Formation of Cerebellar Projection Maps: Development of Olivo- and Spinocerebellar Projections

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In spite of the apparent homogeneity of the cerebellar cortex, which is composed throughout of the same neuronal populations and the same types of afferent and efferent systems, the anatomical work of Voogd (1) and the electrophysiological studies of Oscarsson (2) have shown that the cerebellum has a longitudinal-zonal organization determined by the successive apposition of structurally and functionally distinct longitudinal strips. The specificity of each of these cerebellar compartments results from the precise pattern of its afferent and efferent connections, which constitute the cerebellar projection maps.

The study of the formation of cerebellar projection maps should provide some insight into the mechanisms underlying the acquisition of the longitudinal-zonal organization. This is the aim of a research program started a few years ago in our laboratory and carried out with ML Arsenio-Nunes, F. Bourrat, and M. Wassef. A summary of this research will be presented here, in relation to the following topics: (a) the establishment of the olivocerebellar projection; (b) the formation of spinocerebellar topography; and (c) the arrangement of Purkinje cells during their migration and subsequent segregation.

The rat was chosen as the experimental animal since most of the electrophysiological information concerning the development of olivocerebellar (3-5) and spinocerebellar systems (6,7) has been obtained from this species. Analysis of the immature organization of projections was based on tracing systems using autoradiography and peroxidase techniques. Purkinje cell compartmentalization was studied by means of immunohistochemical techniques with antibodies that specifically stain all cells belonging to this neuronal population in the adult cerebellum.

TOPOGRAPHIC ORGANIZATION OF THE IMMATURE OLIVOCEREBELLAR PROJECTION

The electrophysiological study carried out in Paris by Crépel, Mariani, and Delhaye-Bouchaud (4) indicates that synaptogenesis between climbing fibers (the nerve
endings of all olivocerebellar projections) and Purkinje cells occurs postnatally in the rat, since the first functional synapses to be identified were observed in 3 day old animals (P3). Although of longer duration, these climbing fiber responses are already very similar to adult ones. However, they are not all-or-none in nature, as in the adult cerebellum (8), but are graded in parallel with increasing intensity of stimulus. By P15, the responses acquire their all-or-none adult character. The grading of early responses has been considered as demonstrating that during a transient period Purkinje cells have multiple innervation from climbing fibers. The analysis of intracellular recordings as these synapses mature (9,10) has allowed the determination of the evolution of multiple innervation. The peak of multiple innervation is reached at P5, from P7 to P10 an abrupt decrease occurs, followed by a slight decrease between P10 and P15. At this age, all Purkinje cells are monoinnervated.

In order to evaluate the presumptive influences of both synaptogenesis and regression (theoretically more important; see ref. 11) of the multiple innervation on the establishment of the olivocerebellar topography, we analyzed their organization in newborn rats (PO) before the onset of synaptogenesis, and up to 5 days (P5) when multiple innervation is maximal (12).

Before reporting the results, a brief description of the broad organization of the olivocerebellar system in adult rats will be given. As in the cat (13), this projection is organized in such a way that neurons in restricted sectors of the olivary complex innervate corresponding sagittally aligned strips of cerebellar cortex (14). First, the entire projection crosses the interolivary commissure; then the olivary axons ascend in the lateral aspect of the brain stem and enter the cerebellum mainly through the inferior peduncle. The caudal half of the medial accessory olive (MAO) projects almost exclusively to the vermis, whereas its rostral half projects to the flocculus, the paraflocculus, and a restricted portion of the paravermal zone (intermediate cortex). The ventral and dorsal lamellae of the principal olivary nucleus (PON) mainly supply innervation to the hemispheric cortex. The lateral portion of the dorsal accessory olive (DAO) projects to the paravermal zone (intermediate cortex) of the anterior lobe, and its medial portion innervates the intermediate cortex of the posterior lobe.

The results obtained from an experimental study using anterograde and retrograde transport techniques led to the following conclusions: Olivocerebellar fibers reach the cerebellum during intrauterine life, since these fibers are already present in the cerebellum at birth. At this age the small dimensions of the brain and its high water content make it difficult to restrict tracer injections to a single cerebellar lobule or to a defined olivary subnucleus. This situation allows only a rough estimation of the degree of organization of the projection, but it is sufficient to determine the distribution of olivary fibers within the vermal, intermediate, and hemispheric cortical regions. Thus, the vermal cortex receives olivary projections from neurons located in the caudal half of the contralateral MAO; the intermediate cortex is innervated mainly by neurons in the contralateral DAO, and to a lesser extent in the contralateral rostral half of the MAO. Finally, the hemispheric cortex receives most of its olivary projections from the contralateral PON and from the contralateral rostral half of the MAO (Fig. 1). This highly organized distribution is already identical to the adult olivocerebellar projection.
In newborn rats, the bulk of the olivocereellar fibers are arrested in the prospective white matter and only a few have invaded the overlying gray matter (Fig. 1). Therefore, these afferent axons reach their proper cerebellar territories before their appropriate targets, the Purkinje cells, have matured. As in other systems, such as the visual pathway (5), the arrival of a projection near a target structure is asynchronous with its definitive distribution to cellular targets. At PO, Purkinje cells have a smooth perikaryon and one or two long dendritic processes which, without penetrating into the external granular layer, can follow a long trajectory parallel to it. At P2
FIG. 2. Darkfield micrographs obtained from autoradiograms of rat cerebella. The rats received left olivary injections on the fourth day after birth and were fixed 20 hr later. A: Olivary injection partially involving the PON and the DAO. The MAO was almost unaffected. The most caudal and rostral poles of the olivary complex were free of labeling. Labeled fibers are absent from the medial regions of the vermis, but they are numerous within the hemispherical cortex, where they form bands of unequal width (arrowheads). (Rat, R8b-P4-5, x 34.) B: Left olivary injection involving almost the entire left caudal MAO and extending through the midline, contaminating the right caudal region. As a result of this contamination, labeled fibers reach the cerebellum through both inferior peduncles (large arrows), and a narrow, lightly labeled band is present at the ipsilateral side of the injection (small arrow). The massive involvement of the MAO explains the fact that most of the vermal cortex contralateral to the injection site contains heavy labeling. (Rat, R1-P4-5, x 34.)
to P3 these neurons lose their apical dendrites while numerous thin processes are formed and emerge in all directions from the cell body. At this stage of maturation, the climbing fibers establish early synaptic contacts with the Purkinje cells. Thus after arrival into the cerebellum and until their target cells attain certain developmental stages, these climbing fibers “wait” in the subcortical white matter.

By the fifth postnatal day (P5) the olivocerebellar fibers have moved from the prospective white matter towards the interface between the molecular and granular layers (Fig. 2), where Purkinje cells have arranged themselves into a monolayer. Although a more precise analysis of the topography of the olivocerebellar projection in these older rats is possible, no changes in the organizational pattern were observed. In P5 rats it is identical to that of adult animals (Figs. 2 and 3).

FIG. 3. Study of olivocerebellar projections by retrograde labeling with horseradish peroxidase (HRP). The zones of dense tetramethylbenzidine (TMB) reaction corresponding to the injection sites are indicated in black on the drawings of the cerebellum, whereas the gray areas represent the extension of the blue reaction. The retrograde labeled neurons are plotted on outlines of the inferior olivary complex. A: The HRP injection involves the vermis on the right side but also partially affects the left vermis. Consequently, labeled olivary neurons are present at both sides of the most caudal regions of the MAO. The rest of the olive is free of labeled neurons. B: In this case, the HRP injection involved the left hemispherical region and spread into the intermediate cortex and most lateral region of the vermis. Retrogradely labeled neurons are present only in the contralateral olive. They are disposed in patches mainly present in the rostral MAO, the ventral lamella of the PON, and the caudomedial part of the DAO.
FIG. 4. Development of the spinocerebellar projections. Microphotographs of lobule III of the anterior vermis, in rats which received lectin-conjugated peroxidase (WGA-HRP) in the spinal cord (T12 to L3). (Polarized illumination.) A: Injection on P4 and fixation 24 hr later (P5). The WGA-HRP labeled fibers have invaded most of the granular layer and are organized in clearly
Therefore, neither synaptogenesis between climbing fibers and Purkinje cells nor the regression of their multiple innervation influence the acquisition of the topography of the olivocerebellar projection. However, this study is too coarse to provide the final cell-to-cell correlation that characterized this projection. It is possible that olivocerebellar specificity could be refined at the synaptic level within the cerebellar longitudinal zones during the selective processes of synapse stabilization and synapse elimination (11).

**THE FORMATION OF SPINOCEREBELLAR TOPOGRAPHY**

The spinocerebellar projection has been studied in various mammals, particularly in cats (1,16–18). Spinal axons end almost exclusively in the vermis, where they are distributed within two distinct areas: the anterior target zone, which is the most important and comprises lobules I to V of the anterior lobe; and the posterior target zone, which is restricted to lobule VIII. All spinal fibers terminate as mossy fibers in the granular layer, where they are segregated into five parallel sagittally situated zones varying in width from 200 to 480 \( \mu \text{m} \) and separated by terminal-free intervals of 600 to 800 \( \mu \text{m} \) (Fig. 4C).

Information on the development of this projection is scanty. Only recently Martin et al. (19) reported the first experimental study of the formation of the spinocerebellar projection. This work was carried out in a marsupial, the opossum, whose ontogenetic calendar differs from that of the rat. Accordingly, spinal afferents reach the cerebellum on P7 and acquire their adult topography on P50. The precise stages followed by the intracerebellar spinal axons to achieve their adult pattern were not analyzed.

Most of the electrophysiological information on the development of this projection is limited to rats. Analysis of field potentials evoked by white matter stimulation and aimed at disclosing the earliest signs of synaptic activity have failed to demonstrate functional synaptic transmission between spinal mossy fibers and granule cells before P10 (6). However, at P7 it has been possible to activate Purkinje cells after limb stimulation by inputs mediated through spinal mossy fibers (7). These results indicate that synaptogenesis between spinal axons and granule cells is achieved by the end of the first postnatal week.

Anterograde tracer experiments carried out in rats aged 0, 3, 5, 7, and 30 days (20) have permitted the conclusion that spinal axons reach the cerebellum during fetal life and that their ultimate organization is qualitatively attained by P7. Between P0 and P7 these axons must pass through the various developmental stages neces-

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defined sagittal columns (1 to 2). Note the presence of some dispersion of labeled axons between the columns (arrows). (Rat, \( \times 72 \).) B: Injection on P6 and fixation on P7. The WGA-HRP labeled fibers are disposed in sharply delimited columns (1 to 2) separated by zones almost completely devoid of labeling. (Rat, \( \times 72 \).) C: Injection on P29 and fixation on P30 (adult pattern). The labeled spinocerebellar axons are confined to five clearly delimited columns (1 to 3). (Rat, \( \times 88 \).)
sary for the establishment of the adult spinocerebellar topography. A summary of these successive stages follows.

The Early Stage of Axonal Growth

During intrauterine life, spinal axons grow rostrally in the ventrolateral aspect of the lower brain stem to the inferior and superior cerebellar peduncles. Once in the cerebellum, these fibers only enter those areas where they are normally present in the adult animal, since transient aberrant projections were not detected. Thus, adult terminal domains correspond nicely with the "attracting" cerebellar zones for ingrowing spinal fibers.

The Intermediate Stage or Waiting Period

From P0 to P3 the ingrowing spinal axons remain in the prospective white matter, where they are distributed more or less uniformly. As is the case for Olivocerebellar fibers, those emerging from spinal neurons also reach their appropriate territories before the proper maturation of their target cells (the bulk of granule cell proliferation occurs during the second postnatal week (see ref. 21), and wait in the medullary zone until a distinct granular layer is formed.

The Protocolumnar Stage

By P3 spinal axons begin to invade the cortical gray matter. Although Purkinje cells have not yet reached their final monolayer arrangement at this age, a nascent granular layer containing a noticeable contingent of granule cells already exists. The invasion by spinal fibers does not occur randomly, as they have a tendency to remain clustered within their ultimate terminal domains. However, the typical columnar organization of the adult projection is not yet observed at this early stage, since labeled fibers are dispersed to an important degree between the nascent columns, hence the term protocolumnar stage.

The Columnar Stage

From P3, concomitant with the rapid increase in the amount of labeled spinal fibers entering the granular layer, intercolumnar dispersion diminishes. At P5 the columns have almost acquired their adult appearance, although a low degree of dispersion is still evident (Fig. 4A). From P7, adult topography is totally achieved; the five columns are situated as in P30 rat cerebellum (Fig. 4B). Besides changes in cerebellar size, the only difference in the adult is quantitative (compare Figs. 4B and 4C).

Experiments with retrograde transport techniques indicate that spinal fibers invade the cerebellum in two successive groups. The first one, formed prenata
contains axons from the central cervical nucleus, Clarke's column, the sacral nucleus of Stilling, and to a lesser degree, some border cells. The second group, which reaches the cerebellum between P1 and P3, emerges from new neurons in these spinal areas, especially from scattered cells of the spinal gray matter. These results indicate that the dorsal spinocerebellar tract matures somewhat earlier than the ventral tract, which mainly originates from the border cells (IF) and are in line with those obtained in the opossum by Martin et al. (1G).

Morphological analysis of synaptogenesis between spinal mossy fibers and granule cells requires the ultrastructural study of immature rat cerebella after tracer injections into the spinal cord, since, contrary to climbing fibers which originate from the inferior olive, mossy fibers are a composite population of which only a small part is of spinal origin. However, ultrastructural examination of the immature lobules II and III of the anterior lobe of the vermis, which permits the analysis of mossy fiber maturation in general, discloses indirect evidence of spinocerebellar synaptogenesis. Using this approach, we have identified mossy fibers establishing synaptic contacts from P5 (20). They appear as axonal varicosities partially covered by one or two postsynaptic dendrites and correspond to the primitive stage of mossy fiber synaptogenesis. By P7 the number of detectable mossy rosettes has increased and maturation has progressed. Here, mossy terminals in the primitive stage and in the cup stage (the most numerous) are intermixed with terminals already in the claw stage (see ref. 22 for the definition of these stages). Thus our results provide indirect evidence that spinocerebellar fibers may start synaptogenesis almost immediately after their invasion of the nascent granular layer, since by P5 primitive mossy rosettes bearing mature synaptic junctions are present in the terminal domains of spinal axons. Comparison of these morphological observations with the results of electrophysiological studies reported above indicates a time lag between the onset of synapse formation and the production of synapse activity, suggesting that synaptogenesis must be rather advanced before global synaptic activity can be detected. More important, the temporal correlation between the columnar organization of spinal axons and the appearance of mossy rosettes with mature synaptic junctions indicates that the process of synapse formation does not interfere with the organization of spinocerebellar topography.

GENERAL CONCLUSIONS

The results of the morphological reading reported here provide the developmental chronology of the olivo- and spinocerebellar projections in the rat. For both systems of afferents, the following conclusions can be made. Extracerebellar afferents invade the cerebellum during intrauterine life. At birth, they are arrested in the prospective white matter in a "waiting" period. Olivocerebellar fibers mature somewhat earlier than spinocerebellar. By P1, the former occupy their ultimate territories according to a topography similar to that of adult rats; thus at this age the longitudinal-zonal organization of the olivocerebellar projection already exists. Later, at P3, the spinocerebellar axons display an almost adult organization. There-
fore, both projections undergo organization from the moment they enter the cerebellum, at a still undetermined fetal age, up to the fifth postnatal day. The ultimate topography of both projections is thus attained before the onset of synaptogenesis, indicating that this process does not influence the acquisition of general cerebellar topography.

**TRANSIENT BIOCHEMICAL HETEROGENEITY OF IMMATURE PURKINJE CELLS AS A PRESUMPTIVE BASIS FOR CEREBELLAR COMPARTMENTALIZATION**

Cytoarchitectonic analysis of the developing cerebellum has shown that the process of Purkinje cell migration and segregation ends in the formation of intracortical clusters. This clustering has been observed in many mammals, such as the mice (23), the rats (24), and humans (25). More recently, Kappel (26) was able to identify in monkey embryos nine longitudinal clusters in each hemicerebellum. She emphasized the topographical similarity between the pattern of Purkinje cell clustering in embryos and the adult longitudinal-zonal organization of corticonuclear and olivocerebellar projections. It is therefore tempting to consider that the embryological clustering of Purkinje cells could be related to the zonal arrangement of the cerebellar afferent and efferent connections in the adult (27).

With the aim of studying this hypothesis, Wassef (28,29) carried out an immunohistochemical analysis of early Purkinje cell clustering and differentiation. Among the known antibodies that selectively stain all Purkinje cells in the adult rat cerebellum, three were used for her study. The antibodies were oriented against: cyclic-GMP-dependent protein kinase (cGK) (kindly provided by Prof. Greengard); vitamin-D-dependent calcium binding protein (kindly provided by Dr. Thomasset); and Purkinje-cell-specific glycoprotein (PSG) (kindly provided by Dr. Zanetta). Between E17 and P5, Wassef observed a period in which each of these markers is expressed in an asynchronous manner by Purkinje cells (Fig. 5). Each antibody gives a reproducible mosaic of positive and negative Purkinje cell clusters, varying with age. Although the pattern of positive and negative clusters differs for each antibody, the clusters have common limits in some cerebellar areas. As in adulthood, all Purkinje cells react with the three antibodies by P5.

These results show that during cerebellar ontogeny a transient biochemical heterogeneity exists among Purkinje cells. Furthermore, they suggest that the synthesis of a whole set of proteins by these neurons is topographically regulated, indicating that the biochemical heterogeneity could represent an intrinsic parcellation of the cerebellar cortex. By definition, a basic cerebellar compartment contains identical Purkinje cells. Owing to the observed overlapping of differently immunostained clusters, the basic compartment must be the result of the intersection of several clusters which are positive or negative for various proteins. Therefore, the algebraic addition of the clusters displayed by the immunohistochemical staining of three proteins reveals that the presumed number of basic compartments must be much
FIG. 5. Immunohistochemical staining of Purkinje cell clusters in the immature cerebellum. A: Frontal section of E19 embryo stained with cGK antiserum. This micrograph illustrates the biochemical heterogeneity of developing Purkinje cells as well as the size and distribution of the cGK-containing clusters. The cortical region is occupied by clusters of immunopositive Purkinje cells separated by immunonegative ones. The positive clusters are indicated by Roman numerals. There are four clusters in each hemicerebellum. (Magnification × 26.) B: Sagittal section of a newborn rat cerebellum stained with PSG antiserum. The biochemical heterogeneity of immature Purkinje cells is obvious: numerous Purkinje cells are immunostained in the posterior region, whereas the anterior one shows none. The arrows point to the border between positive and negative clusters (Magnification × 47.)
higher than the number of longitudinal zones and that their size, although varying throughout the cortex, must be smaller than that of a longitudinal zone. Therefore Voogd’s sagittal zones (1) do not correspond to the intrinsic Purkinje cell compartments.

The fine electrophysiological analysis of Oscarsson (30) has provided evidence for a further subdivision of the longitudinal zones. His analysis, carried out in the cat, showed that according to the functional characteristics of climbing fiber inputs, zone B can be subdivided into five subgroups. Each of them is about 200 μm wide and projects to a different subgroup of Deiter’s neurons. This result prompted Oscarsson (30) to postulate the microzone concept, that is, that the cerebellar cortex is composed of an assembly of sagittal microzones, 200 μm or less in width in the cat, defined by their specific efferent and afferent connectivity. Each microzone would represent a structural-functional unit, corresponding to the columns of Mountcastle (31) in the cerebral cortex. Until now the existence of microzones has been shown only in zone B (30) and in the flocculus (for further references, see ref. 32), but they most probably exist throughout the cerebellar cortex. Because of their average size and relatively high number, the microzones could correspond to Purkinje cell basic compartments.

Information obtained from immunohistochemical studies is still fragmentary and does not allow definite conclusions, but it does suggest the following. (a) A temporal relationship exists between the period of Purkinje cell biochemical heterogeneity and the period of cerebellar projection map establishment. As indicated by the analysis of olivo- and spinocerebellar topographical development, the extracerebellar afferents enter the cerebellum during fetal life and acquire their adult pattern by P5. At this age, the biochemical heterogeneity of Purkinje cells disappears. (b) The biochemical heterogeneity of immature Purkinje cells provides a basis for the intrinsic parcellation of the cerebellar cortex resulting from the differential expression of parts of the same genotype by Purkinje cell clusters. (c) The size and number of basic Purkinje cell compartments, as defined by the combination of the differently immunostained clusters, appear to be compatible with the organization of the cerebellar cortex as an assembly of numerous microzones having distinct efferent and afferent connections.

More work is necessary to correlate Purkinje cell compartments and sagittal microzones. However, as a working hypothesis, one can postulate that the intrinsic parcellation of Purkinje cells could provide cues for the afferent axons and orient their growth towards appropriate terminal territories. A possible, although not exclusive, explanation of the mechanism involved in the establishment of cerebellar topography is that the differences observed in Purkinje cell cytoplasmic proteins could indicate differences in plasma membrane proteins that would be reflected on the surface of the Purkinje cells in each compartment in such a way that they would be specifically marked, not only on the somatic plasma membrane but especially on the axolemma. Thus growth cones of the extracerebellar axons in the deep nuclear region could recognize Purkinje cell axons and follow them to their neuron of origin.
This hypothesis considers neither the mechanisms controlling the oriented growth of extracerebellar fibers nor their arrangement in the ascending tracts, the cerebellar peduncles, and the cerebellar central white matter. It supposes, however, that during the ordered growth of extracerebellar axons, they become organized to a certain degree. Once these axons reach the regions occupied either by migrating Purkinje cells or by the specifically displayed bundles of Purkinje cell axons, the somewhat broad topography of the olivo- and spinocerebellar fibers is refined by the above-mentioned mechanism of recognition of appropriate Purkinje cell bundles. Therefore, the ultimate pattern would emerge as the result of the confrontation between two distinct maps, one—related to Purkinje cell parcellation—more precise than the other. In any case, this hypothesis, which attempts to integrate different experimental data, proposes that Purkinje cells are the organizers of cerebellar topography.

REFERENCES


**DISCUSSION**

**Dr. Roig:** Are you suggesting that Purkinje cells can change their own enzymatic machinery, as for instance muscle cells do when differently innervated?

**Dr. Sotelo:** Not at all. What I am saying is that during differentiation of a given category of cells, you have subgroups of cells which express different proteins at different times. When Purkinje cells differentiate, they go through mitotic events and then migrate to achieve their cortical position, and then they develop dendrites. This is one way of looking at differentiation. What I am proposing is that you look at it with a different set of optics—immunological and chemical optics! Using these techniques it has been shown that within Purkinje cells, which form a big population of neurons, one parameter for differentiation is the genetic expression of a particular set of proteins, and this genetic expression is not synchronous for all Purkinje cells. We see then that proteins begin to differentiate for different sets of Purkinje cells at different times, which have nothing to do with the morphological differentiation. The particular interest of the asynchronous expression of these different proteins is that over a
given time period you have biochemical heterogeneity, and this could be very useful in providing cues for the different axons to indicate where they should go in the cerebellum.

Dr. Campagnoni: Could you tell us about the expression of antigens by the Purkinje cells? Are they expressed at a particular time, and then no longer expressed? Or become re-expressed as an adult?

Dr. Sotelo: From postnatal day 5 until death of the rat the three antigens we use are expressed all the time. Our results indicate that when a given cluster of cells becomes positive for protein kinase they stay positive until death. During earlier development we do not know.

From experiments on cultured isolated Purkinje cells in the absence of innervation it can be shown that under these conditions calcium binding is expressed but not protein kinase. It seems that protein kinase is expressed by afferent fibers, mainly climbing fibers.

Dr. Mariani: Immunocytochemistry is a threshold method. Thus it is possible that there could be a quantitative regulation of the level of the protein by the afferents that would not be seen by immunocytochemistry. For example the level of calcium binding increases between postnatal day P5 and P30, but with immunocytochemistry it looks exactly the same.

Dr. Sotelo: If you put an isotope like tritium into an antibody you can use autoradiography and computerized densitometry to give you as effective a degree of quantification as with radioimmunoassay.

Dr. Mariani: It has been proposed that the Purkinje cells are committed by a small number of progenitors very early in development. Do you think that asynchrony in expression of proteins can be related to the different progenitors?

Dr. Sotelo: I think that is too speculative. We are looking at a much later period of development. What we are seeing at this stage is the neuroepithelium of the cerebellum, with much more dispersed stem cells to provide the Purkinje cells. What we want to do next, though I do not yet know how to handle this, is to investigate whether the heterogeneity in expression of these different antibodies has something to do with the columnar organization which is already expressed in the neural epithelium.