Physiological and morphological organization of the cerebellar circuits in various vertebrates

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Since this symposium is dedicated to the study of phylogenetic development of the cerebellum, we consider it appropriate to present a general anatomical and physiological paper on the types of cerebellar circuits so far encountered in the different terrestrial vertebrates we have studied. The main subjects of this paper will be certain varieties of frog (*Rana catesbiana*), alligator (*Caiman sclerops*), pigeon (*Columba livia*) and cat. Although *Rana catesbiana* may not be completely representative of a typical amphibian, we have chosen to study this animal because it is easy to obtain and maintain and has, for an amphibian, a rather large cerebellum. It is our belief that the cerebellum of this amphibian is not only a simple but also a primitive structure, even when compared with that of allegedly more primitive vertebrates such as elasmobranchs (Stensiö, 1963; Jarvik, 1968).

Broadly speaking, the cerebellar circuitry can be said to have changed very little throughout phylogeny as judged by the comparative studies of the different vertebrates available at this time. This regularity of cerebellar circuitry was first noticed by Ramón y Cajal who expressed the view that the constant character of the neuronal organization of the cerebellum in different species made the cerebellar circuit almost a "biological law" (1904). Given this organizational uniformity throughout evolution, we have studied morphologically and functionally the cerebellar cortex of these vertebrates in order to recognize those characteristics which these cerebella have in common as well as those in which they differ. The main purpose of this paper is to attempt to define further the nature of the "basic cerebellar circuit" (Llinás, 1969), assuming that the cerebellar organization is basically the same for all vertebrates and that the differences which arise between species are more the product of adaptations of a basic circuit to particular conditions than an indication of truly different functional properties.

According to our own views on cerebellar neurobiology, an important key to the understanding of the organization of the cerebellar cortex rests on the assumption that the two well known cerebellar afferents—the climbing fiber-Purkinje cell pathway and the mossy fiber-granule cell pathway, which seem to be present in all animals so far studied—function as independent systems.

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Comparative aspects of the climbing fiber-Purkinje cell system

General morphology. As illustrated in color Figs. 1-4, the climbing fibers of the varieties of frog, alligator, pigeon and cat studied here show, in each case, a similar synaptic relationship with the dendrites of their respective Purkinje cells. In every instance the climbing fibers establish a monosynaptic contact of en-passage character which takes place for the most part, as first described by Larramendi and Victor (1967), with small spines of the Purkinje cell dendrites. Also characteristic of this system is the enormous number of synaptic junctions between a single climbing fiber and the dendrites of a Purkinje cell.

A recent calculation of the number of contacts which a climbing fiber establishes on a single frog Purkinje cell has given a total of 300 synapses (Llinás, Bloedel and Hillman, 1969). Similar counts in the alligator (Hillman, 1969b) have yielded a slightly smaller number of contacts between climbing fibers and Purkinje cell dendrites. Further quantitative studies of climbing fiber-Purkinje ceil relations are now in progress for the pigeon and cat. It is our feeling, however, that the number of climbing fiber-Purkinje cell synapses is fairly constant throughout evolution and that only a small reduction in the number of synapses occurs as the animals develop phylogenetically. An interesting variation on this theme, on the other hand, is presented by the rather small climbing fibers in elasmobranchs, suggesting that the climbing fiber input should be much weaker in this fish than its counterpart in terrestrial vertebrates, which indeed seems to be so from an electrophysiological point of view (Nicholson, Llinás and Precht, 1969). The actual length of climbing fibers is not known for teleosts, but their existence has been suggested in mormyrids (Kaiserman-Abramof and Palay, 1969; Nieuwenhuys and Nicholson, 1969) and in other teleosts (Schnitzlein and Faucette, 1969).

Electron microscopical organization of the climbing fiber-Purkinje cell synapse

The close relationship of the climbing fiber to the Purkinje cell dendritic tree, as first described by Ramón y Cajal with light microscopical techniques (1888), is also evident in electron microscopical study. Since the climbing fiber follows the so-called smooth branches of the Purkinje cell dendritic tree, its profiles are seen to lie next to large dendrites (Fig. 5 a to c). At electron microscopical level, the climbing fiber and the Purkinje cell dendrite are enclosed in a glial encapsulation formed by numerous glial processes. The synaptic relationship, previously believed to occur directly between the dendrite and the climbing fiber, has recently been shown to be actually on the spines (Larramendi and Victor, 1967) which stem from the so-called smooth dendritic branches (Fig. 5 d to f). A possible functional meaning for the termination of climbing fibers on the smooth dendrite spines will be discussed in the second part of this paper.

These smooth dendrite spines emerge singly or in groups along the entwining course of this axon, and range in size from mere tuberances to long shafts with a distal club, which in some cases completely circumscribe the climbing fiber. The synapse is characteristic of the type I of Gray with its distinct postsynaptic thickening as shown by Larramendi and Victor (1967) in the mouse. The climbing fiber synaptic vesicles are characterized by their round appearance and by their wide range of size, unlike the parallel fiber vesicles which are uniform in diameter (Fig. 5 d to f).

Dense core vesicles occur more frequently in climbing fibers than in other synaptic boutons of the molecular layer. Usually the synaptic vesicles are arranged closely together in a relatively regular pattern somewhat like those found in parallel fibers. In the four types of animals studied here, the morphological characteristics of climbing fibers are for the most part quite uniform (Figs. 1 to 4 and Fig. 5 d to f). However, certain differences are obvious. In the frog, two or three thin climbing fiber filaments may cover each Purkinje cell dendritic segment (Fig. 5a). In the alligator, the climbing fiber tends to have single (Fig. 5b), or occasionally double, filaments along the Purkinje cell dendritic tree. In contrast, the cat (as shown by Ramón y Cajal, 1911) has numerous axon branches which, as in the frog, ramify along the dendrite (Fig. 4). This branching pattern is evident in electron microscopy by the number of profiles which occur on various sides of a given Purkinje cell dendrite or by the large diameter of the single climbing fiber filament in the alligator in contrast to their smaller size in frog and cat. Additionally, it appears that the smaller axonic arborization tends to contain numerous tubules as in the case of the frog (Hillman, 1969a).

Climbing fiber activation of the Purkinje cell in different vertebrates

As first described in the cat (Eccles, Llinás and Sasaki, 1966a), the climbing fiber-Purkinje cell interaction has so far been found to be excitatory in every animal studied (Llinás, Hillman and Precht, 1969b). In the frog, as in the cat, white matter stimulation of the cerebellum evokes, besides the antidromic activation of the Purkinje cell (Fig. 6A), an all-or-none burst of action potentials in this neurone (Fig. 6B). This burst of spikes is characterized extracellularly by a large positive-negative spike followed by a high frequency discharge of smaller amplitude. In addition to its all-or-none character, the discharge can be typified by the highly stereotyped character of the extracellular potential. In fact, many sweeps can be superimposed with hardly any variation of the waveform of the Purkinje cell response when the microelectrode is located in a stable position with respect to a Purkinje cell. This all-or-none extracellular burst response can also be observed in the alligator, pigeon (Fig. 6 EF, IJ, LM) (Llinás *et al.*, 1969b), and cat (Eccles *et al.*, 1966a). The Purkinje cell burst response was first described by Granit and Phillips (1956) in the cerebellum of the cat and then observed again in the frog (Matthews, Phillips and Rushworth, 1958). These authors concluded that the Purkinje cells could be activated in two general ways: by production of simple spikes which could respond at different frequencies, or in a burst-like fashion. The correlation of these two forms of activation with particular morphological studies in the cerebellum was only recently suggested with appropriate experimental support (Eccles *et al.*, 1966a). In studying the cerebellum of the cat, it became evident that the burst response only occurred following stimulation of particular loci such as the underlying white matter or the inferior olive. As far as the cat is concerned, the final demonstration that the burst response was brought about by the activation of climbing fibers was attained when, following deafferentation of the cerebellar cortex, it was found that only simple spikes could be obtained regardless of whether the stimulus was given to the white matter or to the surface of the cortex (Eccles, Llinás and Sasaki, 1966b). This suggested that a) the bursts were related to a particular afferent system and b) the particular afferent system was extrinsic to the cerebellar cortex. Both of these prerequisites are fulfilled by the climbing fiber system.

The relation between Purkinje cell bursts and climbing fibers was then studied in the frog (Llinás and Bloedel, 1966/67; Llinás, Precht and Kitai, 1967). Again in this system, it was found that they could only be evoked by either white matter or peripheral nerve stimulation and not by stimulation of the surface of the cerebellum, i.e.,

FROG CEREBELLUM

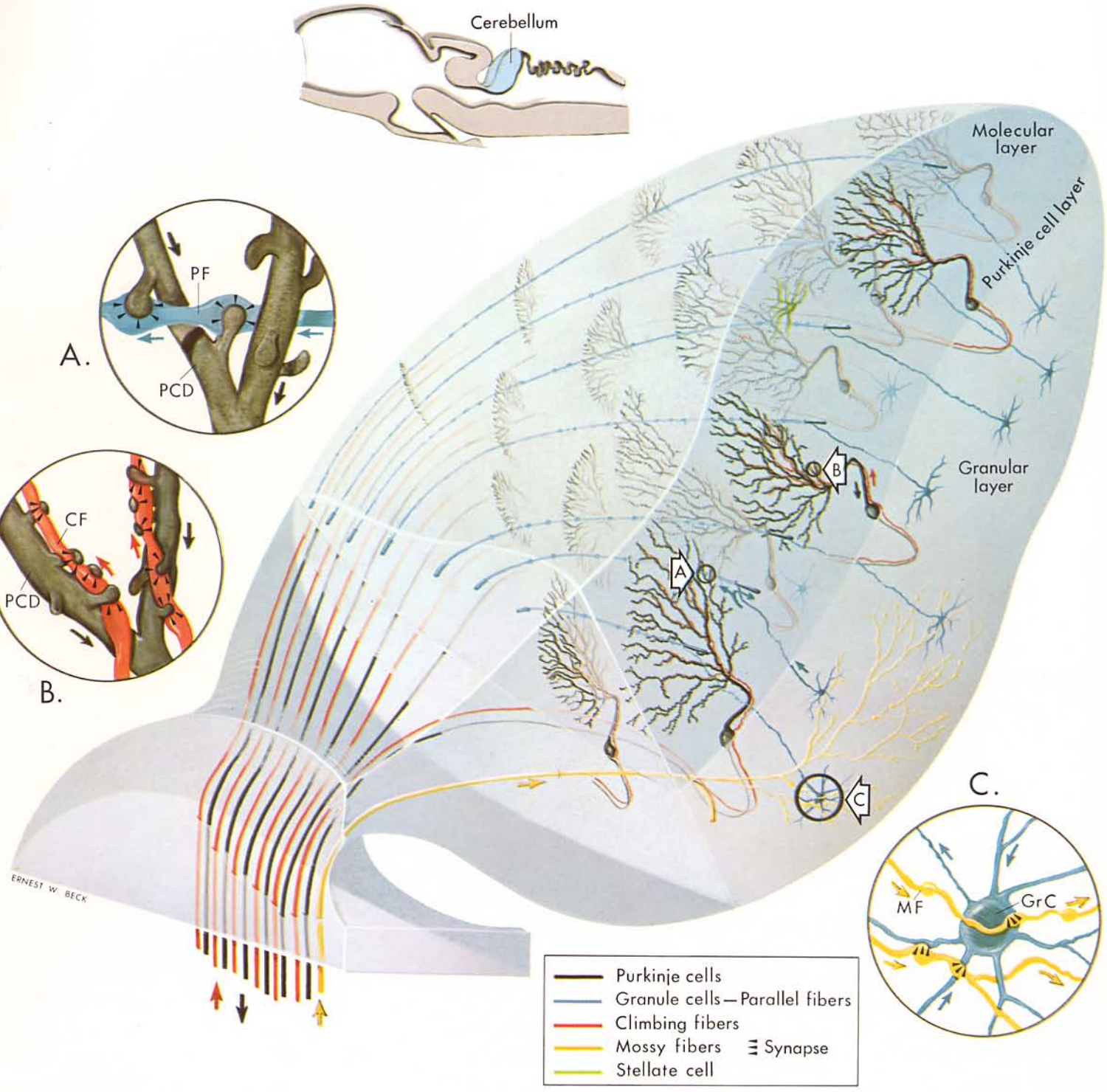

Fig. 1. Frog cerebellar cortex. The location of the cerebellum in the central nervous system is shown in the small diagram in the upper portion of this picture as a light blue region. In the diagram below, the right side of the cerebellum is shown in hemisection. The different neuronal elements are shown in different colors (see color key) and particular loci have been enlarged (call-outs A to C) to illustrate the synaptic relationships between elements at a magnification comparable to that obtained by electron microscopical means. In A: PCD, Purkinje cell dendrite; PF, parallel fiber. In B: PCD, Purkinje cell dendrite; CF, climbing fiber. In C: GrC, granule cell; MF, mossy fiber. Note that the parallel fiber establishes synaptic relation with the spines of the Purkinje cell dendrites (A) and a similar situation is found between the climbing fibers and dendrites of the Purkinje cells (B) while the mossy fiber, which in this animal does not form a glomerulus, ends directly as an en passage type of synapse contacting the dendrites and soma of a rather primitive looking granule cell. In these and subsequent figures, note that in all call-outs the direction of nerve conduction is shown by colored arrows while the actual place of synaptic junction is illustrated by means of small arrowheads.

ALLIGATOR CEREBELLUM

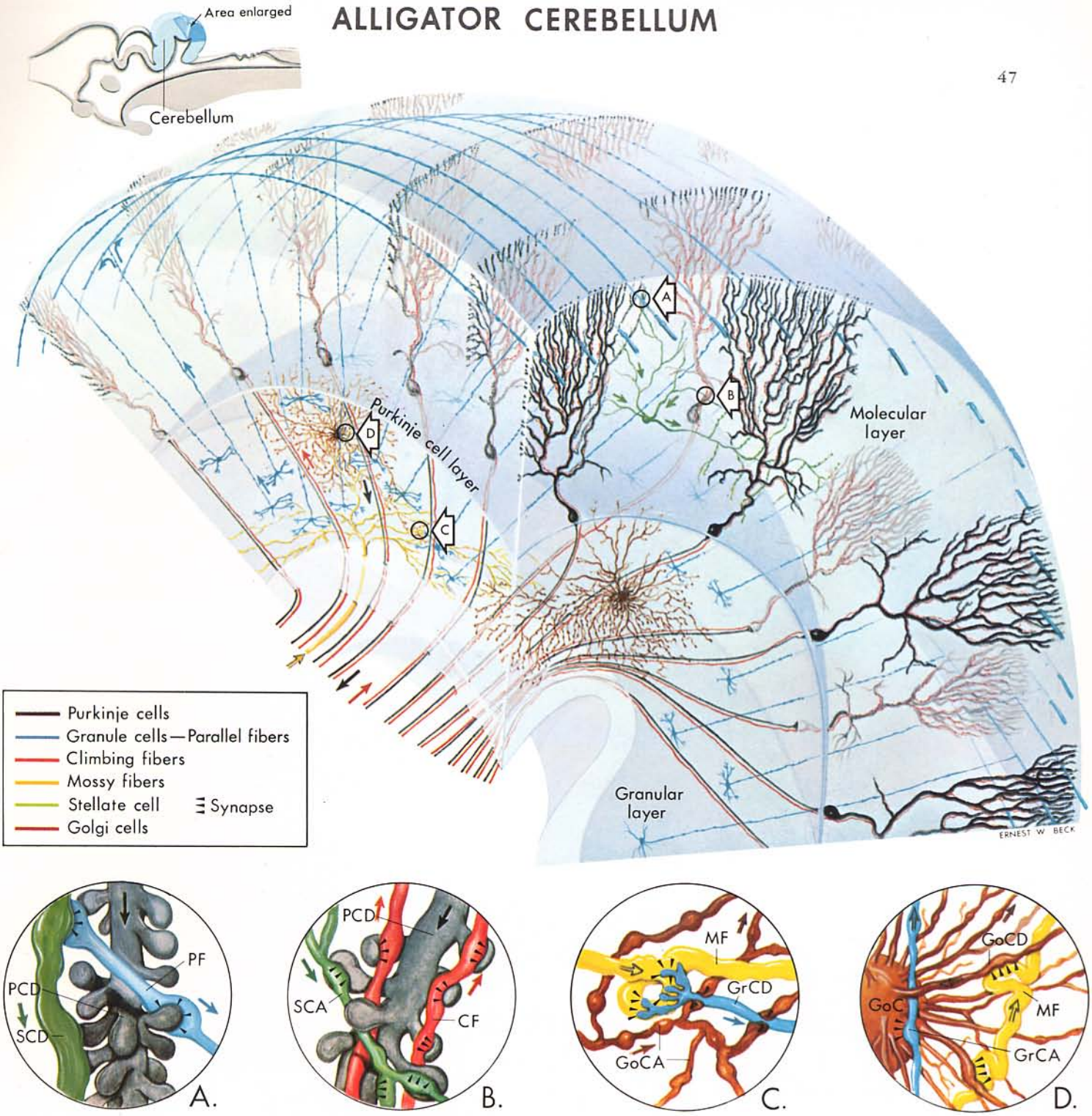

Fig. 2. As in Fig. 1, the cerebellum is shown in a light blue while the area depicted in the diagram below is shown in dark blue. As in Fig. 1, each neuronal element is shown in a different color (see color key). Call-outs A to D point out particular synaptic relationships. In A: PCD, Purkinje cell dendrite; SCD, stellate cell dendrite; PF, parallel fiber. The parallel fiber establishes synaptic contact with the spines of Purkinje cell dendrites and terminates in direct contact with the dendrite of a stellate cell. In B: PCD, Purkinje cell dendrite; CF, climbing fiber; SCA, stellate cell axon. The synaptic organization here is such that the stellate cell axon establishes synaptic contact with the spines as well as with the main stem of the dendrite of the Purkinje cell. Two climbing fibers are shown in contrast with this Purkinje cell branch. This situation is not often found in the alligator cerebellum since in this animal (as opposed to the others illustrated in this series of diagrams) there is for the most part only one climbing fiber filament per dendritic branch. In C: The synaptic organization of the cerebellar glomerulus. The glomerulus is formed by three elements, the mossy fiber (MF), a granule cell dendrite (GrCD) and the Golgi cell axon (GoCA). The mossy fiber-granule cell junction takes place between an expanded portion of the mossy fiber and a claw-like terminal of the granule cell dendrite. In some cases, however, as illustrated here the mossy fiber demonstrates a small convolution at the synaptic site. The Golgi cell axons, on the other hand, quite often terminate at the place where the dendritic claw joins the main dendritic branch, as well as throughout the length of the branch. In D: GoC, Golgi cell; GoCD, Golgi cell dendrite; MF, mossy fiber; GrCA, granule cell axon. The mossy fiber establishes direct synaptic contact with the dendrites of the Golgi cells. Finally, an ascending axon of the granule cell is shown in synaptic apposition with the soma of the Golgi cell as it projects towards the molecular layer. In this drawing the Golgi cell is shown to have two distinct sites for axonal origin.

PIGEON CEREBELLUM

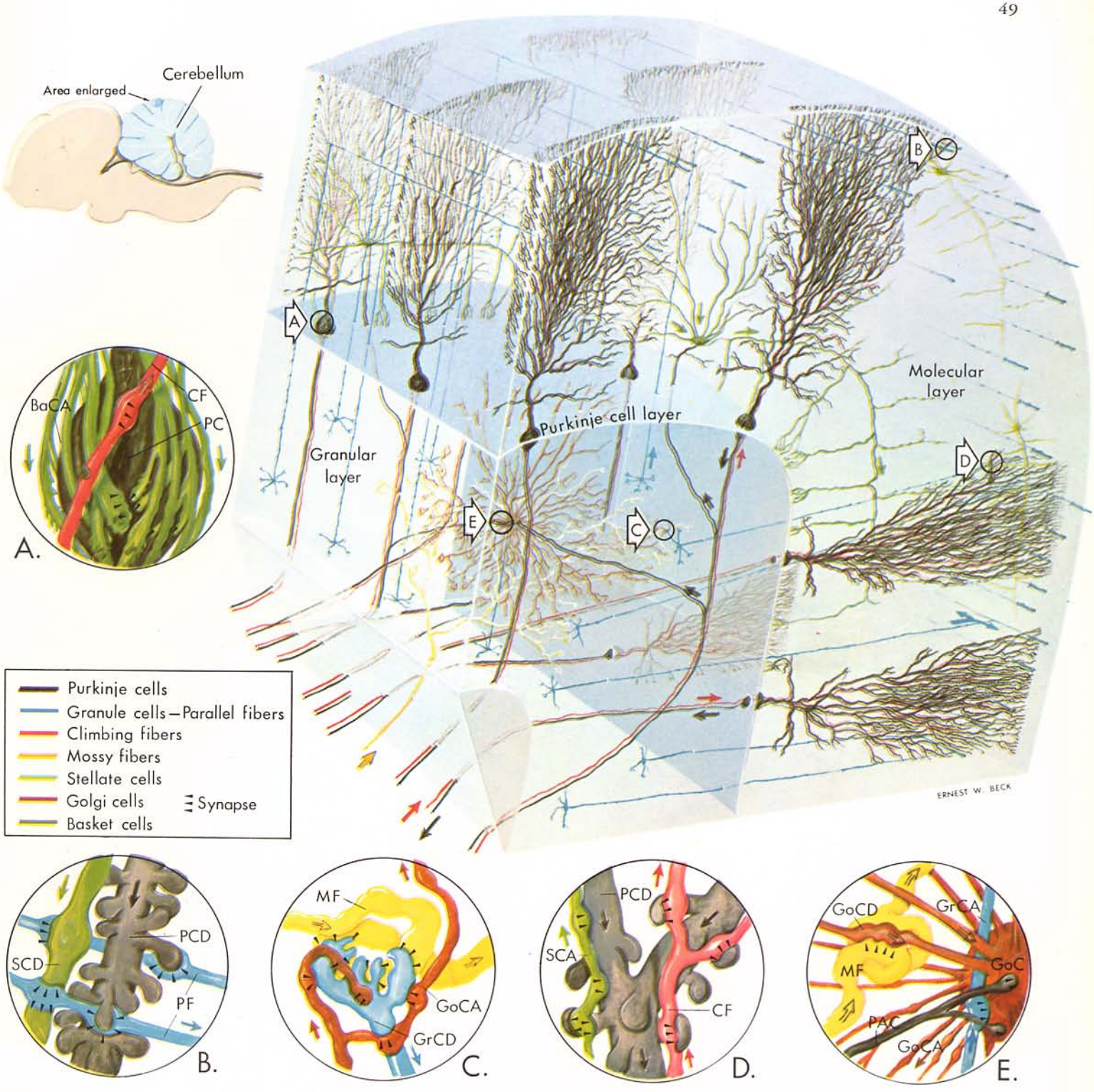

Fig. 3. The upper left corner diagram of pigeon central nervous system showing the cerebellum in light blue and in dark blue the actual area enlarged in the diagram to the right. In this cortex the Purkinje cells are larger than those found in frog and alligator, and basket cells are present. Call-out A shows the body of a Purkinje cell (PC) completely surrounded by basket cell axons (BaCA) which end in contact with the lower soma and axon hillock and have terminal digitiform processes. A climbing fiber (CF) is seen to contact the upper part of the Purkinje cell soma. In B parallel fibers (PF) are seen to join Purkinje cell dendrite spines (PCD) and, directly, the dendrite of a stellate cell (SCD). In C, the cerebellar glomerulus. As in the alligator it is composed of a mossy fiber sac which in this case is formed by a convolution of the mossy fiber (MF) and a rather large and complex granule cell dendrite (GrCD) which is also contacted by the Golgi cell axons (GoCA), both at the claw region as well as the main dendrite of the granule cell. In D a Purkinje cell dendrite (PCD) shows the typical climbing fiber synapse in contact with spines of Purkinje cells and the stellate cell axon (SCA) contacting both spines but chiefly the main stem of the dendrites. In E the Golgi cell (GoC) is contacted by an ascending portion of a granule cell axon (GrCA), by a convolution of the mossy fiber (MF) and also by Purkinje cell axon collaterals (PAC).

CAT CEREBELLUM

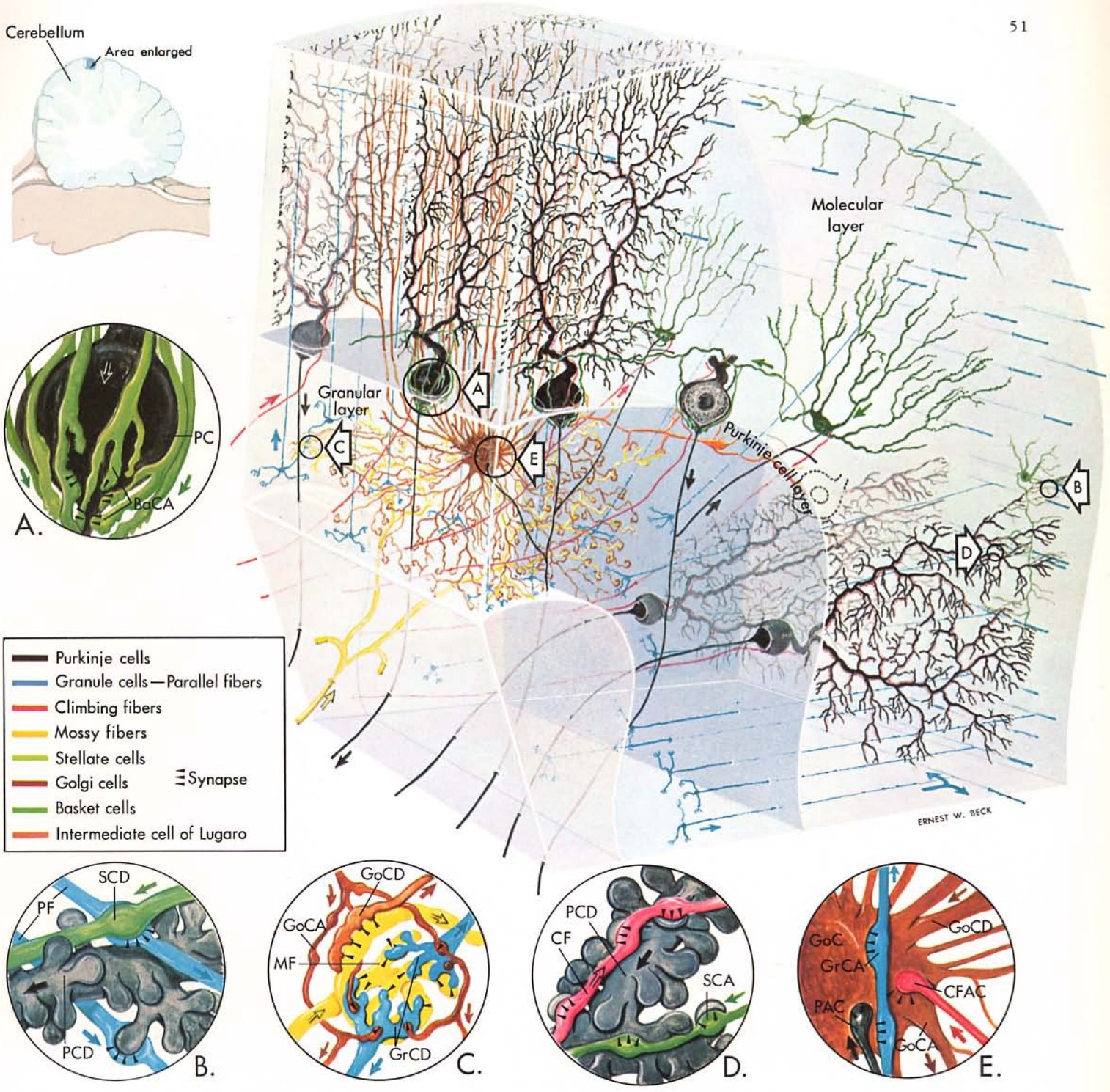

Fig. 4. In the upper left corner a diagram of the brain stem and cerebellum of a cat. The cerebellum is depicted as in previous illustrations in blue and the area illustrated to the right is shown as a dark blue cube just anterior to the fissura prima. This diagram represents the neuronal organization of the typical mammalian cerebellum. Note the large size of Purkinje cell somas as well as the overall richness of the interneurones of the molecular and granular layers. A new cell is shown in this diagram—the intermediate cell of Lugaro which receives, according to Fox (1959), synaptic input from the mossy fibers and the descending axons of the basket cells and in its axon terminates on the basket cells. For the sake of clarity, supra- and infraganglionic plexus have been omitted from this diagram. However, the central connections of these collaterals are illustrated. A shows a Purkinje cell soma and initial segment (PC) surrounded by the terminal axons of basket cells (BaCA). As in the case of the pigeon, the basket axon forms a thick and intricate neuropil around the lower region and initial segment of the Purkinje cell. In B: parallel fiber (PF) contacting a spine of the Purkinje cell dendrite (PCD) and a dendrite of a stellate cell (SCD). In C: cerebellar glomerulus showing a highly convoluted mossy fiber rosette (MF) contacting the terminal claws of the granule cell dendrites (GrCD) and the expanded terminal of the Golgi cell dendrite (GoCD). The Golgi cell axon (GoCA) is shown to terminate directly on the claws and main dendrites of the granule cells. In D: the Purkinje cell dendrite is contacted through its spines by a climbing fiber (CF). A stellate cell axon (SCA) establishes synaptic junction with main dendrites and occasionally also with the spines of these cells. In E: Golgi cell (GoC) receiving synaptic junctions from Purkinje cell axon (PAC) from an ascending granule cell axon (GrCA) and from a collateral of a climbing fiber (CFAC).

stimulation of the parallel fibers. In this case, therefore, it holds true that burst responses are related to climbing fiber activation while single spike activity is related to mossy fiber-parallel fiber activation. However, in other animals such as alligators (Llinás and Nicholson, 1969) and elasmobranch fishes (Nicholson *et al.*, 1969) it is evident that parallel fiber activation can generate burst responses, and thus that in these cases extracellular recordings from single units cannot indicate, *per se*, the mechanism generating the spike bursts. Furthermore, it was found in both alligators and elasmobranchs (Llinás and Nicholson, 1969; Nicholson *et al.*, 1969) that, even in the cases where Purkinje cell potentials were recorded intracellularly, parallel fiber stimulation was able to evoke burst potentials in many ways similar to climbing fiber bursts recorded intracellularly in frog and cat. This, of course, suggests that the single spike or burst characteristic of a Purkinje cell discharge is related not only to the magnitude of the synaptic contact between the afferent fiber and the Purkinje cell, but also to the particular electrophysiological properties of the Purkinje cells (cf. Llinás and Nicholson, 1969).

Besides the burst response which can be recorded intracellularly following climbing fiber activation, an even more constant finding regarding climbing fiber-Purkinje cell activation is the presence of an all-or-none large excitatory postsynaptic potential (EPSP) (Eccles *et al.*, 1966a). Extensive work on this system was done in the cat (Eccles *et al.*, 1966a) where it was found that, for the most part, climbing fibers originated in the inferior olivary nucleus (Szentágothai and Rajkovitz, 1959) and projected to the contralateral cortex via the inferior peduncle. By means of elaborate stimulation techniques involving multiple bipolar electrodes located at different sites (Eccles, Llinás, Sasaki and Voorhoeve, 1966f), it was demonstrated that climbing fiber EPSPs could be generated either from the inferior olive level or from the white matter level. Collision experiments (Eccles *et al.*, 1966f) also demonstrated that these EPSPs were generated by the activation of a single fiber. It became quite evident at that time that the all-or-none character of the EPSP was only a reflection of the all-or-none character of the action potential in the presynaptic fiber. However, given that the potential was all-or-none, the possibility of its being the product of a Purkinje cell spike generated at a distance from the recording site and conducted to the recording site electrotonically, had to be considered. This difficulty was finally overcome with the demonstration that this depolarization could be reversed by the application of a depolarizing current through the recording microelectrode, which implied that this response had an equilibrium potential and thus was the product of the activation of a chemical synapse. Similar types of demonstrations have been carried out for the climbing fiber activation of the Purkinje cell in the alligator (Llinás and Nicholson, 1969).

The very characteristic all-or-none EPSPs produced by the climbing fiber-Purkinje cell synapse have also been observed in alligator and pigeon, in addition to frog and cat, (Fig. 2 CD, GH, NO) and thus can be postulated to be a constant feature for the climbing fiber-Purkinje cell relation. It is quite evident that with the rather large number of synapses which every climbing fiber establishes on the dendrites of Purkinje cells, the activation of a climbing fiber should produce a large EPSP and a very strong activation of the Purkinje cell, especially given that the "shower" of transmitter which must be released on the surface of the Purkinje cell dendritic tree is almost simultaneous throughout the extent of the cell. Thus, a highly synchronized depolarization takes place across the Purkinje cell membrane. Even in elasmobranchs, where the climbing fiber-Purkinje cell function does not seem to be as rich in contacts (Nicholson *et al.*, 1969) as it is in other species, clearcut all-or-none EPSPs can always be observed intracellularly from Purkinje cells (Nicholson *et al.*, 1969).

Mossy fiber-granule cell synapse

Another constant feature of the cerebellar mass in vertebrates is the presence of a second afferent system to the Purkinje cell, the mossy fiber-granule cell-parallel fiber-Purkinje cell pathway (Figs. 7 and 8). The phylogeny of the mossy fiber is unique in that it seems to have evolved from a simple ramifying axonic arborization with relatively small dilatations or small beads which contact the granule cell dendrites (Figs. 7a and c, 8b and d). In reptiles (Hillman, 1969b) the mossy fiber has large smooth beads in addition to the small processes which stem from the main branch (Fig. 7c). In fishes (Nieuwenhuys and Nicholson, 1969) the axonic beads are present but are, however, larger than those in the frog and have a tendency to show out-pocketings or finger-like projections. In birds the mossy fiber terminals become much more complicated and begin to form the coil-like structures (Fig. 8b) named "rosettes" by Ramón y Cajal (1911).

Electron microscopically the mossy fiber can be characterized by its large axonic dilatations which are filled with synaptic vesicles and are clasped throughout their surface area by finger-like processes, the dendritic digits of the granule cells (Figs. 7b and d, 8a and c). The synaptic vesicles of the mossy fiber are distinctly round and of a uniform diameter. Among these vesicles numerous mitochondria can be observed, usually close together in scattered patches. Also characteristic of the mossy fiber ultrastructure is the presence of axonal tubules, which are found in varying numbers,

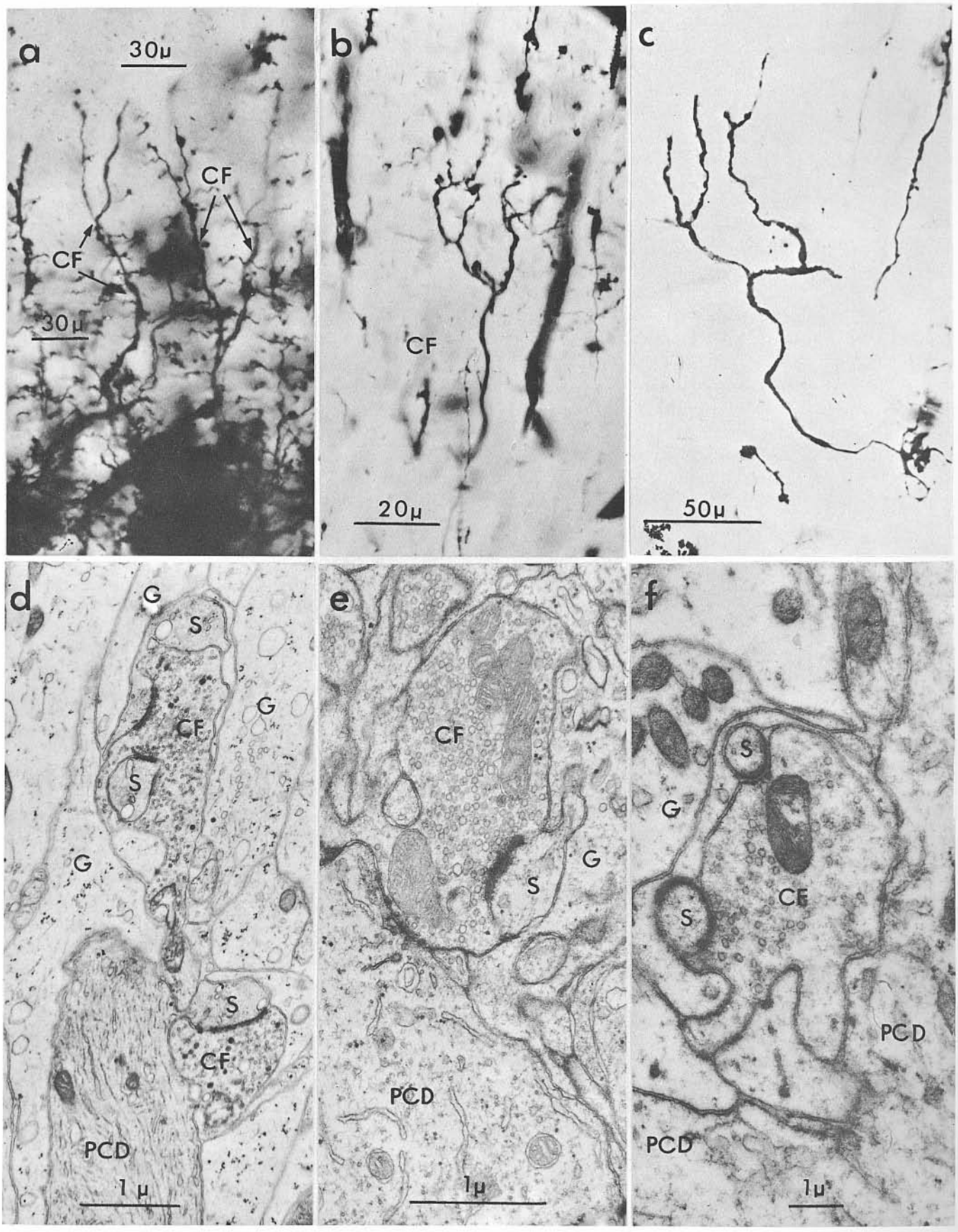
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Fig. 5. Climbing fiber morphology in frog, alligator and pigeon. a,b,c: Golgi stains of the climbing fiber of frog, alligator and pigeon, respectively. In all cases the fiber is seen to arise from the granular layer and distribute following the dendrites of Purkinje cells in the molecular layer. Although the dendrites of Purkinje cells are not visible, the particular distribution of these fibers agrees well with the distribution of the main dendrites of these cells. In d, e, f: electron micrographs of the climbing fiber-Purkinje cell synaptic junctions in frog, alligator and pigeon, respectively. Note that in every case the climbing fibers (CF) establish synaptic contacts with dendritic spines (S) of Purkinje cell dendrites (PCD). This synaptic junction is in in every case encapsulated by glial (G) elements.

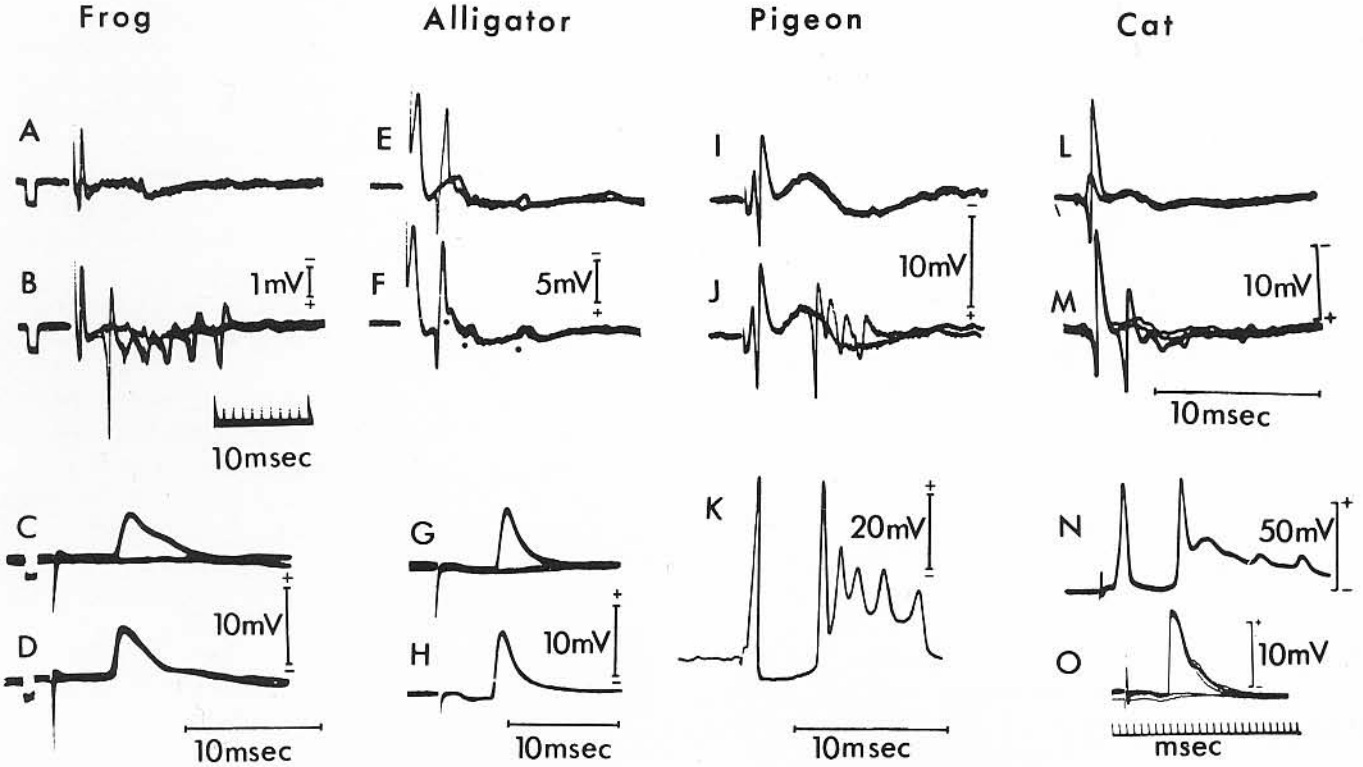
as well as neurofilaments. The contact with the granule cell dendrite is marked by typical synaptic specialization (Palay, 1958) with a distinct postsynaptic membrane thickening and a short, uniform synaptic cleft.

In the frog cerebellum no rosette formations are visible (Fig. 7b). Here the arborizing axon contacts the granule cell dendrites along their course rather than at their terminal dendrite claws (Hillman, 1969b). Multiple synaptic clefts occur between the mossy fiber bead and the granule cell dendrites. This multiple connection is also somewhat evident in alligator cerebella where two or three synaptic clefts can be seen on a single dendritic digit.

Granule cell-Purkinje cell pathway

Another constant feature of the cerebellar cortex is the presence of the granule cell-parallel fiber pathway and the synaptic relationship between parallel fibers and the

Fig. 6. Climbing fiber activation of Purkinje cells recorded extra- and intracellularly. A to D: Climbing fiber responses in frog Purkinje cells. In A, extracellular recording from a Purkinje cell which is identified by its all-or-none antidromic activation from the underlying white matter. As the stimulus is increased, an all-or-none burst of six spikes is recorded (B), which has a very regular amplitude and time course (3 superimposed traces). In C and D, intracellular records from another frog Purkinje cell showing climbing fiber EPSP following a stimulus to the white matter. In C, the all-or-none nature of the EPSP is shown by electrical stimulation at threshold level. In D, two EPSPs are superimposed to show its regular latency and time course. In E and F are extracellular recordings from alligator Purkinje cell shown in a manner similar to that of A and B. As in the frog, the alligator Purkinje cells are activated antidromically from the white matter (E). The all-or-none climbing fiber burst of spikes is shown in E, generating a large action potential followed by three small spikes. The regularity of this response is illustrated in F, where two sweeps have been superimposed. The small spikes are pointed out by three dots. Intracellular records from another Purkinje cell (G and H) show the all-or-none climbing fiber EPSP (G) as well as two superimposed with stimuli at supra-threshold level. Records I and J are extracellular potentials from pigeon Purkinje cell following white matter stimulation. In I, the antidromic invasion of the Purkinje cell. In J, the all-or-none climbing bursts of spikes. In K, the same cell was penetrated and the intracellular records are shown. Following the antidromic action potential, the climbing fiber activation generates a long-lasting burst of spikes. Records L and M (Eccles et al., 1966a) are extracellular potentials from cat Purkinje cells. L shows the all-or-none antidromic Purkinje cell spike. In M, the all-or-none climbing fiber spike burst. Intracellular records from another Purkinje cell following activation of the underlying white matter (N) shows the antidromic action potential followed by the large climbing fiber polarization. In O, the all-or-none climbing fiber EPSP generated by stimulation of the contralateral olive. Time and voltage calibration as indicated. (From Llinás et al., 1969b).



Purkinje cell spines in the molecular layer (Ramón y Cajal, 1888; Ramón y Cajal and Illera, 1907; Fox and Barnard, 1957). Here again there has been almost no change in the character of the synaptic relationship throughout phylogeny which, as will be elaborated in the discussion, seems to have a very important role. The granule cell generates a single axon which ascends to the molecular layer, divides in a T-like pattern, and produces the parallel fiber system (Fig. 9 b, e, h, k). Although simple changes can be observed regarding the length of the Purkinje cell spines upon which the parallel fibers synapse, the neuronal relation is basically very much the same in these four animals. Quantitatively, on the other hand, a truly remarkable difference can be observed as one goes up the phylogenetic scale: the number of parallel fibers impinging on Purkinje cells increases tremendously. In the frog, their number seems to be in the vicinity of 4,000 parallel fibers per Purkinje cell (Hillman, 1969b) and in the cat the relation might be as high as 120,000 per Purkinje cell (Fox, Hillman, Siegesmund and Dutta, 1967), while the climbing fiber-Purkinje cell relation remains quite constant. This fact is depicted in color plates 1-4 where the extent of branching of the Purkinje cells and the number of spines in each cell is shown to increase from that of the frog to that of the cat. Of equal interest, however, is the fact that the diameter of the parallel fibers themselves does not seem to change very much in the four different animals, the diameter size ranging from 0.1 to 1.0μ in all cases.

Electron microscopical organization of the parallel fiber-Purkinje cell synapse

In all the forms so far studied the parallel fiber-Purkinje cell junctions take place on dendritic spines, similar to those described first by Gray (1961) and later by Fox *et al.* (1964, 1967), Hámori and Szentágothai (1964), and Palay (1964). The small parallel fiber axons contain neurotubules ranging from 3 to 10 in number, as seen in cross-cut profiles. Along their course, beads or dilatations occur which in some instances (the frog) may have a diameter up to 2μ. Within the dilatations are synaptic vesicles which are round and of a uniform diameter (Fig. 9c, f, i, l) as described by Larramendi and Victor (1967) in the mouse, Uchizono (1967) in the cat, Hillman (Hillman, 1969b,c; Llinás *et al.*, 1969b) in the alligator, Sotelo (1969) in the frog, and Mugnaini (1969) in the chick. The number of synaptic vesicles in each dilatation varies; however, the arrangement of the vesicles is characteristic in that they lie relatively close to each other, especially over the synaptic site. The parallel fibers contact the "spiny branchlet" spines as they cross through the flattened dendritic tree, forming thereby the typical cruciform-axo-dendritic contact first described by Ramón y Cajal (1911). These Purkinje cell spines usually are club-shaped with a shaft of varying size and small diameter, which we consider to be the most important functional parameter of these spines. (Fig. 9c, f, i, l). Within the spine, numerous membrane profiles appearing as sacs of smooth endoplasmic reticulum can be constantly observed; however, mitochondria are completely lacking. The parallel fiber usually joins the Purkinje cell on one side of its club-shaped spine with a synaptic contact that is characterized by a distinct postsynaptic membrane thickening conforming to the type I synapse of Gray. Surrounding the spine shaft and the remainder of the club is a glial investment from a single glial process.

Functional characteristics of the parallel fiber-Purkinje cell synapse

As demonstrated in the cat (Andersen, Eccles and Voorhoeve, 1964; Eccles, Llinás and Sasaki, 1966c), parallel fibers have an excitatory action on Purkinje cells in all animals so far tested (Llinás *et al.*, 1969b; Llinás and Nicholson, 1969; Nicholson *et al.*, 1969). As seen in Fig. 10, following local stimulation of the cerebellar cortex an EPSP can be recorded from a Purkinje cell with a latency which corresponds to the conduction time of the parallel fibers between the place of stimulation and the recording site. In every case the EPSP is graded in nature as opposed to the climbing fiber-Purkinje cell EPSP which is all-or-none. The graded nature of the parallel fiber-Purkinje cell EPSP is to be expected, given that it represents the summation of the small synaptic potentials produced by the synchronous activation of parallel fibers impinging on a particular Purkinje cell. For this reason, the amplitude of the EPSP is always related to the strength of the stimulus applied to the surface of the cerebellum since the number of parallel fibers activated will be directly proportional to the amplitude of such stimulus. That this depolarization is generated by the parallel fiber-Purkinje cell synapse can be easily shown by recording from Purkinje cells which are not located in the path of an activated beam of parallel fibers, in which case no EPSPs can be evoked.

The graded nature of the parallel fiber-Purkinje cell EPSP explains the graded nature of the Purkinje cell spike response following parallel fiber activation since the larger the EPSP, the faster the membrane potential reaches firing level. Also, as the EPSPs increase in height, the cell tends to respond in a repetitive manner for a very short period of time. Even in cases where the parallel fiber activation of Purkinje cells is able to generate dendritic spikes (such as in the alligator and elasmobranch) the graded nature of this response can always be demonstrated. This is in fact the only way in which the two types of bursts that occur in these animals, the

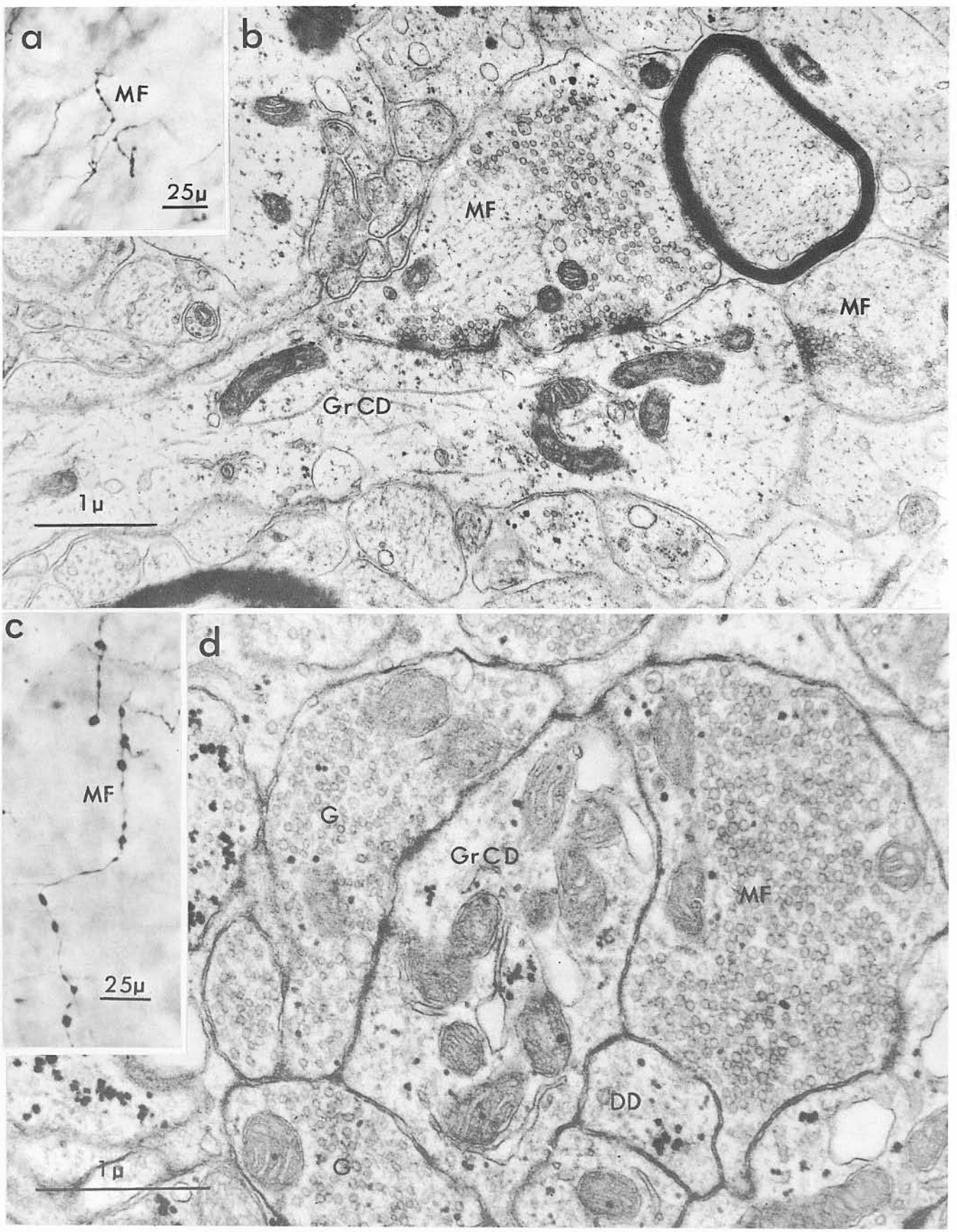


Fig. 7.

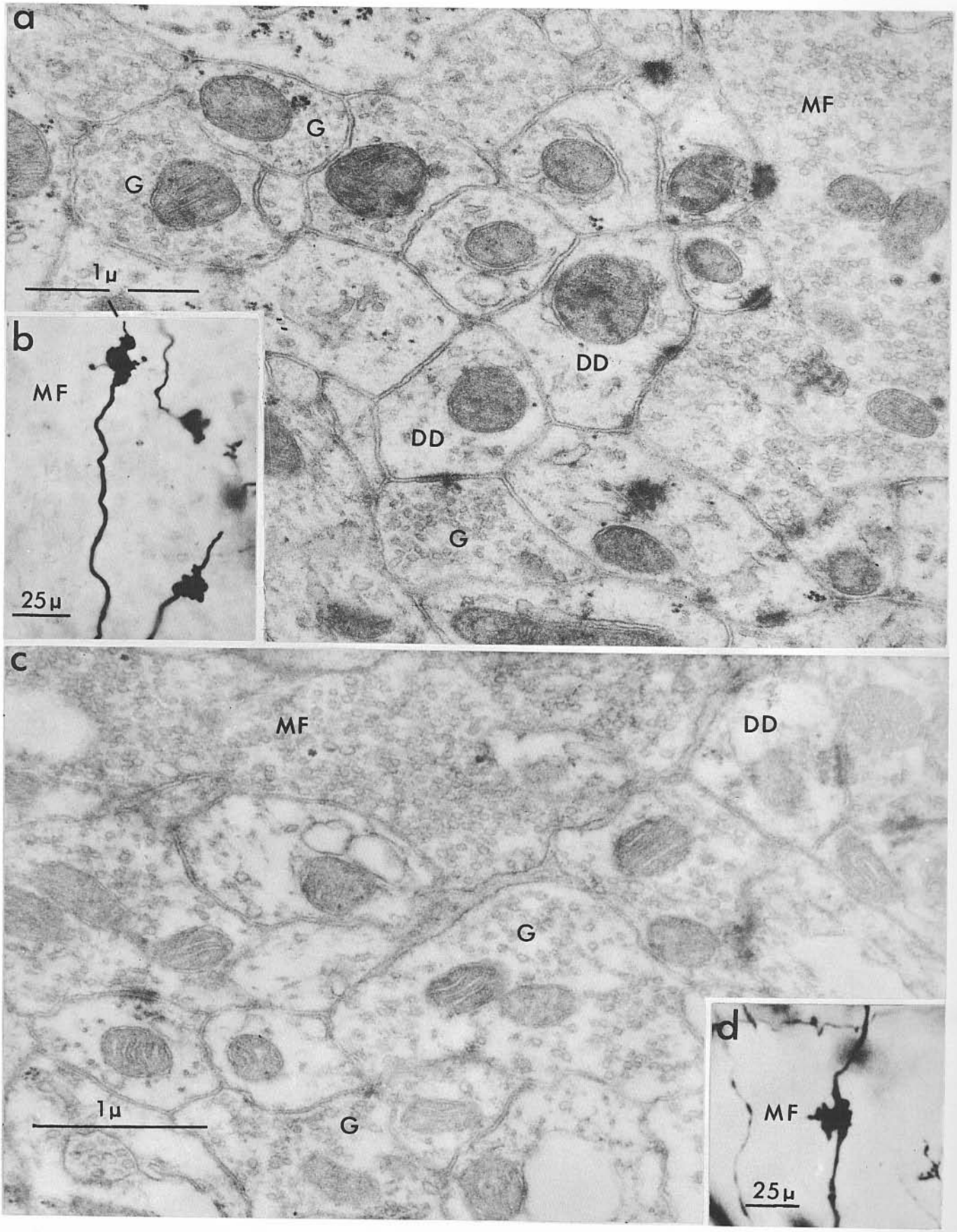


Fig. 8.

Fig. 7. Morphological characteristics of the mossy fiber-granule cell junction in the frog and alligator. a and c: Golgi stain of typical mossy afferents in the frog and alligator, respectively. In both cases the mossy fiber demonstrates bead-like enlargements along its course. These enlargements are known to be the synaptic site with the dendrites of the granule cells. This type of en passage mossy fiber junction is believed to be characteristic of the more primitive cerebellum. In b, a mossy fiber (MF) enlargement and a dendrite of the granule cell (GrCD). Note that in this cerebellum the presence of a glomerulus is not apparent and the junction between the mossy fiber and granule cell dendrite is a simple axo-dendritic contact. In d, cerebellar glomerulus in the alligator. GrCD, granule cell dendrite; MF, mossy fiber terminal; G, Golgi cell terminal; DD, dendritic digits from the granule cells. This picture illustrates a rather simple cerebellar glomerulus where the excitatory mossy fiber input (MF) establishes contact with one side of the granule cell dendrite while the inhibitory Golgi cell terminal contacts the same dendrite from the opposite side. Note that, as in the frog, the mossy fiber contact is still a rather simple expansion of the mossy afferent.

Fig. 8. Cerebellar glomeruli in pigeon and cat. b and d are Golgi cell stains of mossy fibers in pigeon and cat, respectively. Note that in both cases the mossy fiber shows a highly convoluted area which forms the synaptic loci between this afferent and the dendrites of the granule cell. At electron microscopic level in the pigeon (a) the synaptic junction is seen to occur between the convoluted expansions of the mossy fiber (MF) and the dendritic digits (DD) of the granule cell dendrite. The Golgi cell axons also terminate (G) in contact with the granule cell dendritic digits. A similar case is found for the glomerulus of the cat (c) where the mossy fiber contacts the digits of the granule dendritic claw. As in the previous case, the Golgi cell terminates in contact with dendrites of the granule cell.

climbing fiber burst and parallel fiber burst, can be differentiated extracellularly (Llinás and Nicholson, 1969).

General morphology of the interneurones of the molecular and granular layers

The interneurones of the cerebellar cortex represent a very interesting addition to what we have called the "basic cerebellar circuit." Their presence, as well as their complexity, seems to be one of the largest differences which can be found between cerebella of different vertebrates. For example, in certain frogs, such as *Rana catesbiana*, the only interneurones found are the very small and rather scarce stellate cells (Fig. 11a) in the superficial part of the molecular layer (Fig. 2)[1]. These stellate cells, which most probably are inhibitory, do not seem to generate any "long-term inhibition" (Fig. 10A), at least as can be observed in intracellular recording from the soma of Purkinje cells in the frog (Llinás and Bloedel, 1967; Llinás *et al.*, 1969a). On the other hand, it is very clear that neither basket cells (Fig. 11c) nor the large Golgi cells of the granular layer (Fig. 7b) seem to be present in this primitive cerebellum. For this reason (as shown in Fig. 1, inset C) the mossy fiber-granule cell synapse does not form the typical glomerulus but is a simple axo-dendritic type of contact generated by the extensive ramification of the mossy fiber terminals, which for the most part do not end in the typical mossy fiber sacs of other vertebrates. However, in the alligator a very rich stellate cell population can easily be observed at either light or electron microscopical level (Fig. 11b). The axons of these cells, which are generally 400 to 500μ long, run at right angles to the main axes of the parallel fiber system and do not form the typical basket cell synapse around the axon and lower body of the Purkinje cell, but are, for the most part (Fig. 11d), limited to the thick dendrites and lower two-thirds of the dendritic tree as illustrated in color plate 2 and inset.

Golgi cells are definitely present in this animal and can be observed both in light and electron microscopy. They are usually confined to the granular layer and are only very rarely seen sending dendrites to the molecular layer. In comparison to the more elaborate Golgi cells found in pigeon or cat, the Golgi cell of the alligator is similar in size and has fewer dendrites and smaller axonic plexus. Given the fairly deep location of these cells, they must be activated rather strongly by the mossy fiber input (as first suggested by Hámori and Szentágothai [1966] in the cat) as well as by the ascending axons of the parallel fibers as they project toward the molecular layer (Larramendi, 1969). As will be discussed in detail later, the axons of these Golgi cells terminate in contact with the dendrites of the granule cells forming, as in the cat, a feed-forward type of relation (i.e. mossy fiber to Golgi cell to granule cell).

In the pigeon the interneurones of the molecular layer can be divided into two main types, stellate cells and basket cells, and they are very similar to those found in mammals. The stellate cells receive synaptic input from the parallel fibers and distribute at right angles to the axes of this parallel fiber system, as noted for the alligator. Historically the basket cells in the pigeon are of great interest since it was this synapse which led Ramón y Cajal to postulate the neuronal theory. His paper of 1888 demonstrated clearly for the first time that a presynaptic terminal ends in contact with, but not fused to, the target cell. Very much as described first by Ramón y Cajal and later by Estable (1923), it is evident that the basket terminal in the pigeon is formed by a smaller number of axons than in the cat, but on the other hand these fibers not only surround the body, but in many cases descend with the axon of the Purkinje cell almost to the white matter level (Fig. 12a).

The Golgi cell in the pigeon is slightly more complex than that found in the alligator. It tends to have a larger

[1]*This statement may not be valid for all anurans, since Sotelo (1969) has presented evidence for the existence of stellate and Golgi cells in* Rana esculenta. *On the other hand, it is possible that anurans may not be a homogeneous group (Szarski, 1968).*

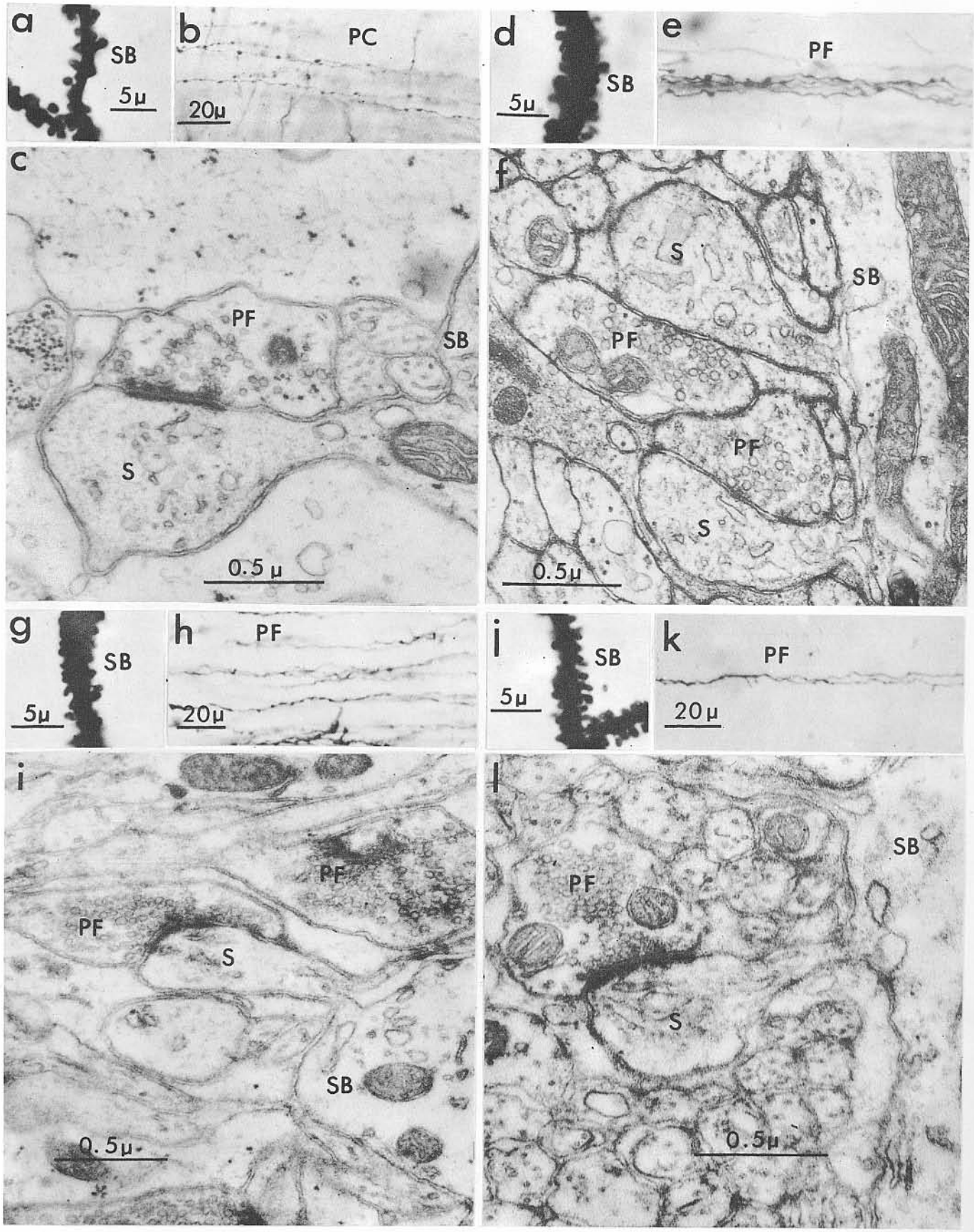

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Fig. 9. Parallel fiber-Purkinje cell synapse in frog, alligator, pigeon and cat. This synapse is formed, in these four species, between parallel fibers and the spinous processes of the Purkinje cell dendritic branchlets. The morphology of the dendritic branchlets can be seen in Golgi stain in a,d,g and j for the frog, alligator, pigeon and cat, respectively. Note that the dendritic branchlet is quite thick in the alligator and pigeon but is definitely thinner in cat and frog. In all four cases the parallel fibers are generated by the bifurcation of the granule cell axons as they reach the molecular layer. Electron micrographs of the actual synapses are seen in c,f,i and l for these four species. In every case the junction occurs between an expansion of the parallel fibers (PF) [as can be seen from the Golgi stains (b,e,h and k)] and a Purkinje cell spine. On all occasions the parallel fiber expansion is filled with rounded vesicles of very uniform diameter (c,f,i, and l) and contacts the spine near the terminal bulb, but never at the very thin neck formed at the junction of the spine with the spiny branchlet (SP) or directly on the spiny branchlet itself.

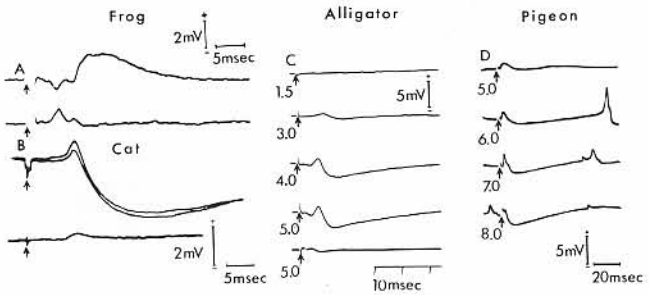

Fig. 10. Intracellular records from Purkinje cells following activation of parallel fibers. A: Intracellular EPSP from a frog Purkinje cell following parallel fiber activation via the mossy fiber-granule cell synapse. Note the lack of IPSP following the parallel fiber EPSP. The lower trace is the extracellular field recorded immediately outside the Purkinje cell at the same gain. For this record, negativity is upward. B: Intracellular records from a cat Purkinje cell (Eccles et al., 1966e) following direct activation of the parallel fibers. The EPSP is cut short by the very large and prolonged IPSP. Lower record as in A. C: Intracellular records from alligator Purkinje cells. The potentials were evoked by local stimulation of the surface of the cerebellum with currents of increasing amplitude (the relative stimulus strength is indicated by the numeral at the left of each record). As the stimulus is increased, the amplitude of the EPSP and the IPSP increases in a graded manner. Note that the duration of the IPSP is shorter than that of the cat. The last record is the extracellular field at the highest stimulus strength. In D, synaptic potentials recorded intracellularly from pigeon Purkinje cells following parallel fiber activation of increasing magnitude. As in C, the amplitude of the EPSP and the IPSP which follow is graded and related to the stimulus strength. In the last three records a small local response follows the prolonged IPSP. Note that the duration of the IPSP is longer than that of the alligator IPSP, and comparable to the IPSP in cat. Time and voltage calibrations are indicated. (From Llinás et al., 1969b).

size and its synapse seems to project more readily to the molecular layer. The interneurone, as in the alligator and cat, represents a negative, a feed-forward, and a feedback system in contact with the dendrites of the granule cells.

In the cat we find the more evolved and complicated interneuronal system so characteristic of the mammalian cerebellar cortex. The neurones of the molecular layer can be divided into at least three different groups (Scheibel and Scheibel, 1954; Eccles, Ito and Szentágothai, 1967) comprised of small superficial stellate cells, large stellate cells, and very large basket cells (Fig. 12b). All of these receive synaptic input from the parallel fibers and establish synaptic contact with the surface of the dendrites or soma of the Purkinje cells. The extent of these basket cells in the cat has been calculated to be as long as one millimeter (Eccles *et al.*, 1967).

Regarding other inputs to the molecular layer, the interneurones in the alligator, pigeon and cat seem to receive (in addition to the input coming from the parallel fibers) synaptic inputs from collaterals of climbing fibers (Scheibel and Scheibel, 1954) (as illustrated in Fig. 4). Other inputs to the pigeon and cat interneurones are the rather large Purkinje cell recurrent collateral plexus that seems to have developed in parallel with the development of the basket cells (as first shown by Scheibel and Scheibel, 1954) and the axonic terminals from other interneurones.

Electron microscopical organization of the molecular and granular layer interneurones

The stellate and basket cells differ morphologically from the Golgi cells, in that the cell bodies of the latter have more rough endoplasmic reticulum. The molecular layer stellates have a relatively light cytoplasm with rough endoplasmic reticulum being displayed especially in the basket cell cytoplasm. The dendrites of the stellate and basket cells extend over a large parallel fiber field, and are, for the most part, smooth with only occasional spines in some mammalian forms. These dendrites do not contain the numerous, distinct and well-organized tubules found in the main dendritic tree of Purkinje cells, but appear more like the spiny branchlets where few dendritic tubules are present. The surface of these dendrites is contacted by varying numbers of parallel fibers which also synapse on the cell bodies. As reported by Scheibel and Scheibel (1954), climbing fibers contact the stellate cells. More recently, quantitation of these parallel fiber connections in relation to climbing fiber, Purkinje cell collaterals and other basket cells has been made in the mouse (Lemkey-Johnston and Larramendi, 1968).

Emerging from the cell body of these stellate neurones is a small conical process, the axon, which thins to a very small diameter at about a cell body width or so away from the point of origin. Beyond this point, the process enlarges in diameter as it courses the molecular layer in a direction transverse to the folium. The molecular neurones have been classified as belonging to three types according to their axonic patterns (Scheibel and Scheibel, 1954.) One type of stellate neurone can be

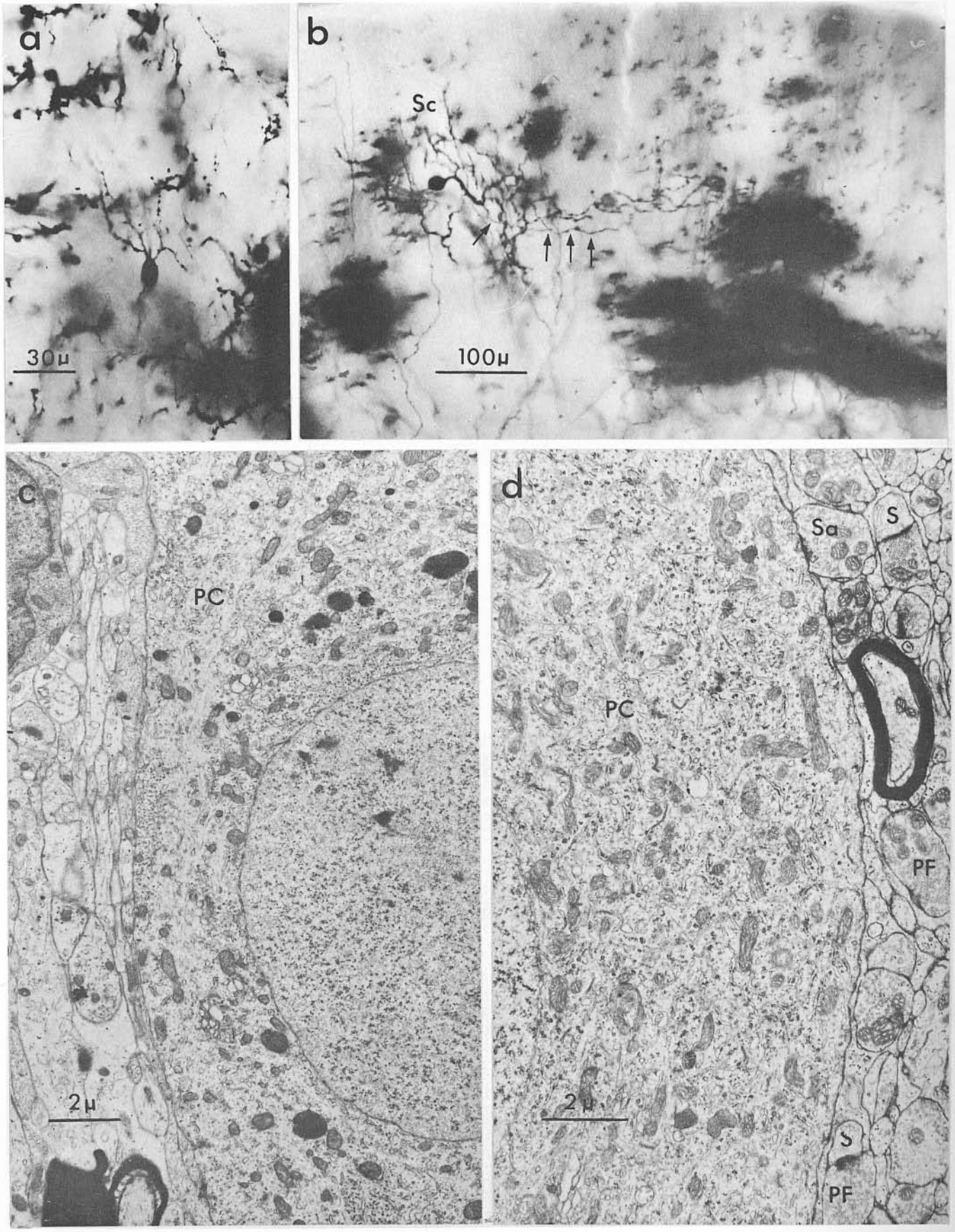

Fig. 11.

Fig. 12.

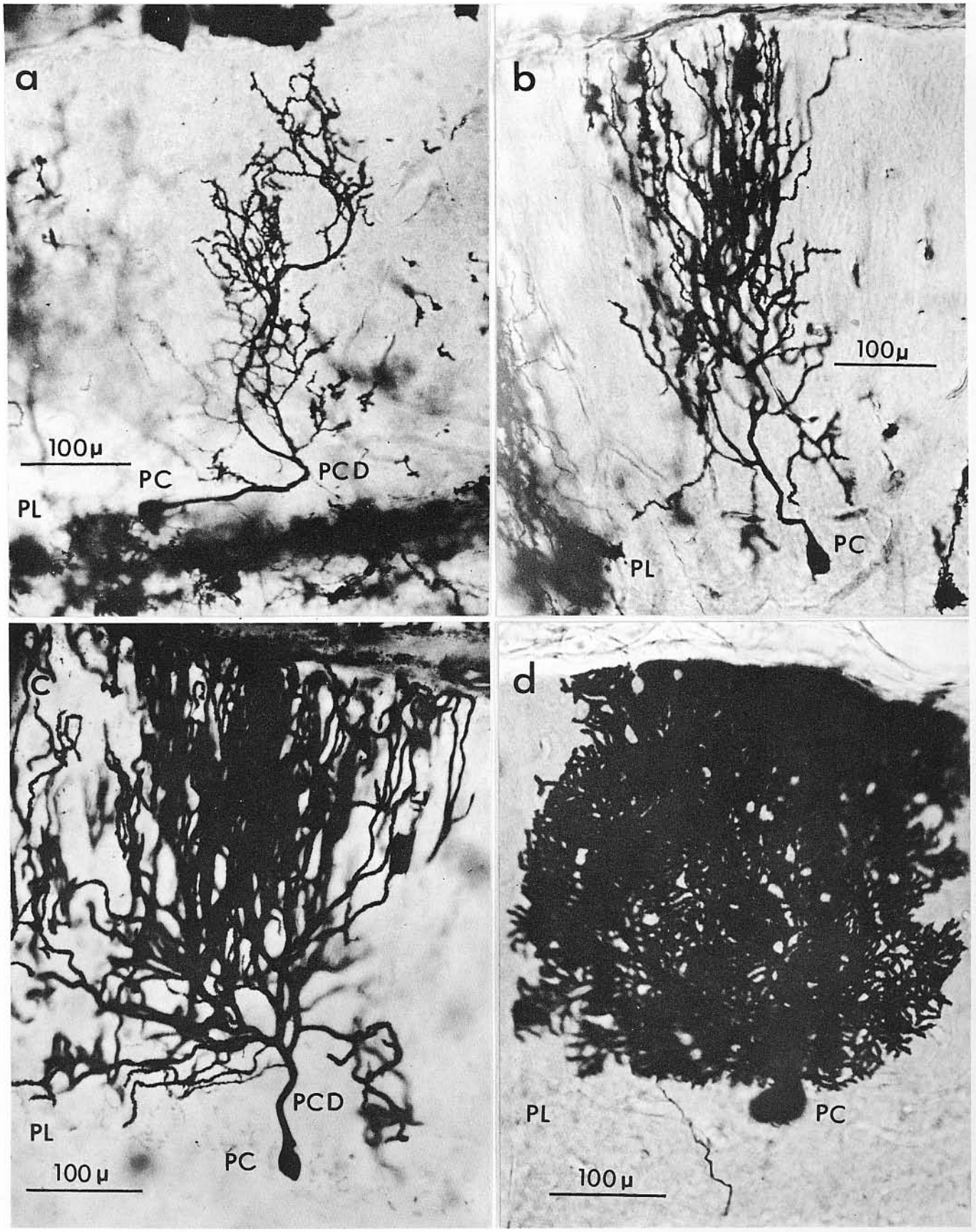

Fig. 13.

Fig. 11. Molecular layer interneurones of the frog and alligator. a: Golgi cell stain of small molecular layer interneurone of a frog. These interneurones have generally a small axon which distributes to the immediately surrounding area and terminates in contact with the dendrites of Purkinje cells. b: Large stellate cell (Sc) of alligator molecular layer. These interneurones are very similar to the large stellate cells found in higher vertebrates. Their axons are distributed in such a manner as to run perpendicular to the direction of the parallel fibers. In the case illustrated the stellate cell axon is seen to emerge from a dendrite (arrows) and to distribute to the surface and deep levels of the molecular layer. c and d: electron micrographs of Purkinje cell bodies in frog and alligator respectively. The frog Purkinje cell has a total lack of interneuronal terminals in the cell body. A similar statement can be made, for the most part, for somas of alligator Purkinje cells (d). On some occasions, however, small stellate cell axons can be seen to contact the upper region of the body of the Purkinje cell (Sa). In the same picture parallel fibers (PF) contact spines (S) of Purkinje cells.

Fig. 12. Molecular layer interneurones in pigeon and cat. a: Typical basket cell (Bc) in avian cerebellum. Note the basket terminals emerging from rather highly located transverse axons of the basket cells (Ba). These descending axons as well as their ascending branches contact the dendrites of Purkinje cells as they progress towards the Purkinje cell soma. Also characteristic of the avian basket cell, especially in pigeon, is a rather long and thick peri-axonal plexus formed by the basket cell terminals along the axons of the Purkinje cells. On some occasions this "axon-cap" formation extends to the level of the white matter. b: Basket cell in cat cerebellum. Note the transverse axon and the basket formations (Ba) which surround the body of the Purkinje cells. c: Electron micrograph of the basket axon terminal on the soma of pigeon Purkinje cell. The basket axon (Ba) is characterized by the filamentous structure and the rather small and elongated presynaptic vesicles. The sites of contact with the Purkinje cell soma (PC) are indicated by arrows. d: Basket cell terminal (Ba) in contact with a Purkinje cell soma (PC) in cat cerebellum. Note in c and d the rather peripheral location of rough endoplasmic reticulum of the Purkinje cells.

Fig. 13. Comparative aspects of Purkinje cells in frog, alligator, pigeon and cat. These four pictures were obtained by means of Golgi stain and are shown at approximately similar magnification. The increase of the complexity of the Purkinje cell dendritic tree from frog to mammal is very apparent. a: In a Purkinje cell of a frog the dendrite (PCD) stems as a single element which runs parallel to the Purkinje cell layer (PL) and then moves sharply upwards towards the molecular layer. In b a Purkinje cell of an alligator. As in the case of the frog, the dendritic tree arises from a single stem and then proceeds to branch to form the Purkinje cell tree. In c and d, Purkinje cells of pigeon and cat. While in the pigeon a Purkinje cell dendritic tree stems from a single element, in the cat it has generally two and as many as three places of origin.

characterized as having a small cell body, a limited dendritic field, and a short axon which remains relatively close to its cell of origin. The second type has a cell body of similar size or larger, is found frequently in the upper three-fourths of the molecular layer and is characterized by an axon which extends transversally in the folium for varying distances up to 500μ. The transversal axon of this second cell type and the short axon of the first type are beaded and branch into a plexus within the molecular layer. The third type, the basket cell, which is found in a significant number only in birds and mammals, has an axon which extends transversally in the lower one-fourth of the molecular layer in cats and lower one-half to one-third in birds. From this transverse axon, which is rather large in diameter, a series of processes extend towards the Purkinje cell to form the classical basket terminal arborization around the soma of this neurone. In avian species these axons extend down into the granular layer following the emerging axon. Also arising from the transverse basket cell axon are ascending and descending processes which do not take part in basket formations but are distributed within the molecular layer as has been shown by Fox *et al.* (1967) in Golgi preparations. This is especially true in the pigeon where relatively superficially located basket cells (Fig. 12a), besides sending long descending processes to the Purkinje cell bodies, also have an extensive axonic plexus within the molecular layer. This finding is suggestive of a transition from the stellate cells of lower forms, such as amphibians and reptiles, into basket cells. This transition must have begun in reptilia such as the lizards, where stellate cells have numerous axons ending near the Purkinje cell bodies (P. Ramón, 1896).

Electron microscopical studies show that the large transversal fibers of the basket axons are identifiable by their diameter and the numerous neurofilaments along their course (Fox *et al.*, 1967). Likewise, the descending axon which forms the basket around the Purkinje cell also contains neurofilaments (Fig. 12 c and d).

The synaptic contact between the basket axon and the Purkinje cell body is distinguishable by its small number of synaptic vesicles which have polymorphic shapes and a diameter smaller than those of the round parallel fiber vesicles (Fig. 12 c and d). The synaptic junction is a type II of Gray which is characterized by equal thickening of the pre- and postsynaptic membranes. As pointed out by Fox *et al.* (1967), basket axons also contact the spines of the Purkinje cell dendrites. Similarly, beaded axons of the stellate molecular neurones contact the Purkinje cell dendrites of the main dendritic tree directly on their surfaces and on rare occasions on their spines also, as in the alligator. These boutons are characterized by the shape and distribution of the synaptic vesicles. Again the vesicles are polymorphic, as viewed in the electron microscope, and their distribution is irregular within the axonic dilatations. The synaptic cleft is typically type II as described by Gray. The axons on which these beads occur are frequently seen to contain neurofilaments, although their diameters are considerably smaller than those of the transversal fiber.

Golgi cells

The granular layer stellates appear as a large neurone

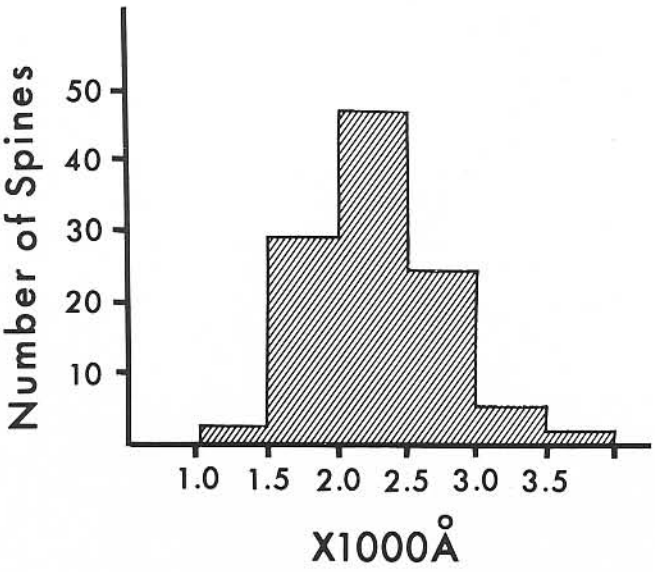

Fig. 14. Histogram of spine shaft diameter in alligator and pigeon. Abscissa, diameter in Angstrom units; ordinate, spine numbers. Only those spines having a very clear profile were measured.

which usually is slightly smaller in size than a Purkinje cell body. In contrast to the more rounded nucleus of the Purkinje cell, the nucleus of this cell is highly indented. Golgi impregnations show numerous processes emerging from the cell. Large processes extend for long distances into the granular layer and up into the molecular layer. However, not all cells appear to have molecular layer dendrites. On the other hand, in higher forms such as birds and mammals, numerous Golgi cell dendrites extend into the molecular layer. This appears to be particularly true in the flocculo-nodular region which contains numerous Golgi cells.

The dendrites and the cell body are contacted by small beaded processes (Figs. 2-4) which appear to be those of granule cell axons as they course into the molecular layer (Hillman, 1969b; Larramendi, 1969). This finding is in agreement with the fact that the granule cell axon contains beads along its granular layer course. The Golgi cell dendrites are also contacted by the mossy fibers as has been demonstrated by Hámori and Szentágothai (1966a) in Golgi preparations. Electron micrographically this junction is characterized by multiple synaptic clefts between a single dendrite and the mossy fiber rosette (Hillman, 1969b). Also contacting the Golgi cell are climbing fibers, as has been described by Scheibel and Scheibel (1954) and further indicated by Hámori and Szentágothai (1966b).

The Golgi cell axons emerge from the cell body and give rise to a profuse arborization in the form of a beaded plexus. This plexus arises from comparatively large axons which thin as they give off the beaded ramifying processes. According to Fox *et al.* (1967), these beaded axons are restricted to the cerebellar island regions, i.e. the same region in which the mossy fiber rosettes contact the dendritic claws of the granule cells. In electron microscopical studies, similar beaded axons are found in the periphery of the cerebellar island (Figs. 2-4). Here they contact the outer rows of the dendritic digits and the granule cell dendrite directly (Figs. 7d, 8 a and c), as was first shown by Fox *et al.* (1967). These beaded processes appear distinctly different from the synaptic vesicle-filled, mossy fiber rosette in that the synaptic vesicles are irregularly positioned and less concentrated in numbers. In addition they are polymorphic in shape in contrast to the round, relatively uniform diameters of the mossy fiber synaptic vesicles (Uchizono, 1967). These beaded axons contact the dendritic digits and dendrites of the granule cells with a synaptic cleft which, in most cases, is a type II junction; however, slight postsynaptic thickenings have been observed.

Electrophysiological characteristics of the interneuronal system

On every occasion where a strict testing procedure has been possible, the conclusion reached has been that the interneurones of the molecular and granular layers are inhibitory in nature. As far as the molecular layer interneurones are concerned, the only point which is not very clear at present is the inhibitory nature of the stellate interneurones in the frog cerebellar cortex. However, it is very possible that the difficulty in demonstrating this inhibition is related to the comparative scarcity of these interneurones as well as to their rather remote distribution on dendrites of Purkinje cells. In terms of evolution it is interesting to note that elasmobranchs, which appear to be more primitive than frogs, show a very clear inhibitory action on Purkinje cells following parallel fiber stimulation (Nicholson and Llinás, 1969; Nicholson *et al.*, 1969) which might suggest that the cerebellar cortex of the frog is a simplified version and that, rather than a primitive cerebellar cortex, it represents a specialization of the circuit of the cortex. Although this possibility is present, for reasons to be given in the Conclusions we believe that the cerebellum of the frog does in fact represent a more primitive type of cerebellum.

In alligators and pigeons (Llinás *et al.*, 1969b), as in cats (Anderson, Eccles and Voorhoeve, 1964; Eccles, 1966c), it has now been demonstrated that following local stimulation of the parallel fiber system an inhibition lasting 60 to 100 msec can be demonstrated both extracellularly and intracellularly on Purkinje cells. In fact the EPSPs generated by the parallel fiber-Purkinje cell synapse are followed by a large IPSP, the amplitude of which is related directly to the size of the parallel fiber volley (Fig. 10 B, C, D). As seen in Fig. 10, such hyper-

polarization cannot be observed in the frog Purkinje cell (A), suggesting that the hyperpolarization which is seen in the Purkinje cell soma of other vertebrates must be generated, for the most part, by synapses located in the soma or nearby dendrites of the Purkinje cell. Following this assumption the remote inhibition (as postulated by Karl Frank, 1959) which could be taking place on the peripheral dendrites of the Purkinje cells of the frog would not generate a large hyperpolarization intracellularly due to the spatial distribution and scarcity of the presumed inhibitory junctions (Llinás *et al.*, 1969a).

As in the cat (Eccles, Llinás and Sasaki, 1966d), Golgi cells were found to produce inhibitory action on alligator granule cells (Llinás and Nicholson, 1969) which they in turn contact and, in this way, tend to regulate the activity of the mossy fiber-granule cell synapse. On the other hand, as has been recently reported, in *Rana catesbiana* such Golgi cell-granule cell inhibition is not present in the cerebellar cortex (Llinás, 1969; Llinás *et al.*, 1969a), very much in agreement with the finding that Golgi cells have not been observed in this animal (Hillman, 1969b).

General morphology of Purkinje cells

Finally, we would like to comment on the morphological characteristics of Purkinje cells in our four vertebrates. As stated above, Purkinje cells are one of the characteristic and constant elements of the cerebellar cortex. In all cases described here (Figs. 1-4, 13) Purkinje cells are restricted to a particular level of the cortex, the Purkinje cell layer. The dendrites of the Purkinje cells project toward the surface and their axons project inward into the white matter. Although Purkinje cells are remarkably similar in all species, clearcut differences between them can be observed morphologically. In general terms the Purkinje cell seems to become very complex in organization as vertebrates go up the phylogenetic scale. In the frog the Purkinje cell dendrites are rather scarce and receive few parallel fibers (Fig. 13a). Although the dendritic tree seems to be arranged at right angles to the parallel fiber beam, the distribution of the dendritic tree is not as neatly organized nor as constant in shape as in higher vertebrates. In the frog, the Purkinje cell dendrite seems to arise from a single branch which then divides into a large number of very thin dendritic branchlets containing numerous dendritic spines (Hillman, 1969a). Characteristically, in the frog cerebellar cortex the Purkinje cell layer is multicellular and as many as two, three and, in some cases, four Purkinje cell rows can be found in particular sites. Typical of these Purkinje cells is the dearth of axon collaterals present in the Purkinje cells of higher vertebrates (Ramón y Cajal, 1911).

The Purkinje cells of alligators and pigeons, on the other hand, can be described together, since they possess many common features. The alligator Purkinje cell, contrary to that of the frog, is characterized by the highly organized nature of its dendritic tree and by the fact that the Purkinje cell layer is limited to a single row of cells (Fig. 13b). The main difference between this Purkinje cell and that of the frog lies in the distribution of the dendritic tree. As in the frog, the Purkinje cell of the alligator stems from a single apical branch situated

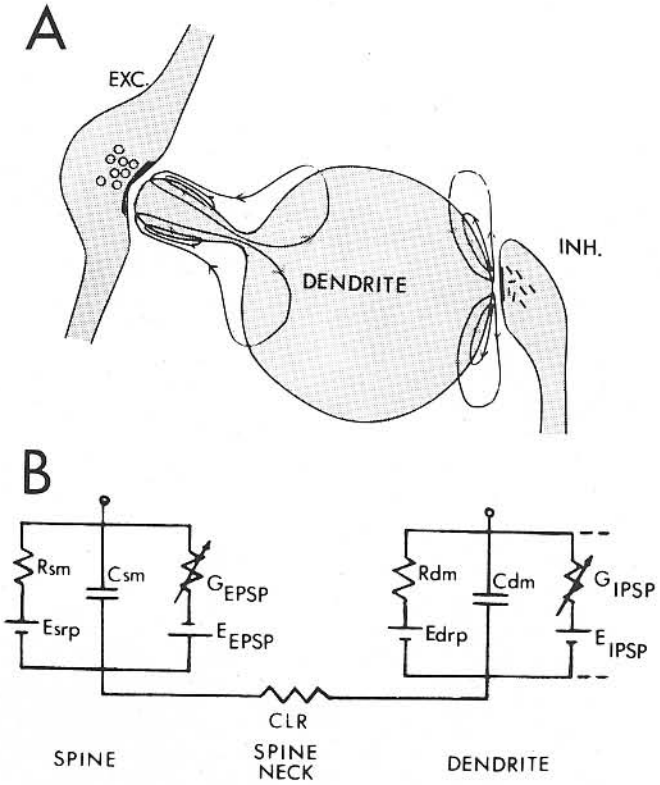

Fig. 15. Diagram of direction of current flow and formal electrical representation of excitatory synapse on a dendritic spine and of an inhibitory synapse on a dendritic shaft in a Purkinje cell. In A the current flow (lines with arrows) generated by an excitatory synapse (EXC) on a spine is inward at the subsynaptic site, outward through the rest of the spine, and reaches the dendritic shaft (DENDRITE) through the neck of the spine. An inhibitory synapse (INH) is shown in direct contact with the dendritic shaft. The direction of flow is outward through the subsynaptic membrane and inward for the rest of the dendrite. B is an electrical representation of the drawing shown in A. The spine is represented by the circuit to the left. Rsm, Spine membrane resistance. Esrp, Spine resting potential. Csm, Spine membrane capacitance. G_{EPSP}, Conductance generated by the excitatory synaptic activation. E_{EPSP}, Driving force for the synaptic potential. CLR, current limiting resistance, represents the large longitudinal resistance of the neck of the spine. At the right of the diagram, electrical representation of the dendrite. Rdm, Dendritic membrane resistance. Edrp, Dendritic resting potential. Cdm, Dendritic membrane capacitance. G_{IPSP}, Membrane conductance generating the IPSP. E_{IPSP}, Driving force for the IPSP. The dashed lines to the right indicate that the dendrite must be regarded as a cable. Small circles at the upper portion of the diagram signify the extracellular fluid.

in the uppermost part of the soma of the Purkinje cell. The length of this dendritic segment is appreciable compared with that of the frog and of the pigeon, although in the latter the Purkinje cell dendritic tree also stems from a single branch (Fig. 13c). Another basic difference between the dendritic tree of the Purkinje cell of an alligator as opposed to that of a frog or cat is the fact that the branches of the Purkinje cell dendrites are much thicker throughout its length and up to the surface. Even the spiny branchlets, which are known to be of a diameter of 1μ and smaller in higher vertebrates (as well as in the frog), seem to have rather large proportions in the alligator. The third important characteristic to be kept in mind is the fact that the parallel fibers seem to contact the Purkinje cell almost throughout its extent and are not restricted to the spiny branchlets as they are in other cerebella. Purkinje cells in the pigeon are rather similar to those of alligators (Fig. 13c). The length of the main dendrite is shorter and the thickness of the dendritic tree is somewhat smaller than that of alligator Purkinje cells (see Fig. 3).

Finally, the Purkinje cell of the cat has been described at length in many places and it suffices to say that, as in the pigeon and alligator, the Purkinje cell layer is restricted for the most part to a single row of cells (Fig. 4) and that the dendrites stem from one or more, usually from two, short main dendrites which almost immediately give rise to secondary smooth branches; these branches then divide further to form a very thick dendritic plexus containing an enormous number of dendritic branchlets and a very large area for parallel fiber input (Fig. 13d).

Ultrastructural characteristics of Purkinje cells

From the ultrastructural point of view, the Purkinje cells have four regions of morphological division: 1) cell body, 2) smooth branches, 3) spiny branchlets, and 4) axons. The characterization of the Purkinje cell body has classically been indicated by its size, shape and Nissl pattern. The ultrastructural characteristics of the cell body have been described by Herndon (1963) in mammals as round to oval shaped cell bodies which contain a large nucleus and nucleolus. Aggregates of rough endoplasmic reticulum were noted, but not in great numbers. These endoplasmic aggregates are dispersed randomly throughout the cytoplasm of various mammalian forms. In the frog (Hillman, 1969a) and toad (Rosenbluth, 1966) the Nissl substance consists of a light perinuclear distribution (Fig. 11c). Occasionally individual endoplasmic reticulum membrane profiles can be found distributed throughout the cell and out into the main dendrite. While the frog Purkinje cell is in the range of 10-15μ, in the succeeding phylogenetic stage the reptile has a considerably larger cell (15-25μ) which contains an aggregate pattern of endoplasmic reticulum structures distributed throughout its cytoplasm (Fig. 11d). In birds a similar distribution of granule studded membranes is noted. (Fig. 13c). The cell body size progressively enlarges from that of amphibians to that of mammals where Purkinje cells up to 30μ in size (like those in cat) have been described. The cell bodies of more primitive forms are, for the most part, oval in shape while as the Purkinje cells enlarge, they tend to take on a more rounded appearance.

The dendritic arborization, described by Ramón y Cajal as smooth branches and spiny branchlets, similarly has ultrastructural characteristics which differentiate these two dendritic segments. The smooth branches, usually of fairly large diameter, contain a number of neurotubules and relatively few mitochondria, while the spiny branchlets are lacking in an obvious pattern of dendritic tubules, although they are present and relatively filled with mitochondria (Fox, Siegesmund and Dutta, 1964). The main dendrite, which is also characterized by its encapsulation in glial processes, is separated from the surrounding parallel fibers, contrasting with the naked spiny branchlets which lie next to parallel fibers. In reptiles and birds, however, the tertiary Purkinje cell branches are incompletely encapsulated by glial processes and, as a result, parallel fibers are adjacent to the Purkinje cell dendrite. These large dendrites also have spines emerging from their surface which contacts parallel fibers.

The number of spines emerging from the spiny branchlets varies between species, ranging from 10 to 30 per 10μ length (Fox and Barnard, 1957; Hillman, 1969a). These spines are club-shaped processes (Ramón y Cajal, 1911; Fox *et al.*, 1964) which arise from the entire circumference of the spiny branchlet. Within the distal enlargement is an array of smooth membranes in the form of small sacs, somewhat like those described by Gray (1959) as the spinous apparatus of the cerebral cortex.

The Purkinje cell axon emerges from the cell body as a conical process which rapidly thins to a relatively small diameter before enlarging and becoming myelinated (Fox *et al.*, 1967). Within this process are numerous mitochondria, vacuoles and tubules. In the frog the initial axonic segments contain characteristic, microtubular arrangements. Also present are large tubules of smooth endoplasmic reticulum which in some fixations appear as vacuoles.

Functional meaning of the two forms of synaptic junctions on Purkinje cell dendrites

From the above study it is evident that excitatory and inhibitory synapses contact Purkinje cells at different sites on the dendrites; excitatory terminals on spines (parallel fibers and climbing fibers) and inhibitory

terminals mainly directly on the dendritic shafts. A theoretical study of the electrical characteristics of these two types of endings (Llinás and Nicholson, in preparation) has suggested that these two different forms of synaptic junction have different physical properties, which indicates different functional organization.

a) Synapses on spines; a "current injecting" mechanism. Spines can be characterized almost uniformly as bulbous protrusions which attach themselves to dendrites by means of a rather small stem. The diameter of these stems has been calculated from 111 spines to be 1,000 to 3,000 Å thick with an average of 2,020 (Fig. 14). Given the small diameter of the junction between the spine and the dendrite, a longitudinal resistance of about 10 MΩ has been calculated for the spine assuming a conductivity of 60Ω cm for the cytoplasm (Hodgkin and Rushton, 1946). This very high longitudinal resistance would behave as a current limiting resistor having two main functions, a) to convert these synapses into a close to constant current system, and b) to protect the length constant of the dendrite by preventing a large variation of input resistance during synaptic transmission. Although the amount of current injected through the small neck of the spine would be small (Fig. 15), given the extremely high input resistance of a spine even a comparatively small number of quanta of synaptic transmitter would tend to approximate the maximum synaptic potential amplitude (E_{EPSP}). Thus the potential generated across the membrane of the spine would be close to 60mV (depending on the spine's resting potential and the equilibrium potential for the EPSP). In this respect, therefore, synaptic transmission through a spine can be viewed as a current injecting system, especially in those dendritic segments where the input impedance may be lower than 10 MΩ. On the other hand, since the amount of current which this system could introduce would be dependent on the potential difference between the inside of the spine and inside of the dendrite, as shown by the fact that climbing fiber EPSPs can be reversed by the application of an extrinsic current (Eccles *et al.*, 1966a; Llinás and Nicholson, 1969), the magnitude of the current injection will be, of course, dependent on the membrane potential as well as on the input impedance of the dendrite. The existence of this current injecting device, however, means that activation of parallel fibers would produce a more linear summation of the synaptic potentials across the dendrites since the conductance change produced by the synaptic transmission would be minimized, as far as the input resistance of the dendrites is concerned, by the large longitudinal resistance of the spine. A second interesting consideration is that if synaptic activation can occur without great conductance changes in the dendrite, the distance a synaptic potential can travel within dendrites (length constant) would not change so much during synaptic transmission, thus ensuring the summation of synaptic potentials which arise from different dendrites. Another important aspect is the generation of dendritic spikes, since the presence of synapses on the spines would tend to prevent the blockage of local and dendritic action potentials due to synaptic shunts. From this latter point of view, it can therefore be stated that dendritic spines aid the generation and conduction of dendritic spikes.

b) Synapses on dendritic shafts as a spike blocking device. Very much in agreement with the previous hypothesis is the fact that inhibitory synapses for the most part establish contact with the shaft of the dendrites (Ramón y Cajal, 1911; Gray, 1961; Hámori and Szentágothai, 1965; Fox *et al.*, 1967; Uchizono, 1967; Hillman, 1969b; Larramendi, 1969; Mugnaini, 1969; Sotelo, 1969). This location of inhibitory synapsis would ensure both a reduction of electrotonically conducted potentials and a blockage of actively conducted spikes. The mechanism of action would be twofold, a) a hyperpolarization of the dendrite to near the equilibrium potential for the IPSP and b) the shunt produced by the large reduction of the dendritic input resistance generated by this synaptic transmission which would reduce the amplitude of the synaptic potentials as well as the distance which these can travel. As far as the dendritic action potentials are concerned, the location of this synapse on the dendritic shaft would be most effective in blocking the conductance since the dual mechanism of hyperpolarization shunt, combined with the rather low safety factor for dendrite spike conduction, is a most efficient system (Llinás and Nicholson, 1969). Furthermore, given the evidence for functional independence of dendrites, it is easy to imagine that an inhibitory volley on a dendrite would produce a "functional amputation" of the dendrites located immediately above the site of the inhibitory synapse, but would not have as great an influence in other dendrites. This view is in agreement with the concept of the Purkinje cell dendritic tree as a complex unit composed of several semi-independently integrated systems, the main dendritic branches. Since the presence of spines is restricted not only to Purkinje cells but is evident in many cells in the central nervous system throughout phylogeny, this form of junction might in fact represent a general solution for effective summation of synaptic potentials. A preliminary analysis of this hypothesis on a mathematical model (Llinás and Nicholson, in preparation) has in fact shown that while synapses on spines tend to sum more linearly, excitatory synapses terminating directly on the dendrites, as in the case of the interneurones of the molecular layer, would summate in a more nonlinear fashion. This difference

may be of great importance in the regulation of Purkinje cell activity by these interneurones since the different dynamic properties of these two sets of neurones may overlap to increase neuronal stability. Another conclusion regarding synapses on spines concerns the relation between the area of synaptic apposition nad synaptic efficiency. Since the amount of current which can be injected it limited by the diameter of the spine shaft, the area of a particular synaptic junction may be more related to functional aspects, such as time course of transmitter depletion during repetitive firing, than to the amplitude of the synaptic potentials which a given synapse may evoke.

CONCLUSIONS

The present comparative study of neuronal circuitry of the cerebellum strongly suggests that the cerebellar cortex of different vertebrates is basically similar in morphology and in function—at least at the neuronal level—the main difference being more related to the mossy fiber input than to the climbing fiber input. It is quite evident that the climbing fiber system shows very little variation throughout the phylogenetic scale as regards its general morphological distribution and its powerful excitatory action on Purkinje cells. The constancy of this climbing fiber-Purkinje cell system suggests, therefore, that this relation must be more primitive than the granule cell-Purkinje cell type of pathway. This is, of course, very reasonable given that the connection is a simple monosynaptic type of relation involving very little neuronal complexity. Assuming that the ontogeny of the cerebellum in some ways mirrors the phylogenetic development of this organ, an interesting correlate is suggested by the finding that ontogenetically the climbing fiber-Purkinje cell synapse is the first to occur in the cerebellar cortex (Ramón y Cajal, 1911). In agreement with this view is the fact that the climbing fiber system in the frog seems to be rather similar morphologically to the embryonic climbing fiber in the mouse (Larramendi, personal communication).

Assuming that cerebellar ontogeny mimics phylogeny, one can further assume that the climbing fiber system possibly develops as a specialization of an excitatory collateral which in very primitive vertebrates utilized the primordial Purkinje cell as a small inhibitory interneurone somehow related to the octavo-lateral system. As in the case of Renshaw cells, the primitive Purkinje cell could be regarded as a simple inhibitory neurone which tends to fire repetitively in order to produce the inhibition of its target cells. It is easy to imagine that at the primitive stage more than one type of fiber ended on the surface of these inhibitory neurones and that with further specialization the one-to-one relation so very typical of climbing fiber-Purkinje cell synapses finally arose. We can now present a simple hypothesis regarding "*la raison d'être*" of the two forms of inputs to the Purkinje cell (Llinás, 1969). One input, the climbing fiber, would utilize the Purkinje cell as the rather primitive and specific inhibitory interneurone which would generate a simple inhibitory pattern, resembling in some ways the hypothetical ancestral Purkinje cell before it became specialized by the presence of the mossy fiber-granule cell system. The other form would generate large population discharges through the mossy fiber-granule cell input.

Study of the evolution of the cerebellum very clearly demonstrates that most changes which have occurred at the level of the cerebellar cortex are strongly related to the mossy fiber-granule cell input. Thus with phylogenetic development one finds that the number of parallel fiber-Purkinje cell synapses increases enormously. It is also evident that the Purkinje cells become highly organized spatially in order to attain maximum divergence with maximum convergence by spreading out at right angles to the direction of the parallel fibers.

Also related to this system is the presence of molecular layer and granular layer interneurones, the development of which seems to be closely related to the great increase in numbers of parallel fibers and the large spread of the Purkinje cell dendritic tree. It seems therefore reasonable to conclude that given the large number of excitatory inputs which Purkinje cells of higher vertebrates have, an inhibitory regulation would be most appropriate in order to increase the proper channeling of the large amount of information which must be reaching the cerebellar cortex per unit of time. In this manner the stellate and basket cells would tend to produce strong inhibitory action at dendritic and somatic levels respectively, while the Golgi cell, by its inhibitory action on granule cells (Eccles *et al.*, 1966d), would regulate the activity of the granule cell layer. In addition to the simple inhibitory feedback system postulated by Eccles *et al.* (1966d), a recent series of experiments (Precht and Llinás, 1969) suggests that the Golgi cells might function also as a complex gating system which would rechannel information to specific areas in the cerebellar cortex.

Another correlation between mossy fiber-granule cell input and cerebellar development is the remarkable parallel between the presence of basket cell neurones and the presence of Purkinje axon collaterals which seem closely related anatomically and strongly related functionally (Llinás, 1967; Llinás and Precht, 1969). Whether or not the quantitative changes which occur in the cerebellar cortex with evolution bring about changes in the functional pattern in the cerebellar cortex is as yet impossible to determine. It seems reasonable to assume, however, that the cerebellar cortex represents a very specific solu-

tion to the problem of motor control and that its similarities of circuitry in different vertebrates are in fact related to similarities at the level of functional organization. It also seems possible to assume that the understanding of the complex organization of the cerebellar cortex would profit greatly from further study of a primitive cerebellum such as present in *Rana catesbiana*, where the cerebellar circuit seems to be very close to the postulated basic cerebellar circuit (Llinás, 1969). The problem as to whether the cerebellum of *Rana catesbiana* represents a primitive rather than a simplified nervous system has of course to be left open (cf. Romer, 1969). However, Jarvik (1968) in a recent symposium on vertebrate phylogeny came to the following tentative conclusion, "The anurans are in certain respects conservative descendents of one of the most primitive groups of vertebrates [osteolepiforms]."

An intriguing corollary which resulted from this comparative study relates to the tendency of excitatory inputs to Purkinje cells to be located in spines. This strongly suggests that dendritic spines function as a current injection mechanism which would allow fine gradation in the summation of parallel fiber action on Purkinje cells and would act to protect the generation and conduction of dendritic spikes. In contrast, the particular location of inhibitory synapses would tend to ensure maximum inhibition both by the production of a hyperpolarization as well as by the generation of large conductance changes. Thus there would be a reduction of the amplitude and spread of synaptic potentials generated immediately above the place of synapse and a most effective blocking of dendritic action potentials.

SUMMARY

A comparative study of the morphological and functional characteristics of the cerebellar cortex in different vertebrates is presented. It is concluded that while the climbing fiber system must represent a very primitive input to the cerebellar cortex which has not changed very much with evolution, the mossy fiber-granule cell system has on the contrary undergone a rather large number of changes with phylogenetic development. Related to this latter system is the large development of the molecular layer in higher vertebrates and the enormous increase in the number of parallel fiber-Purkinje cell synapses, as well as the development of the large inhibitory neurones of the molecular layer, i.e. the stellate and basket cells, which are shown to be inhibitory in all animals tested. Additionally related to the mossy fiber-granule cell system is the development of the inhibitory system of the granule layer, the Golgi cells, which represent both a feedback system and a complex gating system. In parallel with the generation of these inhibitory interneurones is the large development of the Purkinje axon collateral, the supra- and infra-ganglionic plexus of Cajal. Finally, the functional meaning of synapses on dendritic spines as opposed to synapses ending directly on dendritic shafts is discussed.

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