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The Length of Parallel Fibers in the Cat Cerebellar Cortex. An Experimental Light and Electron Microscopic Study

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Summary. Experimental light and electron microscopic studies were carried out to determine the length of parallel fibers in the cat cerebellar cortex. Using a fine surgical knife, vermal and hemispheral folia were cut perpendicular to their long axis. The animals were sacrificed 1–10 days after the operation. Sections of the transected folia were then stained with a Fink-Heimer procedure. The resulting degeneration appeared as fine dots that extended lateral to the lesion, as predictable from the course of the parallel fibers. Densitometer readings indicate that the density of degeneration declines gradually lateral to the lesion. The specificity of the silver impregnation was checked by processing silver stained sections for electron microscopy. This confirmed the location of the silver precipitate on degenerating parallel fibers. The pattern of parallel fiber degeneration in the molecular layer has a trapezoidal configuration centered on the lesion. The shorter parallel fibers are located at the base of the molecular layer and extend for 5 mm. The parallel fibers become progressively longer as they approach the pial surface where they attain a maximum length of 7 mm. Our studies suggest that in folia longer than 7 mm parallel fibers are 6 mm long on the average.

In addition, it was determined on Golgi sections that the average center-to-center distance between en passant boutons of individual parallel fibers is 5.2 μm. The data indicate that an average parallel fiber, 6 mm long, forming approximately 1100 boutons, may synapse with each Purkinje dendritic tree it traverses.

Key words: Cerebellum – Silver impregnation – Parallel fibers – Operated cats.

Morphological and functional studies have indicated that the efferent and afferent connections of the cerebellar cortex are organized in longitudinal zones (Jansen and Brodal, 1940, 1958; Voogd, 1964, 1967, 1969; Voogd et al., 1969; Rossum, 1969; Oscarsson, 1969, 1971; Miller and Oscarsson, 1970;

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Korneliussen, 1972). The Purkinje cells, which are the principal neurons of the cortex, have their flattened dendritic trees oriented in parallel with these zones. The dendrites and the axons of the stellate cells in the molecular layer also conform to this longitudinal order. On the other hand, the parallel fibers, which relay afferent excitatory inputs to Purkinje and stellate neurons, have a strictly transversal orientation (Ramón, 1911).

In order to fully appreciate the relationship of the parallel fibers to the predominantly longitudinal bias of the cerebellum, it is essential to know the length and number of synapses of the parallel fibers. These measurements will aid in stereological computations and theoretical and computer models of the cerebellar cortex.

Previous studies of parallel fiber length were done using different methodological approaches. The results of these studies were contradictory (Dow, 1949; Fox and Barnard, 1957; Palkovits et al., 1971c; Smolyaninov, 1971).

Since the number of parallel fibers is enormous (Fox et al., 1964, 1967; Eccles et al., 1967), we thought that a reliable evaluation of their length could be based on a light microscopic bulk method. The parallel fibers run in the direction of the folia and originate from a T-division of the granule cell axons, ascending to the molecular layer from the granular layer with a direction perpendicular to the cerebellar surface. Transection of the folia perpendicular to their long axis, therefore, should provoke degeneration of the parallel fibers. A fairly accurate measurement of their length could be achieved with silver impregnation methods for degenerating fibers. The method of Wallerian degeneration has been almost exclusively applied to the study of long neuronal connections. Nevertheless, due to the almost strict geometrical order of the cerebellar cortical elements, and to the extensive knowledge of their ultrastructural features (Hámori and Szentágothai, 1964; Fox et al., 1967; Eccles et al., 1967; Larramendi, 1969; Llinás and Hillman, 1969; Sotelo, 1969; Mugnaini, 1972; Palay and Chan-Palay, 1974), the silver impregnation technique, accompanied by electron microscopic control, can be applied to the study of short neuronal connections.

As presented in this account, our measurements of parallel fiber length in adult cats differ markedly from those obtained with other techniques. Our data also show that, contrary to what was previously thought, the parallel fibers are progressively longer from lower to higher levels of the molecular layer.

To determine the total number of synaptic connections formed by the average parallel fibers, the spacing of synaptic boutons along parallel fibers was determined on Golgi sections from normal animals. The thickness and interdigitation of the dendritic tree of Purkinje cells were measured in Golgi sections and in experimental material as described in a separate publication (Brand and Mugnaini, in press).

Methods

Over 60 adult cats of both sexes were used in this study. The animals were operated upon in an attempt to produce a neat transection of some of the folia in the following areas: anterior and pos-

terior lobes of the vermis and hemispheres. A few of the animals were used for electron microscopic controls and for Golgi impregnations. Additional Golgi sections were obtained from a collection available in our laboratory. The cats were anesthetized with 0.13 mg/kg, i.m., of Ketaject (Bristol Labs, Syracuse, N.Y.) followed, after a few minutes, by 0.03 mg/kg of sodium pentobarbital, i.p.

Operative Approaches

In 7 of the animals a pretentorial approach was used. This approach, exposing the anterior part of the cerebellum, allowed transection of some folia under visual control but occasionally resulted in excessive bleeding and required considerable postoperative care of the animal.

In most of the animals folial transections were made blindly via a posterior approach. After separation of the occipital muscles, a hole was made in the supraoccipital bone, using a dental drill and dripping cold saline on the burr's head to prevent heat damage to the cerebellar cortex. The dura was then carefully hooked and cut with little or no bleeding. A Smith-Green cataract knife or a Castroviejo keratome was then inserted ventrally into the cerebellum and toward the anterior vermis or the hemispheres, and retracted with a smooth, dorsalward, semicircular movement. Usually one single lesion was performed in each animal.

Neat transection of the folia with relatively little bleeding was obtained in the following regions of the cerebellar cortex: i) lobules III to VII of the vermis at the midline, in the pars intermedia, and in the pars lateralis; ii) crus I and crus II of the hemispheres.

Animals were sacrificed from 6 hours to 10 days after surgery.

Histological Techniques

For light microscopy the cats were perfused through the ascending aorta with 4% formaldehyde in physiologic salt solution. The cerebella were stored for 1 week in the same fixative, then transferred for another week to a similar solution with 30% sucrose added and finally embedded in albumin-gelatin. Care was taken as to obtain a plane of section parallel to the course of the severed folia.

Frozen serial sections were cut on a sliding microtome at a thickness of 26 μ, keeping the block frozen with powdered dry ice. Several techniques for silver staining of degenerating fibers were used. These included the original Nauta method (Nauta, 1950) and several of its modifications, as the Nauta-Gygax (1954), the Chambers, Liu and Liu (or Nauta-Laidlaw) (1956), the Fink-Heimer (1967) and the Eager (1970) procedures. All the procedures revealed some parallel fiber degeneration, with the exception of the Nauta-Laidlaw. The latter involves the use of a carbonated ammoniacal silver solution. However, selective staining of the degenerating fibers in the molecular layer of the cerebellar cortex required great care since the parallel fibers are thin and their fragments become quickly engulfed by the abundant glial elements. Overall, the best results were achieved using a slight modification of the Fink-Heimer method. This improved the specificity of the staining, at the same time maintaining a light background, thus enabling us to determine the extent of the degeneration with greater precision and reliability.

The following schedule proved valuable:

1. Rinse in distilled water for 5 min.
2. Transfer sections for 15 min to a mixture of:

0.5% uranyl nitrate	10 ml
2.5% silver nitrate	12 ml
distilled water	28 ml

3. Rinse in distilled water
4. Transfer sections for 15–20 sec to a freshly prepared ammoniacal silver solution composed of:

2.5% silver nitrate	10 ml
2.5% sodium hydroxide	0.5 ml
strong ammonia water	13 drops (Pasteur pipette)

This solution has to be replaced after processing about 10 sections.

5. Transfer sections without rinsing to a Nauta-Gygax reducing solution consisting of:

distilled water	900 ml
95% ethanol	75 ml
10% formalin	17 ml
1% citric acid	20 ml

6. Rinse and transfer sections for 1 min to 1% sodium thiosulfate, rinse well, mount, dehydrate and cover.

In cases where this procedure gave unsatisfactory results because of weak impregnation, unspecific precipitate or background staining, improvements were obtained by one or a combination of the following alternations: i) storing the unstained sections in 2% formaldehyde for one week or more; ii) increasing time in step 2 up to 1 hour, altering the number of ammonia drops in step 4 in the range of 10–15; iii) increasing slightly the amount of ethanol and citric acid in the reducer.

Electron Microscopic Controls

Of the operated cats 8 were used for electron microscopy. These were perfused with 150 cc of buffered saline followed by 2–7 liters of a solution containing 2% formaldehyde and 1% glutaraldehyde in 0.12 M phosphate buffer, with traces of 0.045 M $CaCl_2$ added. The pH of saline and fixative solutions was adjusted at 7.2–7.4. After 4–16 hours from the end of the perfusion, the cerebellum was removed and the lesioned folia were dissected out, postfixed in buffered 1% osmium tetroxide, dehydrated in ethanol-propylene oxide and embedded flat in a 50–50 Epon/TAAB resin mixture. Selected areas at known distances from the lesion were trimmed for ultrathin sectioning on an LKB Ultrotome with the "mesa" technique, under the guidance of semithin sections stained for light microscopy with toluidine blue. The ultrathin sections were stained with uranyl acetate and lead and micrographed on a Hitachi HU-12 electron microscope at 75 KV.

Some of the transected folia from animals perfused for light microscopy were separated after the perfusion. These folia were postfixed overnight in formaldehyde and sliced on a Vibratome (Oxford Instruments, Inc.) at 50 μ thickness. The slices were stained with the modified Fink-Heimer procedure, postfixed in buffered 1% osmium tetroxide, and processed further for electron microscopy as above (Brand and Mugnaini, 1975). Flat embedding of these slices was done with a flat bottomed prepolymerized capsule. This same procedure was repeated with transected folia from animals perfused and postfixed with buffered aldehydes with comparable results.

Golgi Impregnations

For Golgi impregnation of normal parallel fibers we used unfixed cerebella, as well as cerebella from normal cats perfused with buffered aldehydes according to the procedure for electron microscopy. In both cases the steps of the metal impregnation procedure were those known as the "rapid Golgi method" (Valverde, 1970).

Measurements

All measurements were done in the microscope using a Zeiss calibrated ocular and then repeated on the micrographs. In order to accurately measure the extent of degeneration along the course of the transected folia, we selected Fink-Heimer sections where the plane of section was perfectly parallel to the course of the transected folium. The extent of the lesions, the bleeding, the edema and the necrosis were also carefully evaluated.

For measurements of the average distance of the en passant boutons of Golgi impregnated fibers, we chose budded reddish-brown fibers from the middle of the molecular layer. We avoided sections where the stellate cell dendrites displayed the "état moniliforme" (Ramón, 1911).

The densitometer readings were made with a Hewlett Packard photometer mounted to a light microscope.

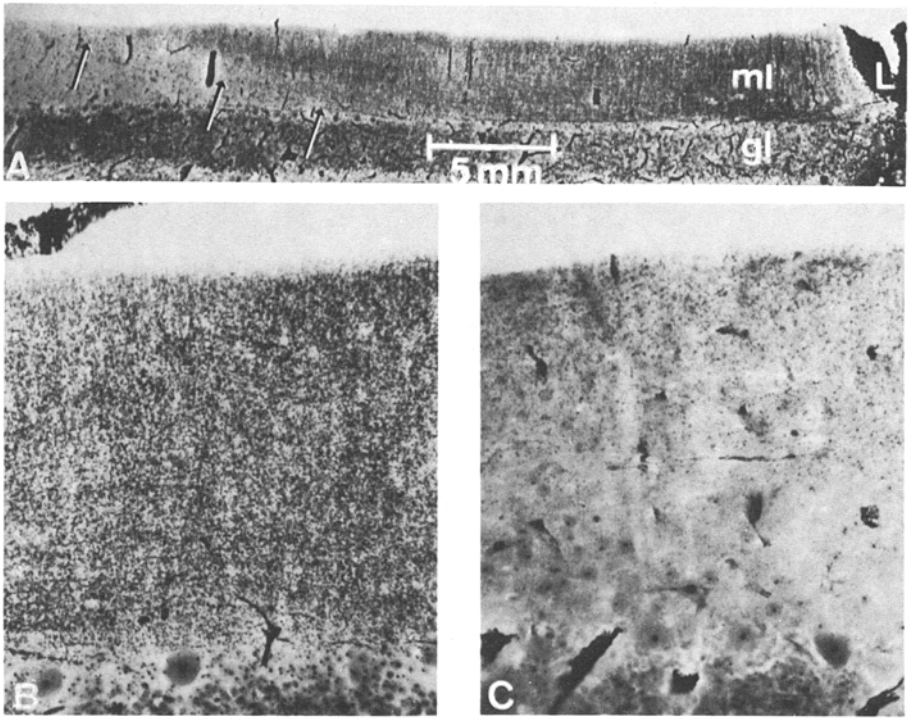

Fig. 1. A Horizontal section of a cerebellar folium transected at *L* and stained with a Fink-Heimer procedure. The degenerating elements lateral to the lesion extend for a progressively longer distance from the deeper to the superficial portions of the molecular layer *(ml)*, thus forming a trapezoidal pattern. The lateral border of the degeneration pattern is indicated by arrows. At *gl*, granular layer. (117 ×). **B** Light micrograph of the molecular layer taken within 1 mm of the lesion, showing the high density of degenerating, silver impregnated elements. (375 ×). **C** Light micrograph of the molecular layer taken 3 mm from the lesion. The density of silver impregnated elements is greatly reduced compared to Fig. 1B. The generating profiles are restricted to the superficial half of the molecular layer. (375 ×)

Results

Silver Impregnation of Transected Folia

At the outset of this study we considered that the length and the pattern of degeneration might vary in different portions of the cerebellar cortex. For this reason lesions were placed at various points in the cerebellar cortex including the medial, intermedio-medial, intermedio-lateral and lateral zones of lobules III–VIII, in crus I and II. In all of these regions the results were similar.

Figure 1A shows the typical pattern of degeneration following transection of a folium. Two features are immediately apparent. First, the degenerating elements extend further from the lesion in superficial than in progressively deeper levels of the molecular layer (Fig. 1B and C). The field of degeneration

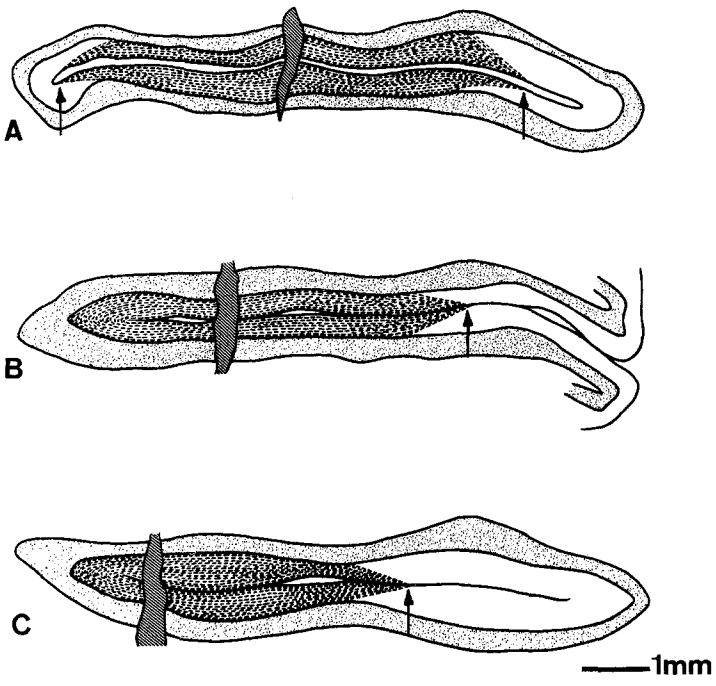

Fig. 2. Drawing of horizontally sectioned vermal folia demonstrating the pattern of parallel fiber degeneration after lesions placed at various locations along the folia. The midline lesion **A** results in a symmetrical degeneration pattern. The two more lateral lesions **B** and **C** result in asymmetrical patterns due to the curvature of the folia. The arrows mark the lateralmost extent of the degeneration

has, therefore, a trapezoidal outline, with the short base toward the Purkinje cell layer and the long base toward the pia.

The short and long bases of the trapezoid measure about 2.5 mm and 3.5 mm, respectively, on each side of the lesion, making the total length of the pattern from 5 mm to 7 mm. The degeneration pattern appeared identical after transections in the medial and intermedio-medial zones of the vermis (Fig. 2A and B). When lesions were placed in the intermedio-lateral zone of a bending folium, the distribution of the degenerating elements follows the curvature (Fig. 3) of the molecular layer. The existence of the curvature, however, does not alter the extent of the degeneration, as the measurements are identical to those in the straight portion of the folium. The trapezoidal pattern is also preserved. When transections were made in the lateral cerebellar zone, the degeneration pattern appeared asymmetrical (Fig. 2C), obviously due to the curvature of the folia, as the degenerating fragments extend toward the midline as far as in the lesions of the midvermal zone (cp. Fig. 2A and 2C). Thus, with the exception of the folia transected near the end, the degenerating elements extend for equal distances on both sides of the lesion.

The second feature to be noticed is that the density of the degenerating elements decreases progressively from the zone of the lesion laterally. This as-

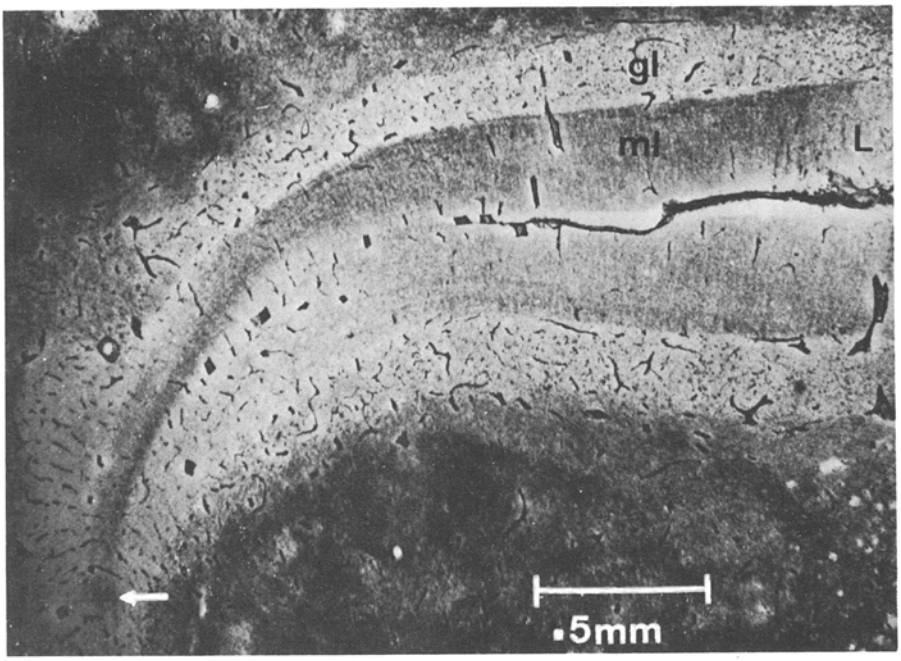

Fig. 3. Light micrograph of the lateral portion of horizontally sectioned cerebellar folia transected within the intermedio-lateral zone of the cortex. The most lateral extent of the degenerating parallel fibers is indicated by the arrow. Note that the degeneration pattern follows the curvature of the folia. *gl* and *ml* indicate the granular and molecular layers. (140 ×)

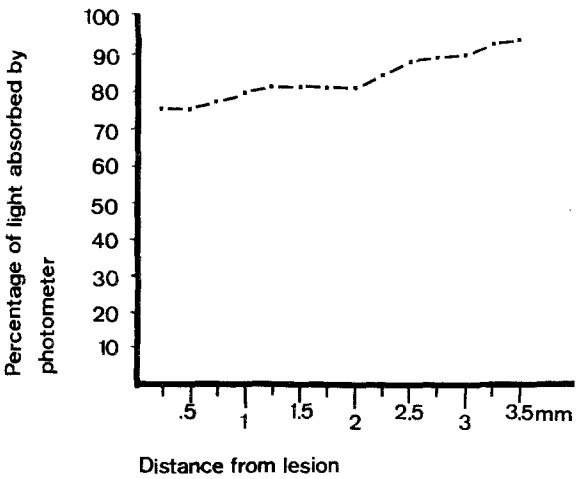

Fig. 4. The graph shows the percent of light absorbed by the photometer as it scans the top of the molecular layer of a transected folium from the site of the lesion laterally in a section stained with a Fink-Heimer procedure. The plane of section is parallel to the course of the folium. Near the site of lesion the large number of silver particles decreases absorption by approximately 25%. The optic density of the molecular layer gradually decreases until it reaches background levels 3.5 mm from the lesion

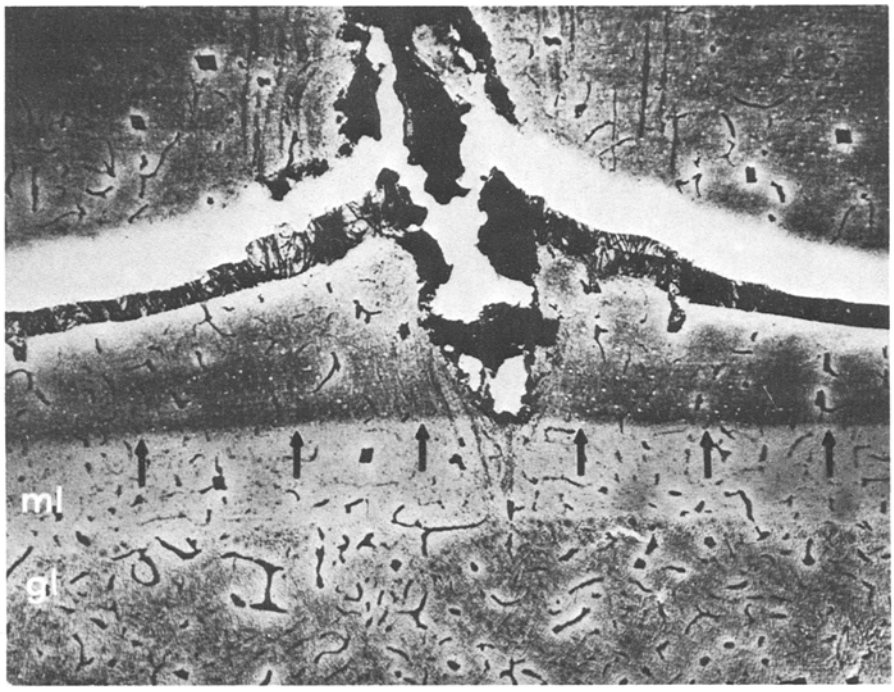

Fig. 5. The tip of the knife has transected completely the folium in the upper part of the picture, but it has entered only the superficial half of the molecular layer in the folium at the center and bottom of the picture. The border line of the resulting parallel fiber degeneration in the partially lesioned folium is sharp (arrows). The molecular layer and the granular layer of the partially lesioned folium are labeled *ml* and *gl*. (140 ×)

pect is easily appreciated by comparing the density of the degenerating elements in Figure 1B and 1C, taken from the same folia, 1 and 2 mm, respectively, from the lesion.

To quantify this density gradient, densitometer readings were made from selected sections. The readings (Fig. 4) show a gradual decrease in the density of silver particles starting at the lesion and going laterally until near the end of the area containing degenerating elements the readings approach the background.

In some of the folia (Fig. 5) the knife has transected the superficial part of the molecular layer sparing the deeper half and the Purkinje cell layer. The silver impregnation of degenerating elements abruptly stops at the end of the knife path. Measurements from these partially cut folia correspond to those in the completely transected ones.

Size and Configuration of Degenerating Elements

Immediately adjacent to the line of folial transection, there is a thin zone that displays a markedly reduced argyrophilia, presumably due to edema. Lateral to

this zone, however, the degenerating elements appear well stained. One notes that their size and configuration vary considerably. Due to the high density of degeneration near the lesion, the nature of the degenerating elements can hardly be established at the light microscopic level, with the exception of the degenerating Purkinje dendrites. These can easily be recognized due to their characteristic branching pattern (Brand and Mugnaini, in press). The size and the configuration of the degenerating elements becomes progressively less diverse more laterally. About 0.5 mm from the lesion the characteristics of the degenerating elements appear homogeneous. In animals killed 2–4 days after the folial transection the argyrophilic material present in this part of the molecular layer is distinctly granular. The granules measure 0.5–2 μ in diameter. They must represent degenerating parallel fibers as these are the only components of the molecular layer extending more than a few hundred microns lateral to the site of transection.

Electron Microscopy

Electron microscopy of tissue blocks from severed folia shows that in the vicinity of the transection site the degenerating elements represent mainly parallel fibers, Purkinje and stellate cell dendrites and climbing fibers. About 50% of the parallel fibers show signs of degeneration (Fig. 6A). In areas 1–3 mm lateral to the lesion practically all of the degenerating profiles can be identified as parallel fibers (Fig. 6B) on the basis of their orientation, their synaptic connections, or both. Electron microscopy of Vibratome sections stained with the Fink-Heimer procedure demonstrates that 1–3 mm lateral to the lesion the silver deposits are selectively located on the degenerating parallel fiber profiles (Fig. 6C). As observed light microscopically, the degenerating profiles become progressively more rare laterally. While 3 mm from the lesion no degenerating element is present at the bottom of the molecular layer, scanty degeneration is still encountered at the top of the molecular layer.

Thus, the trapezoidal pattern of degeneration seen in the Fink-Heimer sections represents parallel fiber degeneration.

Time Course of Degeneration at the Light Microscopic Level

Staining of the degenerating elements in the molecular layer of transected folia was already demonstrable 10 hours after the lesion. The best survival time appeared to be 1–4 days. With longer survival times the number of degenerating elements decreased progressively. Some degenerating elements could still be seen with a 10 day survival time. The elements stainable after such a long interval may belong to fibers newly degenerated as a result of the reparative processes undergoing at the site of the transection; they may represent elements degenerating as a direct result of the transection and still not removed by the glia; or they may be transected processes degenerating with a slower time course. Since the nature of the model and of the lesion makes it impossible to decide about these possibilities, we did not pursue further the analysis of the time course of the degeneration.

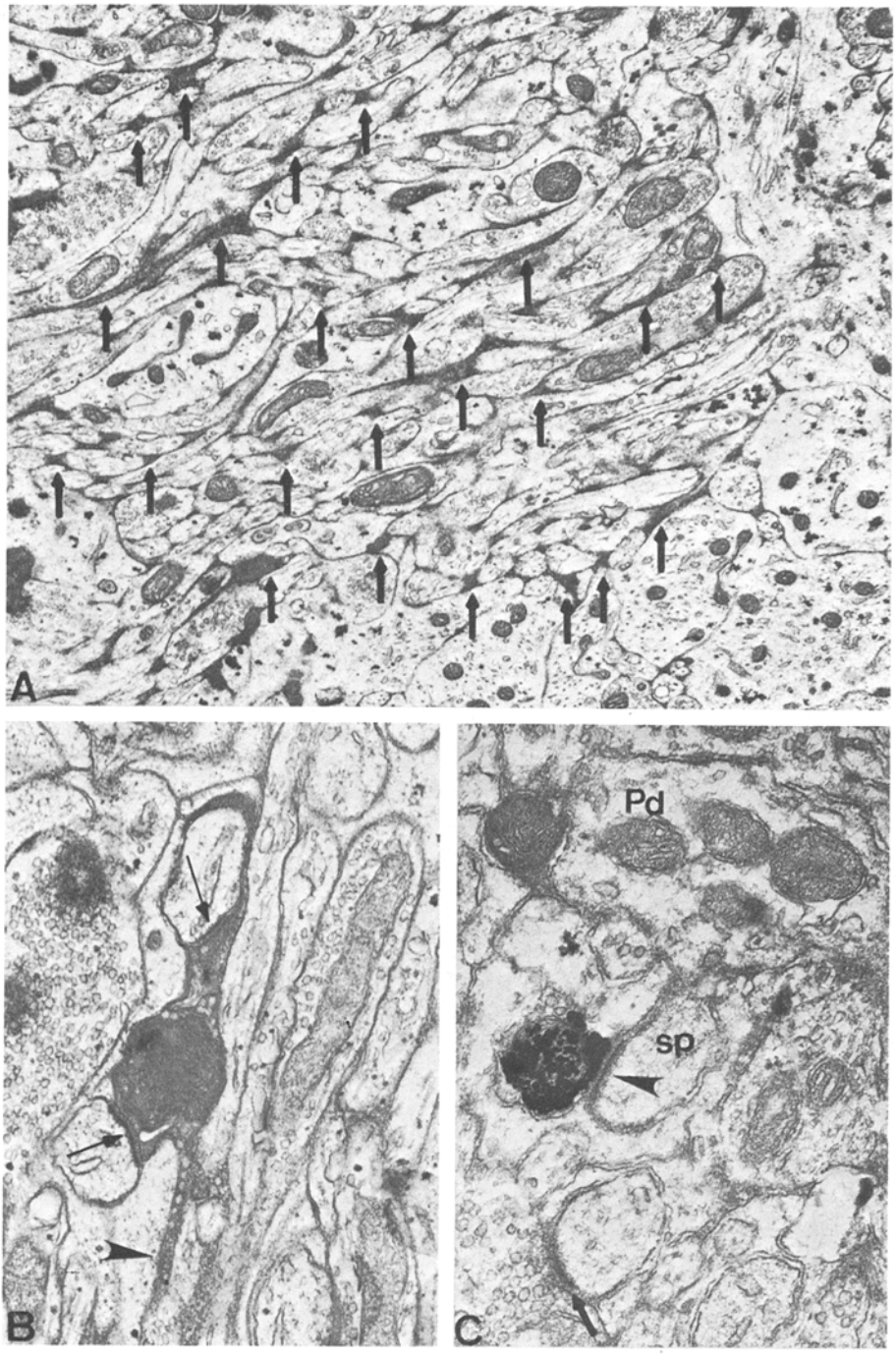

Fig. 6

Time Course of Degeneration at the Electron Microscopic Level

With the exception of the few microns alongside the transection line, where watery profiles are present, all the degenerating elements have an electron dense appearance.

One or two days after the folial transection many degenerating boutons still connected to darkened, thin unmyelinated fibers (the parallel fibers) are attached to normal Purkinje dendritic spines (Fig. 6B) and to stellate cell dendrites. At later stages, the constricted portion of degenerating parallel fibers are no longer demonstrable. Moreover, the degenerating boutons appear progressively smaller and are engulfed by branches of the Bergmann fibers and by "microglial" elements. Their connection with the postsynaptic element is thus lost. The degenerating elements become also progressively more rare, while the residual bodies within the glial cells become more numerous. The ultrastructural events, therefore, are completely in accordance with the time course of degeneration seen in the light microscope.

Golgi Impregnation of Normal Parallel Fibers

As known from previous light and electron microscopic studies (Hámori and Szentágothai, 1964; Fox et al., 1964), the parallel fibers consist of nonsynaptic constricted portions and of en passant, synaptic boutons. Assuming that the swellings seen with the Golgi method correspond to the synaptic boutons in the electron micrographs, we measured the center-to-center distance of the swellings along the parallel fibers in Golgi sections from normal animals. Only beaded parallel fibers in the middle of the molecular layer were considered, as these represented an average between the thicker ones at the bottom of the molecular layer and the thinner ones at the subpial levels. Measurements were taken from sections where the dendrites of stellate and Golgi cells were not beaded and the impregnation was discrete; the center-to-center distance between parallel fiber swellings was found to be 5.2 μ on the average (Fig. 7).

Discussion

Attempts to establish the length of parallel fibers have been made using a variety of techniques including microelectrode studies, direct measurements in Golgi sections, the method of persisting elements, theoretical computations and stereology. The data obtained are diverse. Dow (1949) concluded that in the cat the parallel fibers conduct an artificially applied stimulus in a lateral direction to a distance usually not exceeding 5 mm. Eccles, Llinás and Sasaki (1966) conducted a similar experiment. Although not commenting specifically

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Fig. 6. A Electron micrograph of an area of molecular layer within 1 mm of the lesion from a section parallel to the course of the folium. The electron dense, degenerating parallel fibers (arrows) represent nearly 50% of the parallel fiber population. (15000 ×). **B** Electron micrograph of a typically electron dense, degenerating parallel fiber (arrowhead) with synaptic swelling (arrows) still in synaptic contact with dendritic spines of a Purkinje cell. (30000 ×). **C** Electron micrograph from a section stained with the Fink-Heimer method before osmication, showing a silver impregnated bouton of a degenerating parallel fiber in synaptic contact (arrowhead) with a Purkinje cell dendritic spine *(sp)*. At *Pd* the Purkinje dendritic branchlet giving rise to the spine. The arrow indicates a normal parallel fiber bouton synapsing with a Purkinje spine. (28000 ×)

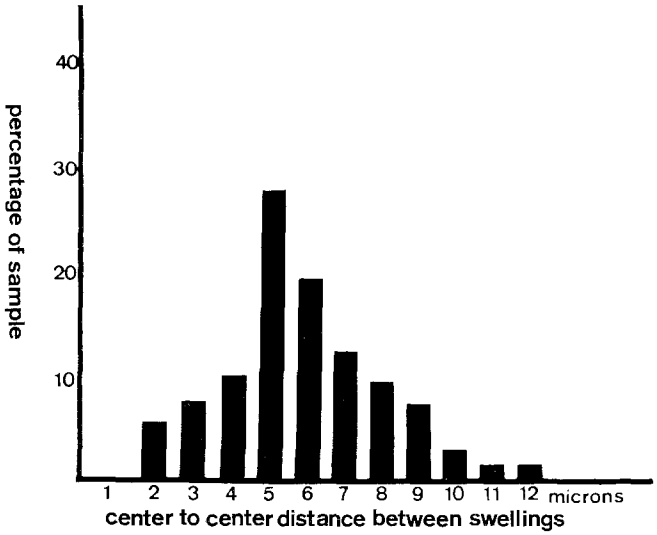

Fig. 7. Frequency distribution of center-to-center distance between parallel fiber swellings. The average distance between the synaptic swellings is 5.2 μ. An average parallel fiber, 6 mm long, can have a total of 1100 boutons

on the length of parallel fibers, they seem to accept the notion that they may be no more than 3 mm long. Fox and Barnard (1957) in Golgi impregnated sections of the monkey cerebellum could follow individual parallel fibers from the point of bifurcation for a distance ranging from less than 1 mm to 1.5 mm in both directions, which indicates a total length of approximately 1.5–3 mm. The longer fibers were located at the base of the molecular layer, and the shorter ones at the top. Braitenberg and Atwood (1958) computed that the human parallel fibers could be from 1–10 mm long. Szentágothai (1965) observed that in chronically isolated slabs of the cat cerebellar cortex, 3 mm long, the density of parallel fibers in the middle of the slab was not noticeably reduced, while if parallel fibers had been longer than 3 mm their density would have decreased significantly. Moureu-Mathieu and Colonnier (1969) removed a portion of a folium in the cat and with the Golgi method and electron microscopy described loss of spines and degeneration of Purkinje cell dendrites as far as 2 mm lateral to the lesion. They interpreted these alterations as due to parallel fiber loss, but did not comment on the length of parallel fibers. Recently, both Smolyaninov (1971) and Palkovits, Magyar and Szentágothai (1971c), approaching the question of parallel fiber length with stereology, estimated the average length of parallel fibers to be 1.6 mm and 2 mm respectively.

Evidently, all the above approaches have their own limitations. With the microelectrode technique one infers parallel fiber length indirectly from the distance at which an above threshold electrical stimulus can be recorded. The Golgi technique has the inherent difficulty of allowing only a few fibers to be

followed for a considerable length, and without the certainty that they have been impregnated along their whole extent. The method of persisting elements, although a very helpful one in certain cases (Szentágothai, 1965), is obviously inadequate for precise measurements. The stereological approach, although a sophisticated anatomical tool, has the disadvantage of inferring the length of parallel fibers from other measurements. As argued below, a silver impregnation bulk method, accompanied by electron microscopic control, furnishes a more realistic estimate of the length of parallel fibers.

Measurement of Parallel Fiber Length

Folial transection in the cerebellum of adult cat brings about a trapezoidal pattern of degeneration in the molecular layer which is revealed by a Fink-Heimer staining procedure. Electron microscopy confirms that, with the exception of an area of molecular layer near the transection site, the pattern seen in silver stained sections is due to parallel fiber degeneration. Thus the generation of parallel fibers must explain the main features seen with the light microscope, namely the trapezoidal pattern of the degeneration and the progressive lateral decrease in the number of argyrophilic profiles. To interpret the pattern of degeneration in the transected folia it is necessary to determine whether or not the granule neurons react to axotomy with anterograde degeneration alone, or with both anterograde and retrograde degeneration.

As known from Golgi studies the parallel fiber is made up of two colinear oppositopolar processes arising from a T-division of the granule cell axon which ascends from the underlying granular layer. Any transection of the folium will therefore intercept the parallel fibers at various points. Two main possibilities exist: (i) the plane of section passes through the T-division itself, disconnecting both oppositopolar branches from the cell body (the latter may be also damaged if it is located exactly, or very close to the transection plane); (ii) the plane of section passes through one of the oppositopolar branches at various distances from the T-division, only one segment of the parallel fiber being severed from the cell body.

If the transected parallel fibers underwent anterograde and retrograde degeneration, all parallel fibers in the vicinity of the transection site would be degenerating. On the contrary, our electron microscopic study showed that the number of normal parallel fibers near the lesion is about 50%. We can then conclude that the granule neurons respond to axotomy in the usual way, i.e. with an anterograde degeneration. Consequently, we may conclude that only those fibers transected at the T-division will show the real parallel fiber length since the parallel fibers severed asymmetrically will have only a portion of one oppositopolar branch undergoing degeneration (Fig. 8). This implies that the number of argyrophilic profiles representing the degenerating parallel fibers should decrease progressively in number from the site of lesion laterally. This is exactly what we observed both in the light and in the electron microscopes. As the number of degenerating fibers indicating the maximum length is small, the lateral boundary of the degeneration will not be sharply defined. Our measurements, therefore, may involve an error in the range of 0.1–0.5 mm.

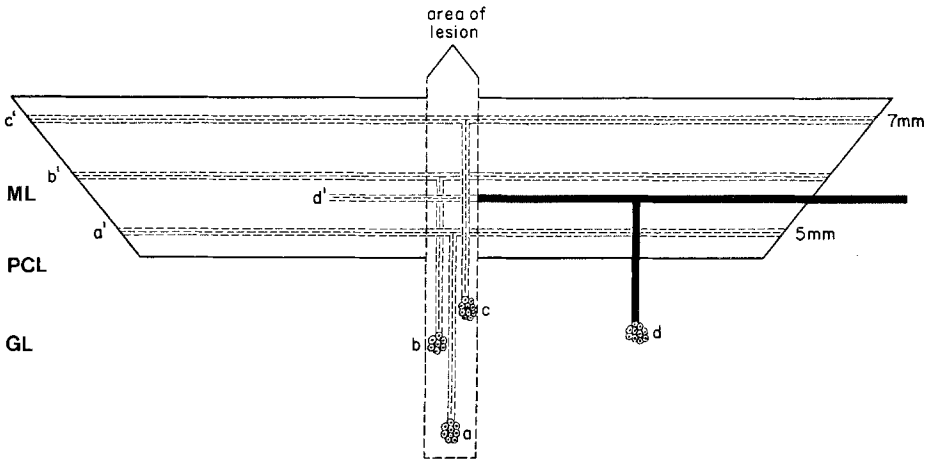

Fig. 8. Schematic diagram showing the effect of a folial transection on parallel fibers originating at or passing through the site of lesion. Acute retrograde degeneration does not occur. *ML*, *PCL* and *GL* designate the molecular, Purkinje cell and granular layers. The stippled lines represent parallel fibers undergoing anterograde degeneration; the dark solid line represents nondegenerating parallel fibers. Granule cells located at the base of the granular layer (a) give rise to the shorter (5 mm long) and thicker parallel fibers (a') in the lower portion of the molecular layer. The granule cells located in the middle of the granular layer (b) give rise to parallel fibers (b') of intermediate length and diameter located in the middle of the molecular layer. The granule cells near the top of the granular layer (c) give rise to the longer (7 mm long) and thinner parallel fibers (c') in the superficial portion of the molecular layer. The gradient of parallel fiber length, thus, determines the trapezoidal pattern of degeneration. As indicated by our electron microscopic studies, parallel fibers that do not bifurcate directly in the path of the lesion undergo degeneration only in the segment (d') severed from the parent cell (d). The density of degenerating elements, therefore, decreases gradually lateral to the lesion

It is evident that the maximum distance from the lesion at which argyrophilic granules are present at a given level of the molecular layer represents the maximal length of the parallel fibers at that level. Hence the parallel fibers must become progressively longer from the bottom of the molecular layer, where they measure 5 mm, to the top of the layer, where they become 7 mm long (Fig. 8).[2]

This gradient is opposite to the one assumed in the previous literature. The commonly held view is that parallel fibers in large laboratory animals like the cat and the monkey are shorter than 3 mm on the average, with the longer fibers located near the Purkinje cell layer and the shorter fibers near the pial surface. As mentioned previously, this view was based on some measurements from Golgi impregnated sections by Fox and Barnard. In a conversation with Dr. Fox, he pointed out to us that their fragmentary data have been given undue emphasis. They presumably measured either partially impregnated fibers or fibers which were not totally comprised within the section.

[2] The numbers given here are probably slightly in deficit as some shrinkage, at least 10%, takes place during the preparation.

It may be argued that while some parallel fibers do reach lengths of up to 7 mm, the average length is still 2 mm as was concluded by Palkovits et al. (1971c). If this were the case there would be a steep decline in the density of silver particles 1 mm lateral to the site of the lesion. Contrary to this the densitometer readings show a gradual decrease in the optical density of the molecular layer. In order to determine the exact statistical distribution of parallel fiber lengths, one needs to carry out a precise electron microscopic analysis of the density of molecular layer components, including degenerating parallel fibers, at various distances from the transection site. Such a study is pending. Nevertheless, due to the apparent homogenity of parallel fiber diameters within the same level of the molecular layer, and on the weight of the consistent results of the densitometric analysis of several sections, we tentatively conclude that our bulk method furnishes a measurement reasonably representative not only of the maximal length of parallel fibers but also of their average length.

In conclusion, we assume that at least in most of the cerebellar folia, parallel fibers are 6 mm long. On the average[3], the value of 5 mm given by Dow (1949), therefore, appears reasonably correct.

If an increase in the size of the cerebellum is accompanied by an increase in length of the parallel fibers (Smolyaninov, 1971), then a maximum length of 10 mm for human parallel fibers, as computed by Braitenberg and Atwood (1958), must also be realistic. Further studies on the length of parallel fibers in other animal species, now in progress in our laboratory, may reveal interesting evolutionary correlations.

In the folial white matter of the cat, longitudinal zones, made up of narrow fiber bands, approximately 1 mm wide have been demonstrated (Voogd, 1969). Thus the average parallel fiber spans a considerable number of these bands.

From a cytological point of view, it is important to note that the gradient of parallel fiber length from superficial to deeper levels of the molecular layer is opposite to that of parallel fiber thickness, which increases in the opposite way (reviewed by Palay and Chan-Palay, 1974). Perhaps the increase in length of the fibers compensates for the decrease in thickness and represents a trend in the population of granule neurons to equalize axonal volume. It should also be noticed that the longer parallel fibers are those which are laid down later in ontogeny. This is contrary to the case of stellate cell axons (Ramón, 1911; Scheibel and Scheibel, 1954; Fox et al., 1967; Eccles et al., 1967; Chan-Palay

[3] Preliminary observations on transected short folia of the paramedian lobule indicate that the trapezoidal pattern and the lateral decrease in density of degeneration are not present. Since the number of normal parallel fibers near the site of the lesion is high, the parallel fibers undergo Wallerian degeneration only, as in the vermis. All parallel fibers in the folia of the paramedian lobule, thus, presumably reach both ends of the folium. They should have unequally long oppositopolar processes, with the exception of those having a T-division exactly in the middle of the folium. Perhaps these folia are shorter than the potential length of the parallel fibers. If our interpretations are correct, the relationship between granules, parallel fibers, and Purkinje neurons in the paramedian lobule and in the vermis must differ considerably. This suggestion of the existence of regional variations calls for extensive new stereological determinations of the composition of cerebellar cortex.

and Palay, 1972), another type of cerebellar cortical interneuron which originates from the same matrix as the granule neurons, namely the external granular layer.

Number of Parallel Fiber-to-Purkinje Cells Synapses

As known the flat face of the espalier-like dendritic trees of the Purkinje cells are situated at right angles to the parallel fibers which pass among the branches of the trees synapsing with the dendritic spines. Estimates of the number of Purkinje trees crossed by a single parallel fiber and of the number of Purkinje cells synapsed by it have been attempted by several investigators. The numbers arrived at are different, depending obviously on the values given for the length of the parallel fiber, thickness and interspacing of the Purkinje dendrites, total number of spines per Purkinje tree. Fox and Barnard (1957), whose Golgi sections indicated an average parallel fiber length of 2 mm, estimated at 310 the number of Purkinje trees crossed by one parallel fiber. A parallel fiber would synapse 1 out of 3 Purkinje cells. After a reevaluation of the number of spines per dendritic tree, Fox et al. (1964, 1967) considered that this ratio may be close to 1:1. Eccles et al. (1967) gave estimates similar to the latter authors, and Marr (1969), Pellionisz (1970), Mortimer (1970) and Albus (1971) used these values for their models. Palkovits et al. (1971c), however, on the basis of stereological reconstruction figured that the average parallel fiber is 2 mm long, but it crosses the tree of 225 Purkinje cells, synapsing only with 1 out of 5 of them. Smolyaninov (1971) estimated the number of spines per Purkinje cell of the cat as low as 18000 and the average length of parallel fibers is 1.6 mm. He concludes that one parallel fiber synapses with only 30 Purkinje cells.

In a related study (Brand and Mugnaini, in press), evidence will be presented that the majority of the Purkinje dendritic trees in the cat are 8–13 μ thick and that there is no "dead space" (or nonsynaptic space) between subsequent trees. In their thickest portions the stacked dendrites may interdigitate in the direction of the parallel fiber course for up to 2 μ. In general a single tree occupies an exclusive section of the molecular layer about 8 μ thick, and an additional 3–5 μ of dendritic space can be shared with the preceding and the successive dendrites. Thus the average parallel fiber, 6 mm long, traverses the dendritic arborization of approximately 750 Purkinje cells.

An exact evaluation of the average number of synaptic sites for unit length of parallel fiber can be obtained only from reconstruction of many series of electron micrographs. Assuming that the en passant swellings seen along the parallel fibers in Golgi sections truly represent synaptic knobs, we checked the center-to-center distance of the swelling in a large number of arbitrarily selected parallel fiber segments. The correctness of the above assumption is debatable as beading of the fibers might be an artifact of procedure. On the other hand, it is known that the core of the fiber may become impregnated while the boutons are not.

Chan-Palay and Palay (1972), using a high voltage electron microscope, have shown that Golgi impregnated swellings along parallel fibers are the synaptic knobs. Electron micrographs of the molecular layer from animals fixed by perfusion often show artifacticious beading of the basket cell dendrites but not of the parallel fibers. Nevertheless, to reduce the chances of an estimate in excess of the center-to-center distance between the swelling, we discarded the sections in which the stellate cell dendrites presented the "état moniliforme". Moreover, sections with massive impregnation and with encrustations on the parallel fibers were avoided. However, we also discarded parallel fibers showing unusually long segments without swellings. This may allegedly have introduced a bias in our measurements.

The average parallel fiber forms a total of 1100 swellings, given that their estimated center-to-center distance of 5.2 μ represents a true average along its entire length.

In the cat (Hámori and Szentágothai, 1964; Mugnaini, 1972), the overwhelming majority of the parallel fiber boutons are synaptically related to Purkinje cell dendritic spines. The relationship is usually one-to-one, but in a number of cases a single bouton forms one synaptic junction with each of two Purkinje spines, or with a Purkinje spine and a stellate cell dendrite. A minority of synaptic junctions are formed with non-Purkinje elements, namely with stellate cell neurons (their perikarya, dendritic trunks, spines and emission cone of the axon) and with Golgi cell dendrites.

Palay and Chan-Palay (1974) have reported that 1 out of 3 parallel fiber swellings form a double synapse in the rat. No reasonable estimate of the frequency of the boutons with double synaptic junctions in the cat is available in the literature. Assuming that double synapses are formed by one-fourth of the parallel fiber swellings, the total number of synaptic junctions in a 6 mm long fiber would approach 1400. Thus, even accounting for an adequate number of synaptic junctions with stellate neurons and Golgi cell dendrites, the cat parallel fibers seem able to form at least one synapse with every Purkinje dendritic tree they traverse. The fact that the center-to-center distance between synaptic swellings is smaller than the thickness of the Purkinje tree suggests the same conclusion.

The stereological data of Palkovits et al. (1971a, b, c; 1972) as well as some of the measurements in the previous literature suggest that there might be no available space in the molecular layer to locate the estimated number of parallel fibers, if they are 6 mm long, as indicated by our measurements. The Purkinje dendritic tree may, furthermore, not be extensive enough to accommodate the number of spines necessary for contact with every crossing parallel fiber.

Further quantitative analysis of the cerebellar cortex is thus required to explain the reasons for this divergence.

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