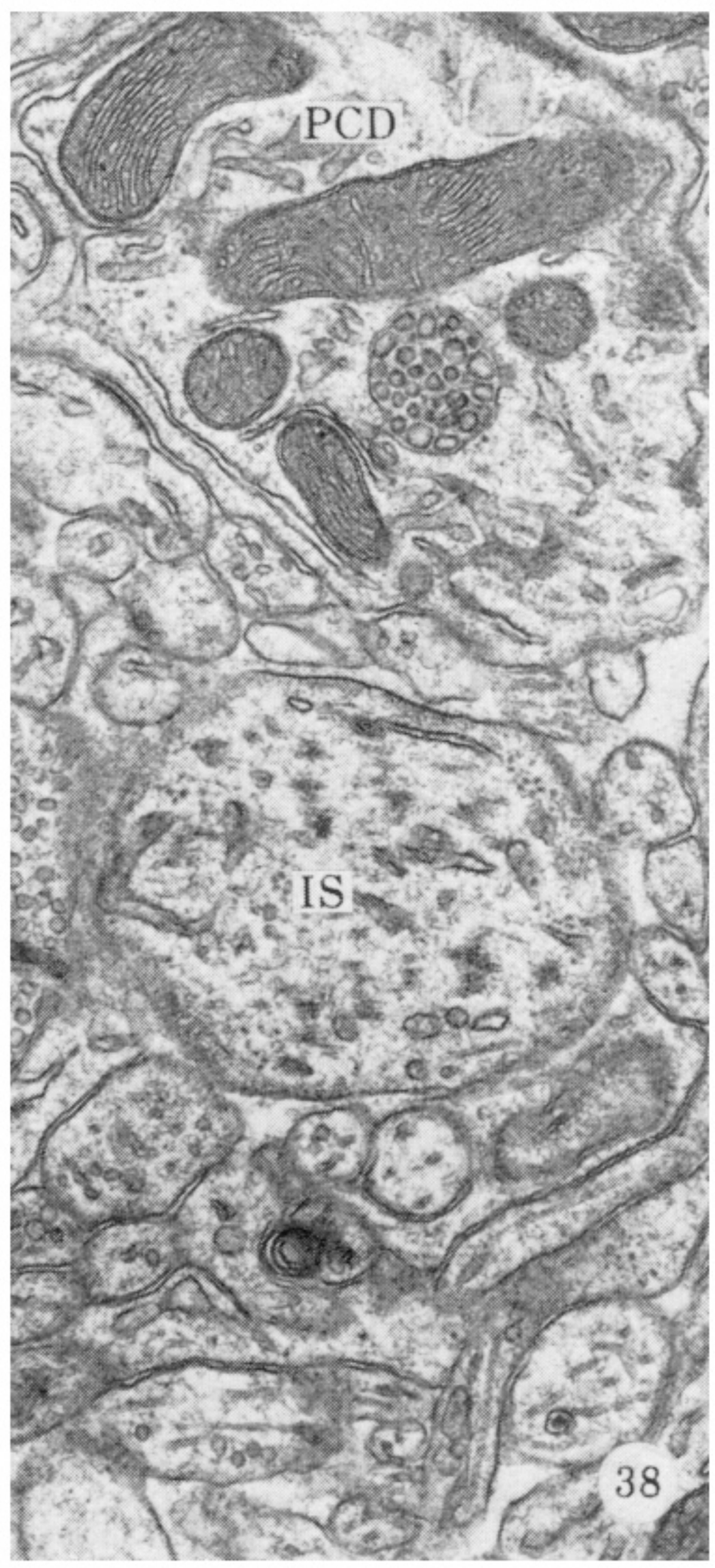


FIGURE 36. Two Purkinje cell perikarya (PC) in the central cerebellar mass. The two perikarya are very close. The arrows indicate a small area in which both perikarya are directly apposed. An axon terminal, probably belonging to a stellate-like interneuron (ST) establishes synaptic contacts on both perikarya. A climbing fibre varicosity (CF) is synapsing on a Purkinje cell spine. (Magn. × 14000.)

FIGURE 37. Axon hillock (AH) of a Purkinje cell located in the central cerebellar mass. Note that these neurons are not invested by basket fibres. (Magn. × 15000.)

FIGURE 38. Oblique section of the initial segment (IS) of a Purkinje cell axon located in the central cerebellar mass. Note the absence of a pinceau formation. (Magn. × 24000.)

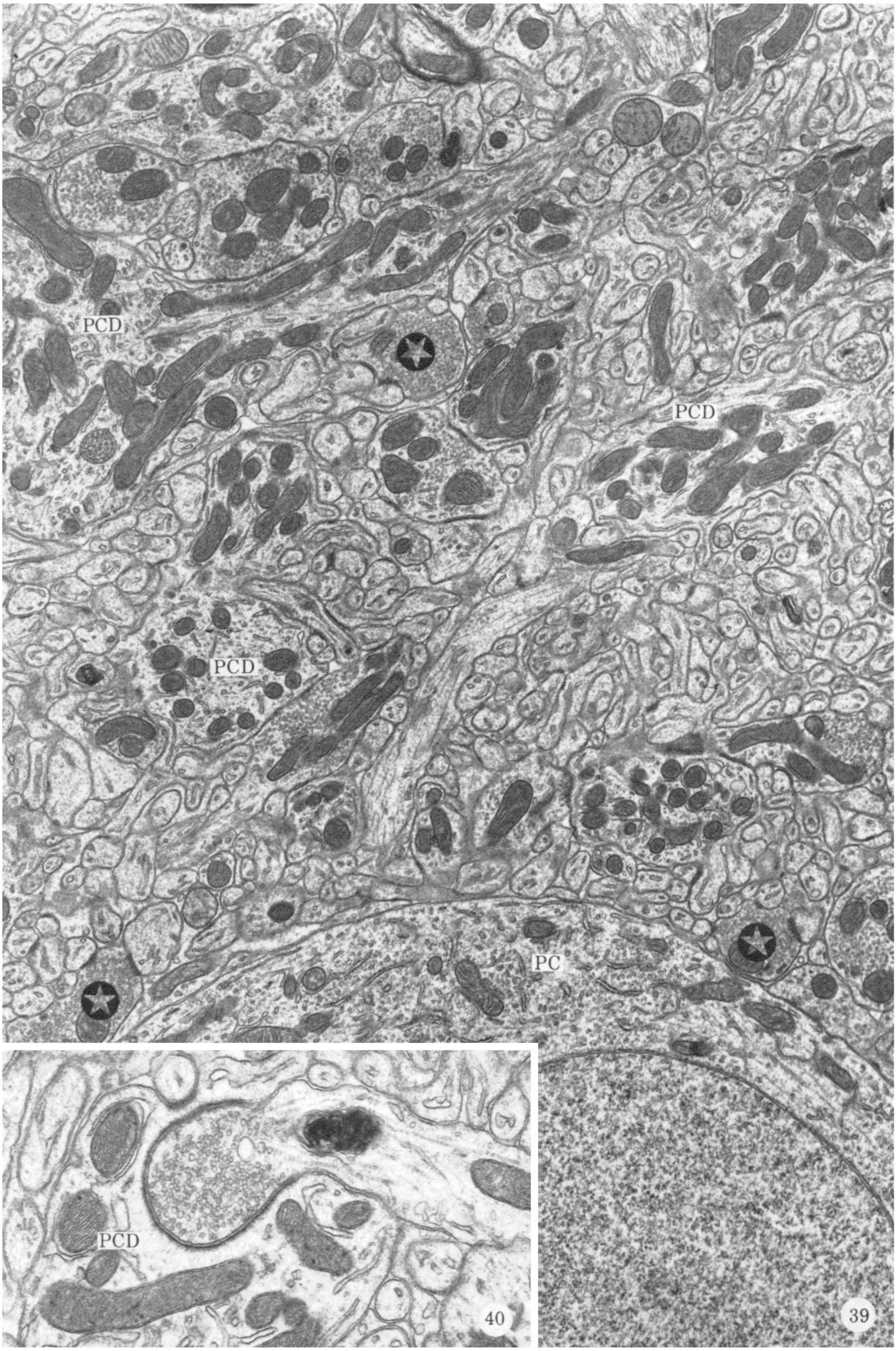
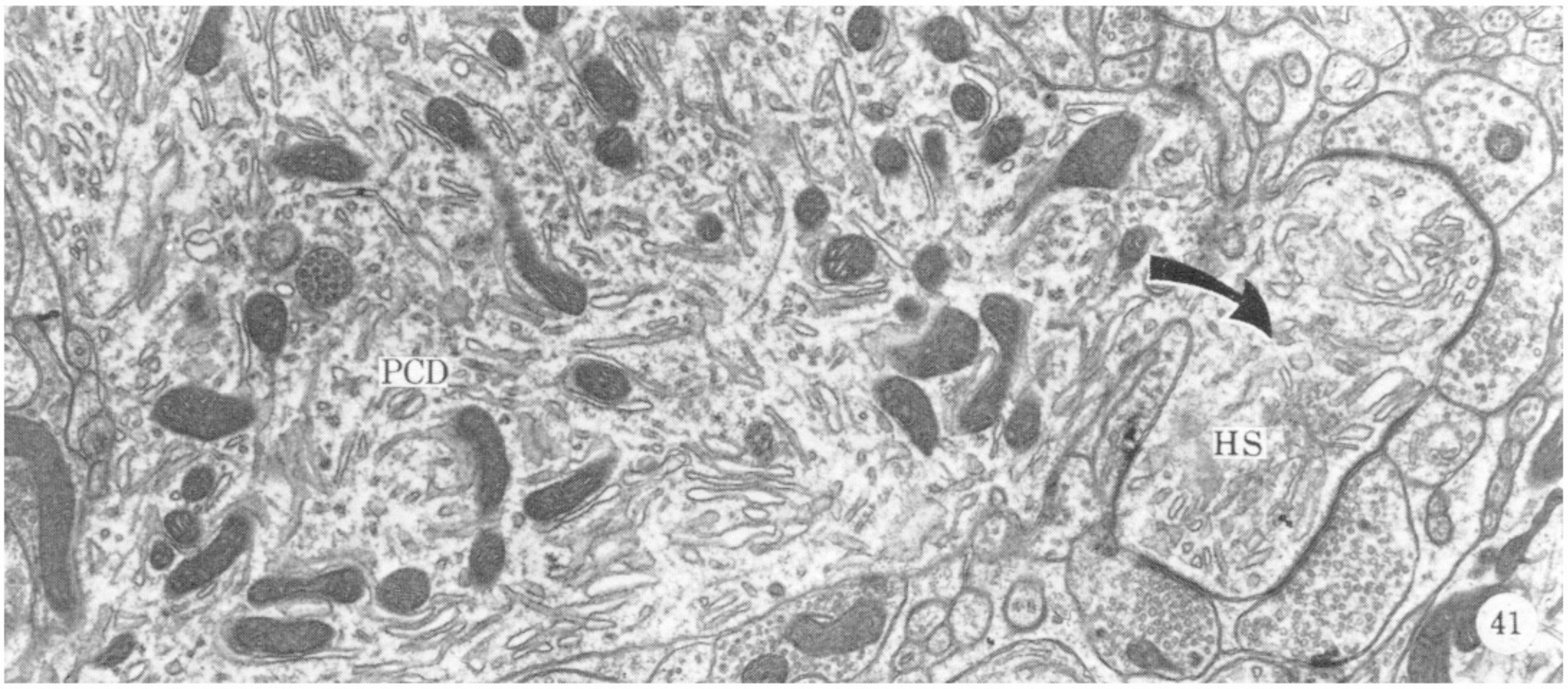


FIGURE 39. Purkinje cell perikaryon (PC) and surrounding neuropil in the central cerebellar mass. This neuropil is almost exclusively formed by Purkinje cell dendritic profiles (PCD) and the innumerables free spines emerging from them. Three climbing fibre varicosities (black circles with transparent stars) synapse on some of these spines. (Magn. × 12000.)

FIGURE 40. Purkinje cell dendrite (PCD) invaginated by an unidentified profile filled with spherical synaptic vesicles. The dendritic membrane invaginated by this profile exhibits a postsynaptic-like differentiation. The unidentified profile does not develop a 'presynaptic vesicular grid'. (Magn. × 23000.)

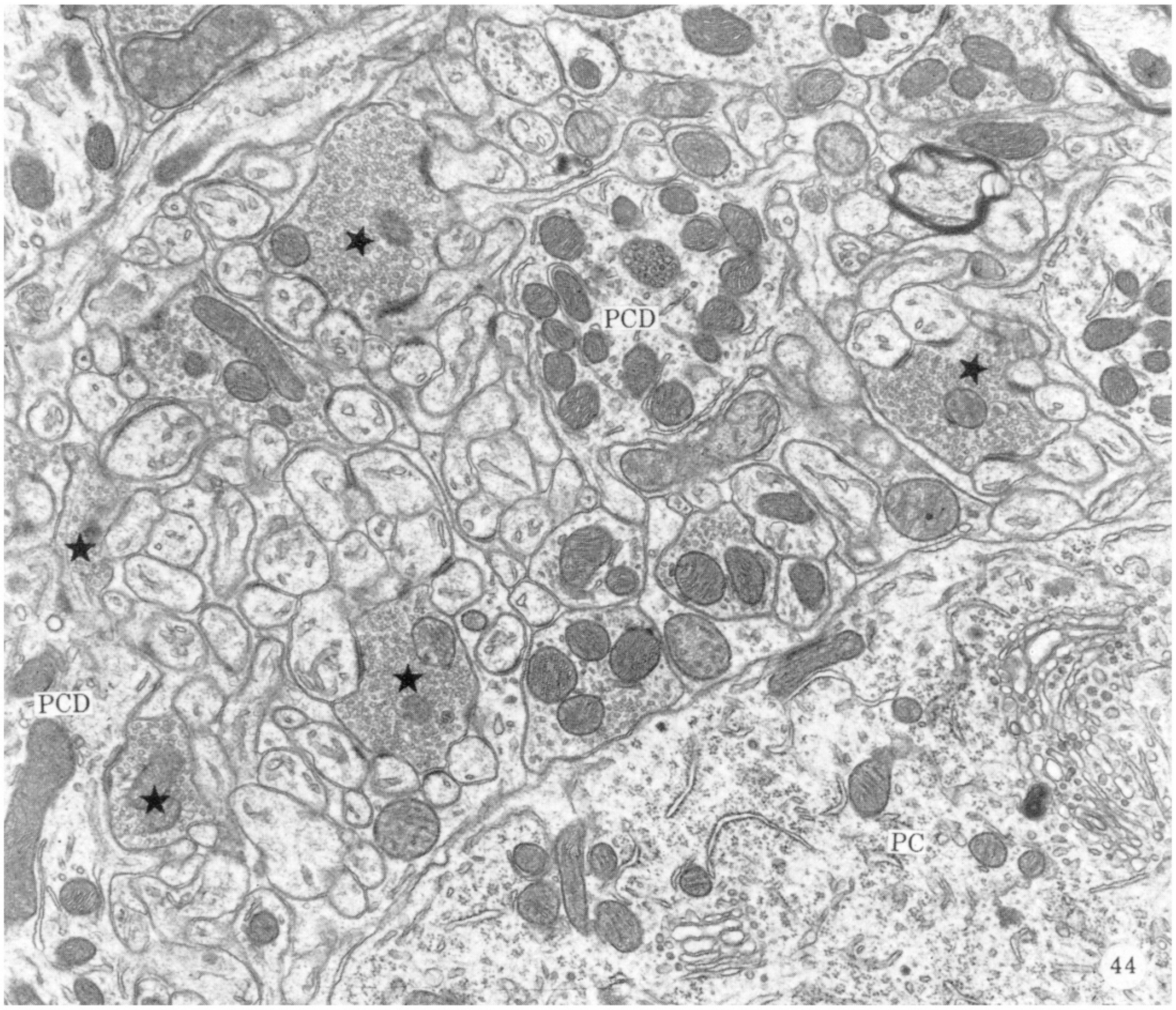


Electron micrographs of hypertrophic spines (HS) or dendritic protrusions emerging from Purkinje cell dendrites (PCD) and observed in the central cerebellar mass.

FIGURE 41. The neck of the hypertrophic spine is visible (arrow). Almost all the cytoplasmic surface of this profile is undercoated by a postsynaptic-like differentiation, which receives numerous synaptic boutons. (Magn. × 14000.)

FIGURE 42. Note the undercoating of the membrane, the fine granular material of the cytoplasmic matrix and the large amount of smooth cisterns of the endoplasmic reticulum, which characterize dendritic spines. (Magn. × 15000.)

FIGURE 43. In this protrusion, in addition to the organelles present on spines, there are some rare microtubules and mitochondria. (Magn. × 19000.)



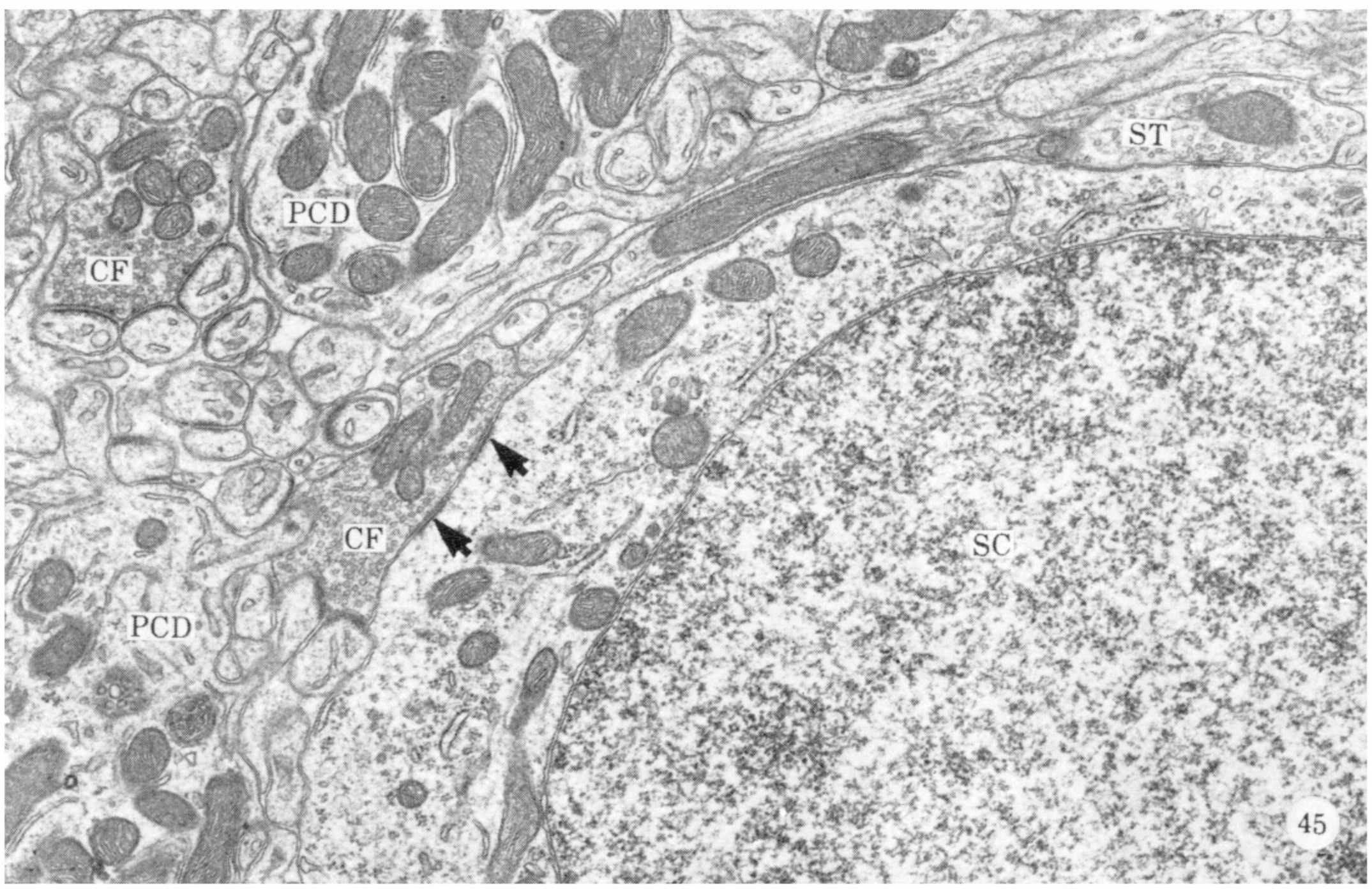


FIGURE 44. Increased density of climbing fibre varicosities (black stars) in the neuropil of the central cerebellar mass. A Purkinje cell perikaryon (PC) with its typical subsurface cistern and associated mitochondrion, occupies the lower right corner of the micrograph. Purkinje cell dendrites (PCD) and numerous free spines characterize this neuropil. (Magn. × 16000.)

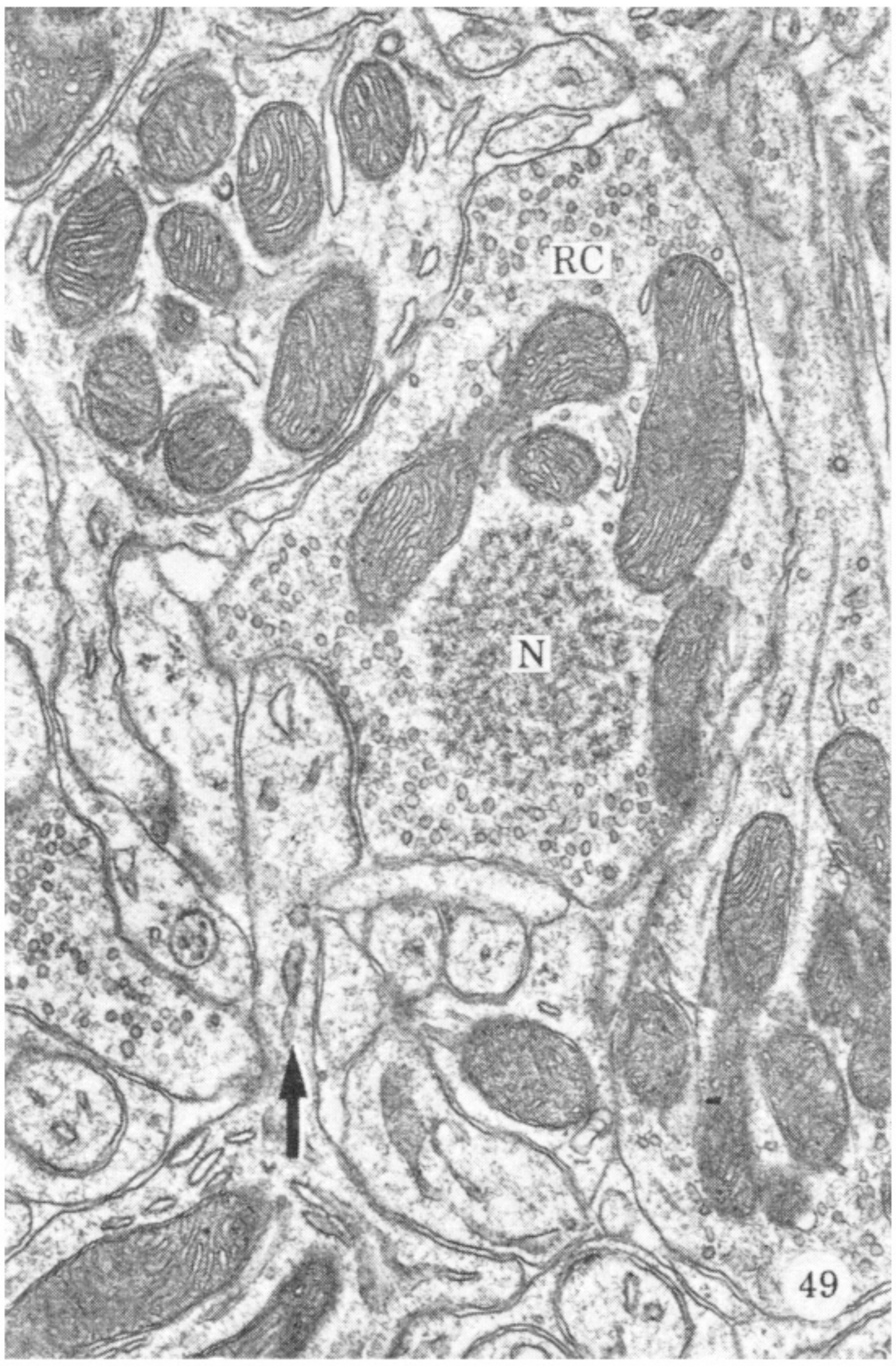
FIGURE 45. Central cerebellar mass. A perikaryon of a stellate-like neuron (SC) receives a stellate axon terminal (ST) and a climbing varicosity (CF). The arrows point to the postsynaptic densities of this Scheibel's collateral. Purkinje cell dendrites (PCD) and their free spines form the neighbouring neuropil. (Magn. × 16000.)

Heterologous synapses between mossy fibres (MF) and Purkinje cell dendritic spines in the central cerebellar mass.

FIGURE 46. A Purkinje cell dendrite (PCD) gives rise to two spines (S) within the plane of the section (arrows). One of them is facing a mossy terminal (MF), which has developed a 'presynaptic vesicular grid' at the zone of contact. The other one (right low corner), although facing an axon terminal, is free of innervation. (Magn. × 25000.)

FIGURE 47. A mossy terminal (MF) synapsing on five spines (black stars), probably arising from nearby Purkinje cell dendrites (PCD). (Magn. × 16000.)

FIGURE 48. A mossy terminal (MF) establishes a synaptic contact on a hypertrophic spine (HS) arising from (arrow) a small Purkinje cell dendritic profile (PCD). (Magn. × 20000.)



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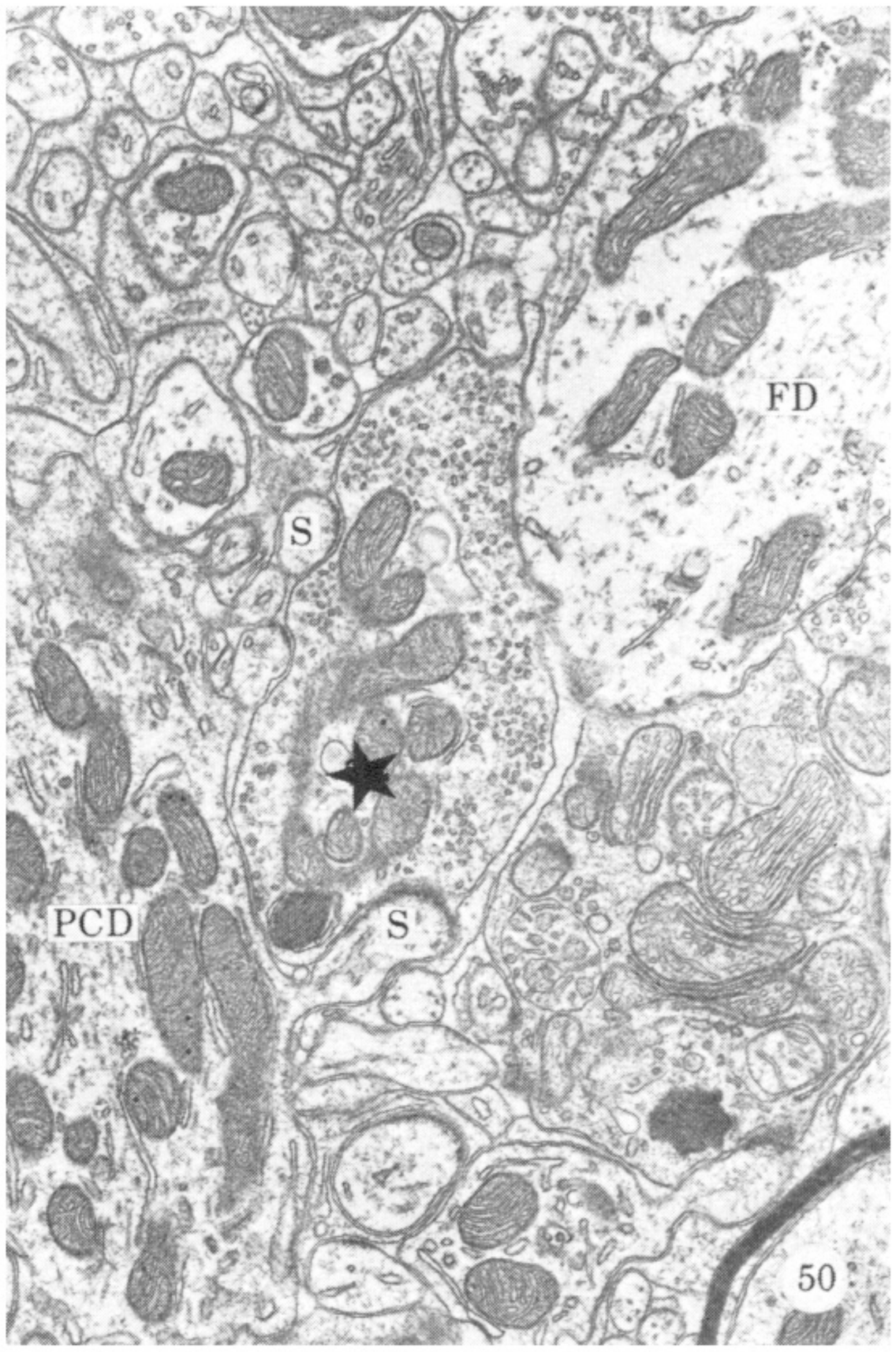

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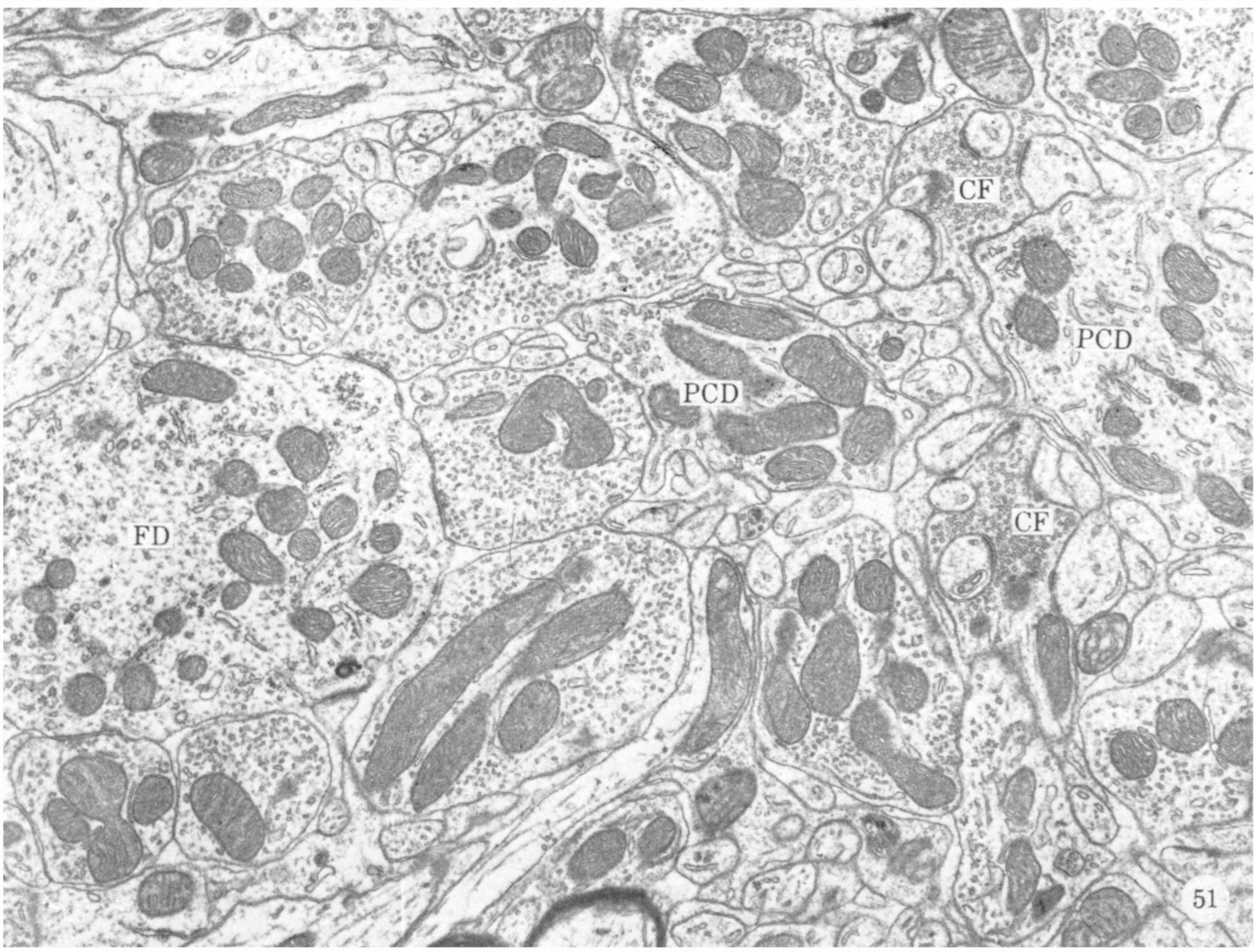

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FIGURE 49. Recurrent collateral of a Purkinje cell axon (RC) synapsing on a spine arising from (arrow) a Purkinje cell dendrite. Note the presence of a nematosome (N) in the axoplasm of the terminal. (Magn. × 23000.)

FIGURE 50. Purkinje cell dendrite (PCD), within the deep cerebellar nuclei, in close relation with a dendrite from a fastigial neuron (FD). An altered axon terminal probably belonging to a Purkinje axon is in synaptic contact with the fastigial neuron. A normal terminal containing pleomorphic synaptic vesicles (black star) is directly apposed to the Purkinje cell dendrite, and two of its spines (S), as well as to the fastigial neuron. (Magn. × 14000.)

FIGURE 51. Purkinje cell dendrites (PCD) and their normal synaptic investment, climbing fibres (CF) are intermingled with neuronal profiles characterizing the fastigial nucleus. This electron-micrograph illustrates the invasion of disorientated Purkinje cell dendrites through the fastigial nucleus. (Magn. × 13000.)