Invited Review

Compartmentation of the granular layer of the cerebellum

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Summary. Numerous studies have demonstrated that the cerebellum is highly compartmentalized. In most cases, compartmentation involves the Purkinje cells and the molecular layer, but there is also substantial evidence that the granular layer is subdivided into a large number of highly reproducible modules. We first review the evidence for a modular granular layer. Compartmentation of the granular layer has been revealed both functionally and structurally. First, tactile receptive field mapping has revealed numerous discrete functional modules within the granular layer. The molecular correlates of the receptive fields may be the compartments revealed by histological staining of the cerebellum for several enzymes and antigens. The structural substrate of the receptive fields is the mossy fiber afferent projection map, and anterograde tracing of various mossy fiber projections shows afferent terminals in parasagittal bands within the granular layer that are topographically aligned with the Purkinje cell compartments. Based on this evidence we argue that the cerebellum consists of many hundreds of reproducible structural/functional modules, and that a modular organization is a prerequisite for the efficient parallel processing of information during motor control.

The complex organization of the adult granular layer implies elaborate developmental mechanisms. In the second part of the review we consider five developmental models to generate the modular organization of the adult granular layer: 1) the external granular layer is heterogeneous, and its topography translates directly into a modular granular layer; 2) granular layer modules are clones, derived from single external granular layer precursors; 3) modules in the granular layers are a secondary epigenetic response to the compartmentation of the Purkinje cells; 4) modules are secondary to the compartmentation of the afferent terminal fields; 5) modules are sculpted by activity-dependent processes.

Key words: Zebrin, Granule cell, Purkinje cell, Pattern formation, Mossy fiber

1. Compartmentation of the adult granular layer

The cerebellum is a major sensory-motor interface in the brain. Afferent information enters the cerebellar cortex via the climbing fiber pathway which contacts the Purkinje cell dendrites directly, the mossy fiber pathway which reaches the Purkinje cells via the granule cell interneuron (and various minor groups of afferent which terminate in all layers of the cerebellar cortex e.g., Dietrichs et al., 1994). The Purkinje cell projections are the sole cortical efferent pathway, via the cerebellar and lateral vestibular nuclei. While the details of the circuitry have been understood for many years (e.g., Eccles et al., 1967; Palay and Chan-Palay, 1974) the topographical organization of the cerebellum has only been appreciated more recently. One of the most impressive features of the cerebellar cortex is its repetitious cytoarchitecture. There are no obvious cytoarchitectonic boundaries and even upon close analysis the cellular organization of the cerebellum is uniform. As a result, the cytology of the cerebellar cortex does not reveal how information processing is segregated. However, the homogeneity of the cerebellar cortex is now believed to camouflage underlying complexity on an enormous scale and, in fact, the cerebellum is now viewed as structurally, biochemically and functionally highly molecular (Hawkes and Gravel, 1991). Most previous reviews of cerebellar parcellation have emphasized the Purkinje cells and their pivotal role in establishing and maintaining topography (e.g., Sotelo and Wassef, 1991; Hawkes, 1992; Hawkes et al., 1992; Wassef et al., 1992; Hawkes and Mascher, 1994). However, all cerebellar laminae are modular, and the granular layer may be the most highly modular of all. Therefore, in this article, we will first provide an overview of the molecular architecture of the cerebellum, and then review in more detail the evidence for compartmentation in the granular layer.

1.1 Molecular markers of regionalization

Molecular heterogeneity within the cerebellar cortex was first revealed by the histochemical demonstration of alternating parasagittal positive and negative bands of 5'-nucleotidase (5'N) activity in the molecular layer of the mouse cerebellar cortex (Scott, 1963, 1964). Subsequently, parasagittal bands of acetylcholinesterase (AChE) activity were described in the molecular layer of the young cat (Marani and Voogd, 1977) and in the granular layer of the adult monkey, rat, mouse, and hamster (Roffler-Tarlov and Graybiel, 1982; Hess and Hess, 1986; Voogd et al., 1987; Boegman et al., 1988). Similar bands of cytochrome oxidase (CO) activity have been reported in both rats and primates (Ingram et al., 1985; Hess and Voogd, 1986; Leclerc et al., 1990).

The development of monoclonal antibody (MAb) technology opened a new avenue to the understanding of cerebellar organization. MAbs have been used for molecular mapping within the brain, and a variety of MAbs have been produced that can be used to distinguish between different cells within the cerebellum (e.g., Hawkes et al., 1982a,b). By the use of MAbs, differences between cerebellar cortical subdivisions have been revealed at the molecular level. Numerous regional differentiation markers have been identified, including glutamic acid decarboxylase, motilin (Chan-Palay et al., 1981), the taurine synthesizing enzyme cysteine sulfinic acid decarboxylase (Chan-Palay et al., 1982), the B1 antigen (Ingram et al., 1985), the synaptic vesicle antigen Q155 (= synaptophysin: Hawkes et al., 1985), antigen B30 (Stainer and Gilbert, 1989), and protein kinase C_δ (PKC_δ: Chen and Hillman, 1993).

The most thoroughly studied molecular markers of compartmentation are the polypeptide antigens zebrin I (Hawkes et al., 1985) and zebrin II (Brochu et al., 1990). Zebrin I is a 120 kDa polypeptide of unknown function; zebrin II is aldolase C (Ahn et al., 1994; Hawkes and Herrup, 1996). *Anti-zebrin* immunoreactivity reveals a symmetrical array of parasagittal bands extending rostrocaudally throughout the cerebellum that is confined exclusively to the Purkinje cells. Zebrin immunoreactivity is intracellular and extends throughout the Purkinje cells, including the somata, dendrites, axons, and axon collaterals (Hawkes et al., 1985; Hawkes and Leclerc, 1986, 1987; Brochu et al., 1990; Doré et al., 1990). Reconstructions of the pattern of zebrin expression reveal a median band of zebrin+ Purkinje cells adjacent to the midline (P1+) and 6 other zebrin+ bands disposed symmetrically on either side of the midline through the vermis and hemispheres (P2+ to P7+: Hawkes et al., 1985; Hawkes and Leclerc, 1987; Brochu et al., 1990; Eisenman and Hawkes, 1993: Fig. 1). Separating the P+ bands are similar bands of Purkinje cells that do not express zebrins (P1- to P7-). Because zebrin immunoreactivity is expressed independently of afferent input (e.g., Wassef et al., 1990; Seil et al., 1995) and is highly reproducible it has proved useful for comparing the distributions of molecular markers. By
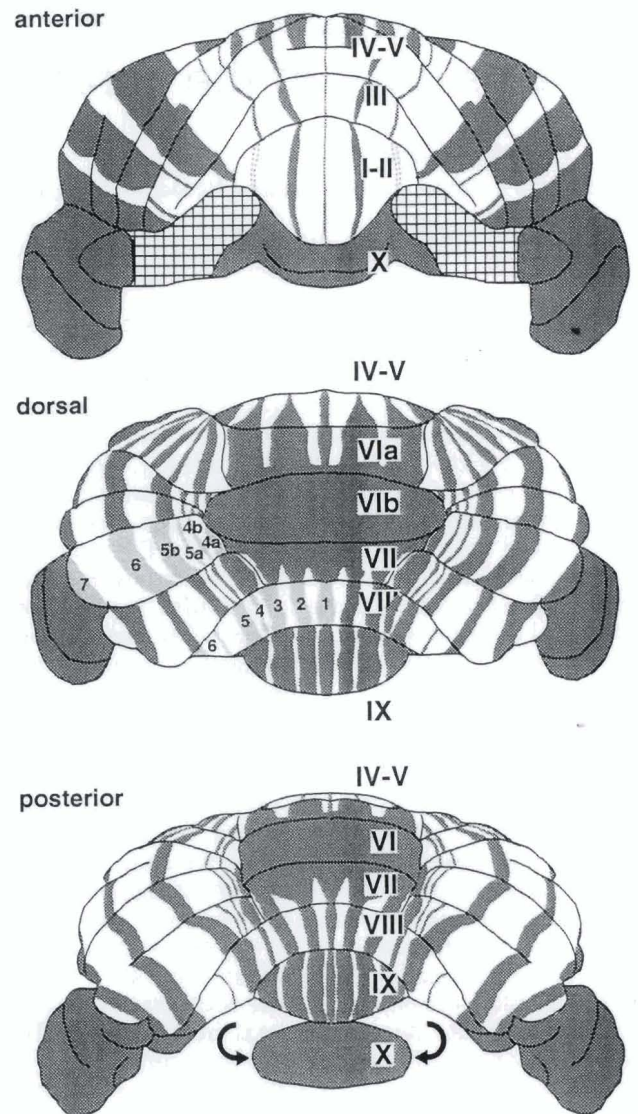

Fig. 1. Drawings of three surface views of the mouse cerebellum - anterior, dorsal and posterior - showing the locations of the zebrin+ bands of Purkinje cells. The Purkinje cell bands P1+ - P7+ are labelled in the dorsal view (for clarity, only the numerals have been used). Note that in the vermis of the posterior lobe the immunoreactive Purkinje cells form five to seven bands (posterior and dorsal views) whereas in lobules VII and VI all vermal Purkinje cells are immunoreactive (posterior and dorsal views). This pattern gradually changes in the anterior lobe to result in three to five very narrow immunoreactive bands (anterior view). In the hemispheres there are three major immunoreactive bands of Purkinje cells on either side (P5b+, P6+, P7+) plus two sub-bands in the paravermal area of the paramedian and ansiform lobules (P4b+, P5a+). Note too that the Purkinje cells are all zebrin+ in the nodulus (lobule X, illustrated as indicated by arrows as reflected out from the ventral surface of the cerebellum), the paraflocculus and the flocculus. Adapted from Eisenman and Hawkes (1993).

using the zebrin I bands as a reference frame, several band markers have been found to codistribute in the rodent cerebellum, including zebrin II (Brochu et al., 1990), 5'N (Eisenman and Hawkes, 1990). AChE (Boegman et al., 1988), the B1 antigen, the B30 antigen, and the nerve growth factor receptor (reviewed in Hawkes, 1992). Other antigens are expressed in parasagittal bands that are either specific to the zebrin⁻ Purkinje cell subset (e.g., Map-1a: Touri et al., 1996) or in the zebrin⁻Purkinje cells plus selected bands of zebrin⁺ Purkinje cells (e.g., P-path: Leclerc et al., 1992). Still further complexity is seen in the molecular layer when sections are stained with an antibody against the human natural killer cell antigen (HNK-1:, Hawkes, 1992; Eisenman and Hawkes, 1993). HNK-1 immuno-reactivity is associated with several cell types but is most prominent on Purkinje and Golgi cells (Eisenman and Hawkes, 1993). In the molecular layer the HNK-1

epitope is expressed in parasagittal bands that both overlap with and subdivide the zebrin bands. In addition to hetero-geneity of the cortex itself, several afferent populations have distinct molecular signatures that are regionally distributed (e.g., enkephalin immunoreactivity in the opossum cerebellum - King et al., 1987; corticotrophin-releasing factor immunoreactivity in the opossum - Cummings et al., 1989).

1.2. Regionalization of the granular layer

Most studies of cerebellar compartmentation have emphasized the Purkinje cells and the subdivisions of the molecular layer (reviewed in Hawkes et al., 1992). Similar studies in the granular layer are less well developed. Nevertheless, both regional variations and modular subdivisions have been described. Detailed histological analysis has shown some subtle regional
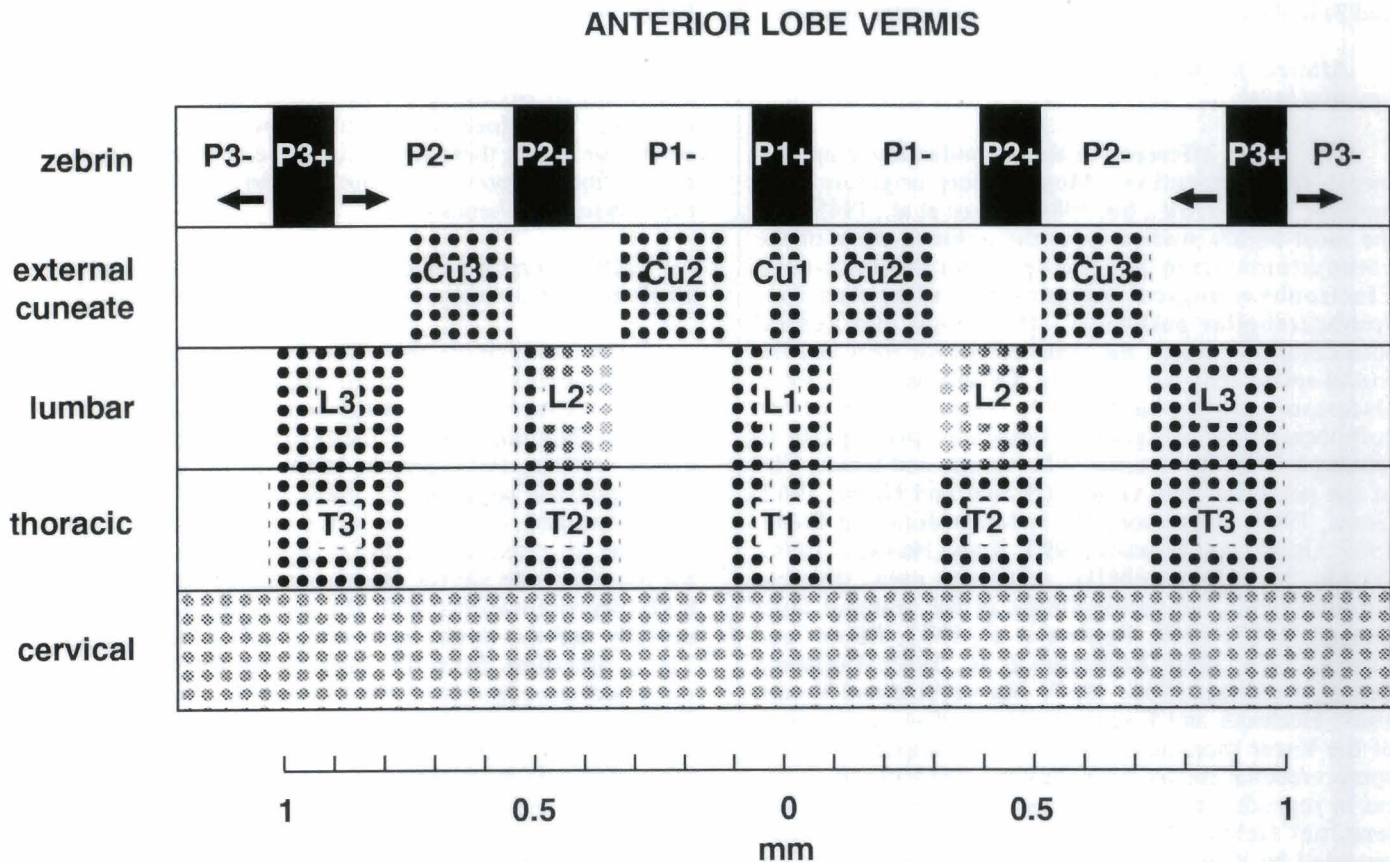
ANTERIOR LOBE VERMIS

Fig. 2. Lobules II/IIII of the rat anterior lobe vermis: a schematic representation of the relationships between zebrin⁺/⁻ bands of Purkinje cells and the mossy fiber projections from the external cuneate nuclei, and lumbar, thoracic and cervical levels of the spinal cord. There is one zebrin⁺ PC compartment at the midline (P1⁺) and two others (P2⁺ and P3⁺) positioned laterally. P1⁺ and P2⁺ are separated by P1⁻, P2⁺ and P3⁺ by P2⁻. More laterally there is also a weak P4⁺ compartments (not illustrated). Terminals of the cuneocerebellar tract are clustered under P1⁺ (the Cu1 cluster), and within P1⁻ (Cu2) and P2⁻ (Cu3). Only the medial edge of Cu3 clearly corresponds to a zebrin⁺/⁻ boundary. The lumbar and thoracic terminals have very similar distributions (the LT1/T12, L2/T2 and L3/T3 fields) and are complementary to the cuneocerebellar projection. Statistical analysis suggests that the lumbar L2 terminal field is narrower than the thoracic T2 and so is illustrated here as fading towards its medial edge. The empty space aligned with Cu1 and splitting L1 and T1 at the midline is questionable: neither a split within L1/T1 nor the presence of Cu1 is unambiguous. The lateral edge of Cu3 abuts the medial edge of L3/T3. More laterally, L3/T3 are drawn as aligned with the lateral edge of P3⁺ (this is speculative as the P3⁺ band cannot confidently be identified in double-labeled sections). The mossy fiber terminals from the cervical spinal cord are shown as spread uniformly across the vermis. Adapted from Ji and Hawkes (1994).

differences in the granular layer across the cerebellar cortex. For example, Lange (1982) reported variations between regions based on the density and shape of the individual neurons: within the evolutionarily younger regions of the cerebellar cortex, granule cell somata are smaller than elsewhere. Similarly, there may be different densities of Golgi cells in different regions of the cerebellum (Lange, 1982). More strikingly, a variant of the Golgi method has recently revealed two novel neuronal types. One is restricted exclusively to the granular layer of the vestibulocerebellum - the unipolar brush cell (Mugnaini and Floris, 1994), and the other is found between Purkinje cell somata within the Purkinje cell layer - the candelabrum cell (Laine and Axelrad, 1994). However, over and above these regional variations the granular layer is also highly modular. Compartmentation in the granular layer is seen by using: 1) afferent and efferent tract tracing techniques; 2) by the electrophysiological mapping of tactile receptive fields; and 3) in the distributions of several molecular markers.

Afferent terminal fields define compartments in the granular layer

The major afferents to the granular layer are the mossy fiber projections. Mossy fibers originate from multiple sources (e.g., Ito, 1984; Voogd et al., 1985), but the most highly studied from the point of view of the compartmentation are the spinocerebellar tracts. Electrophysiological mapping has identified four spinocerebellar pathways - the dorsal and ventral spinocerebellar tracts, the cuneocerebellar tract and the rostral spinocerebellar tract (e.g. Oscarsson, 1973, 1981, Oscarsson and Sjölund, 1977). Mossy fibers of the spinocerebellar projections terminate principally in lobules II-V of the anterior lobe vermis and lobule VIII of the posterior lobe vermis (Brodal and Grant, 1962; Grant, 1962; Oscarsson, 1973; Matsushita and Ikeda, 1987; Gravel and Hawkes, 1990; Ji and Hawkes, 1994). Within the spinocerebellar receiving areas discrete terminal fields run parasagittally in the granular layer (Voogd, 1967, 1969; Hazlett et al. 1971; Ekerot and Larson, 1973, 1980; Robertson et al., 1983; Yaginuma and Matsushita, 1986, 1987; Matsushita and Ikeda, 1987; Heckroth and Eisenman, 1988). The organization of the lower thoracic/higher lumbar component of the spinocerebellar mossy fiber-Purkinje cell pathway in the rat is reproducible (Gravel and Hawkes, 1990). The terminal fields align with the Purkinje cell bands revealed by *anti-zebrin* immunocytochemistry but, in addition, there are mossy fiber terminal field boundaries that have no apparent equivalents in the Purkinje cell layer, suggesting that cerebellar compartmentation is more elaborate than zebrin staining alone would suggest. In a more detailed study, spino- and cuneo-cerebellar mossy fiber terminal fields in lobules II and III of the rat cerebellum were compared to the Purkinje cell compartments revealed by zebrin II immunocytochemistry (Ji and Hawkes, 1994: Fig. 2).

The extent to which mossy fiber compartments can be identified in the spinocerebellar projection varies markedly with spinal level. Projections from the more caudal spinal cord terminate in sharp sagittal bands while projections from more rostral regions are diffuse (Matsushita et al., 1991; Ji and Hawkes, 1994). Comparisons of Purkinje cell bands and spinocerebellar terminal fields confirmed that the granular layer is finely subdivided by the different mossy fiber projections, and that this parcelation does not completely coincide with the overlying Purkinje cell compartments as revealed by *anti-zebrin II*.

Detailed analysis of the spinocerebellar projection showed that the patterns of termination in the anterior lobe are not only sharply defined sagittally but can occur in transverse bands and discrete patches (Tolbert et al., 1993). Small injections of wheat germ agglutinin-horseradish peroxidase into L1 resulted in labeling of terminal clusters and isolated mossy fiber projections. Large injections resulted in a sagittal stripe pattern of organization. However, the more controversial data comes from large intersegmental injections, which appeared to result in a transverse band pattern of labeling. Therefore, within the granular layer of the cerebellar cortex there may exist a much more complex mossy fiber subcompartmentation than is suggested by the Purkinje cell bands.

Tactile receptive field mapping reveals a mosaic of small functional compartments in the granular layer

Early analysis of the afferent projections to the cerebral cortex suggested an organized somatotopy related to a surface map of the body reminiscent of the «sensory homunculus» of the primary somatosensory cortex (Adrian, 1943; Sniper, 1944). In the cerebellum, two organized somatotopic maps of the body surface were identified, one located in the anterior lobe vermis, the other, inverted relative to the anterior map, located in the posterior lobe vermis. Both maps contained complete body representations with the head regions located dorsally and the trunks located medially in the vermis. The projections from the limbs were located in the intermediate regions of the cerebellar hemispheres. However, more detailed physiological mapping studies of tactile responses, conducted by high resolution microelectrode mapping of the granular layer, revealed an elaborate organization of peripheral projections to the granule cell layer that is very different from a simple sensory homunculus (e.g. Joseph et al., 1978; Shambes et al., 1978a,b; Kassel, 1980; Bower et al., 1981; Bower and Woolston, 1983; Kassel et al., 1984; Welker and Shambes, 1985; Welker, 1987; Bower and Kassel, 1990). Instead, they revealed a novel projection pattern in the cerebellar cortex that is commonly referred to as «fractured somatotopy» (e.g., Welker, 1987). In this interpretation, peripheral projections to the granular layer terminate in columnar modules or patches that are oriented vertically to the cortical surface. Each patch

defines a specific tactile receptive field (although intrapatch somatotopy is often found in the larger patches), and the patches are organized in mosaics that represent different regions of the periphery. Receptive fields from a specific location on the body may terminate in multiple patches in one or more folia (Joseph et al. 1978; Shambes et al., 1978a,b; Kassel et al., 1984; Welker and Shambes, 1985). Several afferent projections to the granular layer have been shown to respect the patchy organization of the receptive fields. For example, projections from single trigeminal neurons terminate in a patchy mosaic pattern (Woolston et al., 1981) and trigeminal neuron collaterals have been shown to branch extensively within granule cell patches of the same receptive fields, between two patches located on a single folium, between two different ipsilateral folia, between the hemispheres and vermis, and between the hemispheres. It has been argued that these patches in the granular layer are focal points of information processing for specific body regions, and by organizing and regulating communication between adjacent patches the contributions from different body surfaces can be compared and integrated (Llinás, 1982).

The role of the parallel fiber system remains poorly understood. Parallel fibers are the transverse extension of the granule cell axons in the molecular layer of the cerebellum, and extend mediolaterally through the molecular layer over several millimeters (Mugnaini, 1976), thus traversing the separate parasagittal compartments of Purkinje cells. However, there exists strong evidence that compartments in the granular layer have a direct influence on the overlying Purkinje cells. Bower and Woolston (1983) have shown that there is a prominent vertical organization of tactile projections from the granule cells to the Purkinje cells, such that mossy fiber terminals in the granular layer preferentially
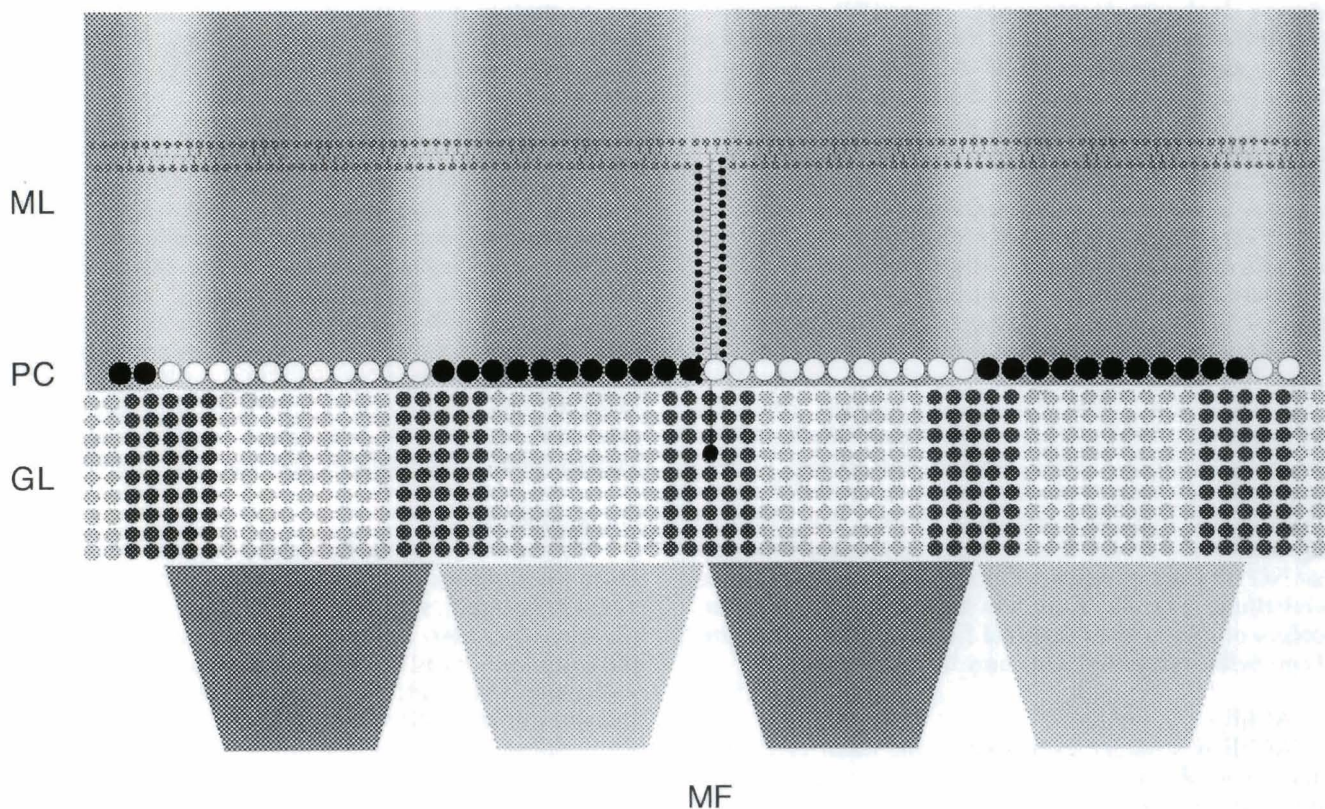

Fig. 3. A diagram to illustrate a role for NO in shaping cerebellar receptive field properties. The topography of the afferent terminal fields, Purkinje cell (PC) bands, and NADPH-diaphorase patches is shown. Mossy fibers (MF) terminate in parasagittal bands: adjacent terminal fields are differently shaded. Their terminal fields are drawn here as coincident with the zebrin bands of Purkinje cells (PC), although this is an oversimplification. The terminal arbor of one granule cell is shown. Because of their local density and preferential distribution closer to the Purkinje cell somata, synapses from the ascending portion of the granule cell axon (darkly shaded) dominate the more diffuse parallel fiber component (lightly shaded), which may explain the congruence of tactile projections to the granule cell and Purkinje cell layers (Llinás, 1982; Bower and Woolston, 1983). Because NADPH-diaphorase (NOS) expression in the granular layer (GL) is concentrated at the boundaries between mossy fiber terminal fields (darkly shaded), mossy fiber-induced nitric oxide release would be highest at the terminal field boundaries. One result of this would be the depression of granule cell synaptic strength at Purkinje cells receiving conjunctive mossy fiber and climbing fiber activation. This would create a zone of attenuated synaptic activity (illustrated by the lighter molecular layer shading) that would sharpen the edges of the mossy fiber receptive fields. Such refinement would be especially influential if adjacent mossy fiber terminal fields should overlap at their boundaries. According to this scheme, there would be less nitric oxide-induced long term depression at the center of the receptive fields because NOS levels are low in the underlying granular layer. Adapted from Hawkes and Turner (1994).

drive the immediate overlying Purkinje cells. Other studies have confirmed the existence of some spatial correspondence between the organization of mossy fiber-mediated tactile receptive fields and the zebrin II compartments (Chockkan and Hawkes, 1994).

Molecular markers reveal compartments in the granular layer

Numerous molecular markers are known for the compartmentation of the Purkinje cell/molecular layers (e.g., reviewed in Hawkes et al., 1992). Markers of granular layer compartments are rare by comparison. To date, five are known: three enzymes (NADPHd, AChE, and CO) and two antigens (synaptophysin and CD15).

NADPHd:
The clearest molecular marker of granular layer compartments is histological staining for nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase (NADPHd) a reliable marker for nitric oxide synthase (NOS) in the brain (Bredt et al., 1991; Hope et al., 1991). In the granular layer of the cerebellum NADPHd is expressed nonuniformly by patches of granule cells and synaptic glomeruli (Yan et al., 1993; Hawkes and Turner, 1994; Schilling et al., 1994). The patches are found in all lobules, are reproducible from individual to individual, and topographically align with the Purkinje cell zebrin II compartments. High levels of NADPHd correlate well with the boundaries of mossy fiber terminal fields and several experiments have concluded that NO is involved with second-messenger systems that potentiate long term depression in the cerebellum (Garthwaite et al., 1988; Crepel and Jaillard, 1990; Ito and Karachot, 1991, 1992; Shibuki and Okada, 1991; Daniel et al., 1992, 1993). Hawkes and Turner (1994) proposed that long term depression of parallel fiber-Purkinje cell synapses mediated by NO may involve only a subset of mossy fiber/granule cell projections, and that NO may act to refine refining receptive fields in the cerebellum (Fig. 3). Thus, via lateral inhibition at the borders of mossy fiber terminal fields, a clear separation of cerebellar receptive fields may be maintained.

AChE:
AChE is strongly expressed in the adult cerebellar cortex, with the highest levels of activity in the granular layer where subsets of large synaptic glomeruli and Golgi cell somata are stained. The functional significance of AChE activity is obscure in that it does not appear to be related to cholinergic transmission. The distribution of AChE staining in the granular layer and the white matter of the adult rat cerebellar cortex is patchy (Boegman et al., 1988). The AChE patches are organized in parasagittal bands running rostrocaudally throughout the cerebellum. Large Golgi cells are AChE-reactive in the more superficial granular layer, while smaller Golgi cells were stained in deeper regions of the granular layer. Profiles of Golgi cell axons were also stained in the granular layer. Elsewhere in the cerebellar cortex there is little or no AChE activity. The boundaries of the AChE patches in the granular layer of the vermis and hemispheres align with the zebrin $I^{+/-}$ boundaries of the overlying Purkinje cell compartments (Boegman et al., 1988). This is interesting as it suggests that molecular layer AChE compartments may be congruent with the Purkinje cell bands in the molecular layer. Unfortunately, there are important species differences in AChE expression, and while a banded distribution of AChE has been documented in monkey, hamster, and mouse cerebella, the cellular basis of the staining is often obscure (Roffler-Tarlov and Graybiel, 1982; Hess and Voogd, 1986; Voogd et al., 1987).

Cytochrome oxidase:
Cytochrome oxidase (CO) activity is compart-mentalized in the granular layer of several species. In the adult monkey, CO is distributed in a longitudinally banded pattern in axons and glomeruli throughout the white matter and granular layer of the vermis and paravermis (e.g. Hess and Voogd, 1986). The distribution of CO is topographically identical to the banded distribution of AChE, with a close correspondence between the AChE and CO-rich zones. Leclerc et al. (1990) extended these findings to show that the boundaries of the CO compartments in the granular layer and zebrin I compartments of the molecular layer are congruent. The complication here is that there is a striking difference in the pattern between rats and primates: the CO^+ and zebrin I^+ zones overlap in the monkey, while in the rat the apposite is observed, CO^+ patches align with the zebrin I^- zones (Leclerc et al., 1990). In contrast to the CO distribution in other brain regions, the pattern of activity in the granular layer does not seem to be a dynamic response to recent patterns of electrical activity (Leclerc et al., 1990).

Synaptophysin:
Regional heterogeneity can be seen in the granular layer by immunocytochemical staining for the synaptic vesicle antigen, synaptophysin. *Anti-synaptophysin* (MAbQ155) also reveals a pattern of rostrocaudal parasagittal bands which encompasses both the molecular layer and, more intensely, the granular layer (Hawkes et al., 1985; Leclerc et al., 1989). The reason for regional differences in staining intensity is unknown. It may be that the high intensity regions contain higher number of synapses per glomerulus, a higher number of glomeruli, a higher number of synaptic vesicles, or some combination of these.

CD15:
The blood antigen 3-α-fucosyl-N-acetyl-lactosamine (CD15) is partitioned in the cerebellar cortex of several vertebrates. In the mature mouse, rat, rabbit, monkey, and human CD15 is expressed in the molecular layer (Marani and Tetteroo, 1983; Marani and Mai, 1992). However, only in the mouse is there a parasagittal banded expression of CD15, and this is co-distributed with 5'-nucleotidase activity.

Granular layer modules can also be revealed by using ethanol fixation

The most extensive example of regionalization in the granular layer has resulted from a novel method for revealing anatomical compartments (Hawkes, 1996). The adult mouse cerebellum was ethanol-fixed, paraffin-embedded and sectioned. Upon rehydration, cerebellar sections wrinkle elaborately to expose an array of modules in both the transverse and sagittal planes of all lobules throughout the vermis and hemispheres. The wrinkling patterns were reproducible between serial sections and between individual animals. More importantly, the modular compartmentation of the granular layer had a clear alignment with the overlying Purkinje cell compartments as revealed by *anti-zebrin II* immunocytochemistry.

1.3. The modular cerebellum

The results summarized above thus indicate that, the granular layer is a highly modular structure. We can speculate that modules are arranged hierarchically into bands. First, there are parasagittal bands in the granular layer, identified through anterograde tracing of various mossy fiber projections. In some cases, these are topographically aligned with the Purkinje cell parasagittal bands identified by using molecular probes. Bands in the granular layer are further subdivided into functional modules, identified by tactile receptive field mapping, and these may have anatomical equivalents in the pattern of granular layer wrinkling revealed in ethanol-fixed sections (Fig. 4).

The elaborate modular segregation of the cerebellar cortex may reflect the need for parallel information processing for motor control (reviewed in Hawkes and Gravel, 1991). It is possible that each module in the cerebellar cortex receives afferent input that represents a single sensory domain (receptive field). Due to physiological time constraints during motor activity, a parallel motor processing system would prove more efficient than a serial one. The cerebellum is a prime example of a parallel processor. Large scale sensory input to the cerebellum is segregated into separate modules where local structural and functional specializations may process the information appropriately. Within each module learning may occur by epigenetic adaptations, such as the modulation of interneuron connectivity and the efficacy of synaptic activity. This neuronal fine tuning allows a module to optimize its activity for processing a specific input modality.

2. Development of granular layer compartmentation

2.1. Development of the granular layer

The cerebellum arises from the ventricular neuro-epithelium abutting the mesencephalic-metencephalic

boundary referred to as the cerebellar plate (Martinez and Alvarado-Mallart, 1989). The different cell populations in the cerebellum are generated sequentially during embryonic and early postnatal life. High levels of mitotic activity commence in the cerebellar anlage at approximately embryo day 12 (E12) in the rat and E9 in the mouse. Neurons are born in two waves. An initial wave of cells is produced that will differentiate into the vestibular nuclei and the crebellar nuclei. The second wave of differentiating cells, which are generated ventral to the deep nuclear neurons, become the Purkinje cells (Altman, 1982). Once the Purkinje cell progenitors cease dividing (at E16 in the rat and E13 in the mouse), the first phase of cerebellar development is complete (Miale and Sidman, 1961; Altman and Bayer, 1978).

In a second phase of development the Purkinje cells move to the surface of the cerebellum and the external granular layer (EGL) forms. The EGL is the germinal epithelium that generates the granule cells. The Purkinje cells migrate radially from their anlage through the population of cerebellar nuclear neurons to reach the surface of the cerebellum. At the same time the cells of the EGL proliferate in the germinal trigone and spread rostromedially, along the basal lamina beneath the pia mater, to cover the entire outer surface of the cerebellum. The EGL migrates primarily rostrally over the surface of the cerebellum. The progenitors of the granule cells were originally thought to arise exclusively from proliferating germinal cells at the
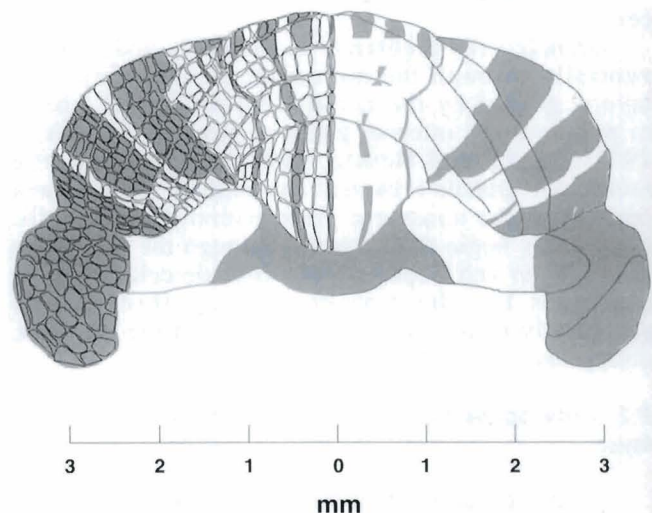

Fig. 4. The hypermodular organization of the cerebellum. A rostral view of the mouse cerebral cortex. Lobules I-V are labeled. The zebrin II+ bands of Purkinje cells are shaded. Overlaid on this organization is an array of modules in the granular layer, that subdivide the Purkinje cell compartments both transversely and parasagittally. The distribution of the modules is schematic, but their sizes are realistic. It is proposed that individual modules in the granular layer are defined by the terminal fields of the mossy fiber afferents and are sometimes equivalent to the patches seen in the tactile receptive field maps. Each patch comprises ~20 Purkinje cells and their associated interneurons and receives a precise mossy and climbing fiber innervation. Adapted from Hawkes (1996).

embryonic rhombic lip, a proliferative region at the caudolateral cornes of the cerebellar plate (Miale and Sidman, 1961; Fujita et al., 1966). Later, it was shown that EGL progenitors are found not just the caudolateral corners of the cerebellum but along the whole caudal edge (Altman and Bayer, 1978; Voogd, 1992; Hallonet and Le Douarin, 1993; Otero et al., 1993; Ryder and Cepko, 1994).

The postnatal development of the cerebellum is dominated by the production of the granule cells in the EGL and their descent to the internal granular layer. During this process the axons of the granule cells remain in the molecular layer to form the mature parallel fiber network, where they synapse with the growing Purkinje cell dendrites (Altman, 1972). Granule cell proliferation occurs throughout the entire EGL for approximately fifteen days after birth (Miale and Sidman, 1961; Fujita et al., 1966; Fujita, 1967; Ryder and Cepko, 1994). Early proliferation causes the EGL to grow in thickness, and during this period all cells of the EGL are mitotically active (Fujita et al., 1966). Subsequently, during the latter stages of proliferation, mitotically active cells are confined to the superficial half of the EGL and the deep EGL is mainly postmitotic. Altman (1972) proposed that proliferation in the EGL gives rise to several cell types of cerebellar interneurons and glia. However, more recent transplantation experiments in quail-chick preparations (Hallonet et al., 1990) and *in vitro* proliferation assays of the immature mouse EGL (Gao et al., 1991) suggest that the EGL produces only granule cells.

Granule cells proliferate in the EGL, and descend ventrally through the molecular and Purkinje cell layers, guided by the radial Bergmann glial fibers, to populate the internal granular layer (e.g., Rakic, 1971; Hatten and Heintz, 1995). In addition, the postmitotic granule cells may also migrate medially and laterally in the transverse plane within the deep EGL before they begin their descent through the molecular layer (Ryder and Cepko, 1994). Granule cell migration continues for about approximately three weeks postnatally in the mouse, after which time the EGL disappears.

2.2. Development of compartmentation in the granular layer

Several developmental mechanisms may combine to generate the modular organization of the mature granular layer. Five different models are suggested, and tested, below:

1) The EGL is heterogeneous, and its topography translates directly into a modular granular layer

Although the entire EGL is proliferative and immature granule cells migrate as a uniform population, it remains unclear whether the cells of the EGL are themselves homogeneous. One line of evidence indicates that the EGL is heterogeneous. Immunocytochemical staining for the CD15 antigen reveals a clear parasagittal banding pattern in the EGL of the embryonic human cerebellum at 16 weeks of gestation (Marani and Tetteroo, 1983; Marani and Mai, 1992) that disappears later in gestation. There is also a transient expression of CD15 in parasagittal bands in the EGL of the developing rat and rabbit (Marani, 1988; Marani and Tetteroo, 1983). In contrast, the NADPHd heterogeneity of the granular layer is not apparent in the EGL, and expression only develops neonatally (Yan et al., 1993; Schilling et al., 1994). NADPHd/NOS activity is first detected at P3. During the first postnatal week of development the granular layer express NOS uniformly (Schilling et al., 1994). Subsequently, clusters of granule cells begin to suppress their expressions of NOS, and from this, a new heterogeneous pattern of NOS expression emerges that persists into adulthood (Yan et al., 1993; Schilling et al., 1994; Hawkes and Turner, 1994). The selective suppression of NOS in granule cell patches may be activity-dependent (see below).

2) Individual granular layer modules are clones, derived from single EGL precursors

Because granule cell migration is directed by the vertically oriented Bergmann glial fibers, the clonal progeny of a single precursor in the EGL would be expected to be clustered together in the adult granular layer. Such is indeed the case. There is evidence that embryonic ventricular progenitors are allocated to produce mouse granule cell populations that aggregate in discrete regions of the adult cerebellum (Miyake et al., 1993). When individual embryonic progenitors were labeled at E13 with a recombinant retrovirus carrying the *E. coli lacZ* gene, the infected cells and their clonal progeny constitutively express ß-galactosidase (ß-gal). While clones of ß-gal+ granule cells indeed formed discrete clusters in the adult granular layer, there is reason to think that granular layer topography does not have a clonal basis. First, within clonal clusters there were also many ß-gal- granule cells, indicating that granule cells descended from a single embryonic progenitor become extensively mixed with granule cells derived from other embryonic progenitors. Secondly, the granule cell clones do not seem to respect the known functional boundaries of the cerebellar cortex.

3) Modules in the granular layer are a secondary epigenetic response to the compartmentation of the Purkinje cells

Purkinje cells may play a role in forming the molecular compartments within the developing granular layer. Several Purkinje cell markers have been identified in rodents that reveal cerebellar compartmentation during early Purkinje cell development (e.g., reviewed in Wassef et al., 1992). These antigens are expressed

selectively by clusters of Purkinje cells and reveal a rostrocaudal compartmentation of the embryonic and early neonatal cerebellum. The different perinatal compartmentation antigens do not share common boundaries, but rather there is substantial overlap between them. Wassef et al. (1985, 1992) proposed that the combinatory expression of the small numbers of antigens may uniquely identify a high number of different zones that might be recognized by different mossy fiber growth cones, and thus organize the projection topography with respect to the Purkinje cell compartmentation.

Is granular layer heterogeneity secondary to compartmentation of the Purkinje cells? There is substantial evidence that granule cells and Purkinje cells are mutually dependent for their normal development. For example, it has been suggested that the Purkinje cell dendrites are stimulated to begin their extension into the molecular layer by the granule cell descend and the development of the parallel fibers (Sidman, 1968). However, there is not the case in humans, where Purkinje cell dendrites begin to grow before the descent of granule cells (Rakic and Sidman, 1970; Sidman and Rakic, 1973). Similarly, numerous studies confirm that the absence of granule cells disrupts normal Purkinje cell dendrite arborization (e.g., Kilham and Margolis, 1964; Purpura et al., 1964; Hirano et al., 1977; Aggerwal and Hendelman, 1980; Baptista et al., 1994). The consequences of Purkinje cell death for granule cell development are even more severe. Parallel fiber contacts with the Purkinje cells seem to be essential for the long-term survival of the granule cells. For example, in the *lurcher* mutant mouse, where there is a massive loss of Purkinje cells after the second postnatal week, ninety percent of the granule cell population dies as a secondary effect of Purkinje cell degeneration (Wetts and Herrup, 1982a,b). Similarly, secondary granule cell death occurs in *Purkinje cell degeneration* (Mullen et al., 1976; Landis and Sidman, 1978) and *staggerer* mutant mice (Sotelo et al., 1974; Landis and Mullen, 1978). Thus, it is plausible that Purkinje cells play an influential role in the development of molecular compartmentation in the granular layer. Finally, this is consistent with the ample evidence showing that molecular compartmentation of Purkinje cells aligns with granule cell compartments (NADPHd-zebrin II: Hawkes and Turner, 1994; wrinkles-zebrin II: Hawkes, 1996; AChE zebrin I: Hess and Voogd, 1986; CO-zebrin I: Leclerc et al., 1990).

4) Modules in the granular layer are secondary to the compartmentation of the afferent terminal fields

The olivocerebellar climbing fiber and mossy fiber afferents have already invaded the cerebellar white matter of newborn rats (Sotelo et al., 1984; Arsenio-Nunes and Sotelo, 1985) and mice (Heckroth et al., 1990; Paradies and Eisenman, 1993). Anterograde transport studies have revealed the pattern of spinocerebellar mossy fiber innervation in the early postnatal rat. Before P1 the mossy fiber growth cones have reached the cerebellar white matter of the anterior vermis and the pyramis. Between P1 and P3, the central white matter in both the anterior and posterior projection areas become more densely populated until, by P3, the granular layer of the anterior lobe is penetrated by the first spinocerebellar axons. The terminal fields are already crudely organized in parasagittal columns («protocolumns») at this stage. Mossy fibers first synapse with the granule cells at P5, and by P7 the spinocerebellar terminal fields are organized in a pattern resembling the adult projection.

The early compartmentation of ingrowing mossy fibers may reflect the Purkinje cell chemical heterogeneity in the late embryo and early neonate (Sotelo, 1987; Sotelo and Wassef, 1991; Wassef et al., 1992). For example, Ji and Hawkes (1995) have shown that neonatal spinocerebellar mossy fiber projections terminate selectively on molecularly distinct Purkinje cell early clusters. The spinocerebellar mossy fiber afferent invade the granular layer at a time when few if any granule cells are present (P3). Therefore, either the mossy fibers are organized into compartments by the few existing granule cells on their topography is independent of their adult targets. If the granule cells do not influence the compartmentation of the mossy fiber innervation, then an alternative is the recognition of Purkinje cell heterogeneity by the developing spinocerebellar axons. To address this issue, the spinocerebellar projections of adult agranular cerebellar cortices were analyzed. The results suggest that deficits in granule cell development *per se* do not alter the normal mossy fiber terminal fields, but that if the Purkinje cells are defective or missing, the normal projection patterns are lost. For example, in models in which the primary defect is in the granule cells and granule cells die early in development, the spinocerebellar projections to the anterior vermis retain their normal columnar organization (Arsenio-Nunes and Sotelo, 1985; Arsenio-Nunes et al., 1988; Eisenman and Arlinghaus, 1991; Sotelo and Wassef, 1991; Vogel and Prittie, 1994). In contrast, in the *staggerer*, granule cell death is secondary to the lack of proper Purkinje cell dendrite formation (Landis and Muller, 1978), and normal spinocerebellar topography is absent.

5) Modules in the granular layer are sculpted by activity-dependent processes

Following the initial establishment of mossy fiber topography there may be a second phase of refinement and error elimination that is activity dependent. When cerebellar N-methyl-D-aspartate (NMDA) or gamma-aminobutyric acid (GABA) receptors are blocked during postnatal development by the topical application of competitive antagonists, the local spinocerebellar topography is disrupted (Tolbert et al., 1994). More recently, the administration of methylazoxymethanol to

the rat pup at the time of birth was shown to severely disrupt the spinocerebellar mossy fiber-Purkinje cell topography (Ji and Hawkes, 1996). This suggests that while Purkinje cell compartmentation is essential for the early organization of mossy fiber topography, granule cell-mossy fiber interactions may regulate the later stages.

Activity-dependent processes may also underlie the differential expression of NADPHd/NOS in the developing rodent cerebellum. When dissociated granule cells form the cerebellar anlage were examined *in vitro* prior to mossy fiber innervation, all the granule cell precursors produce granule cells that express NOS (Schlling et al., 1994). The intensity of NOS expression was increased by suppressing electrical activity. This suggests that compartments in the adult granular layer with different levels of NOS expression are a secondary granule cell response to positional cues in the granular layer that are influenced differentially by synaptic activity. For example, it may be that the compartmentation of NOS expression is secondary to afferent mossy fiber compartmentation (Schilling et al., 1994).

The same might be true for the CD15 antigen. Transplantation experiments in rabbits have shown that CD15 remains expressed in granule cell remnants of the transplants for seven months post-operative (Marani, 1988). Thus, the developmental expression and maintenance of CD15 seems to persist in the absence of developmental cues that may regulate its expression.

2.3 A model of granular layer development

The data reviewed above can be organized into the following, speculative, model of granular layer development:

Stage 1: The cerebellum is compartmentalized from at least E14 into an array of Purkinje cell clusters with distinct molecular phenotypes. The ingrowing mossy fiber afferent growth cones read the Purkinje cell «map» and organize accordingly. The EGL spreads like a blanket over the surface of this highly ordered matrix, but there is little evidence that it is heterogeneous at this stage.

Stage 2: Granule cells are born postnatally in the EGL and migrate through the Purkinje cell layer and immature molecular layer, to reach their terminal resting place in the granular layer. During migration they leave behind axonal processes that from the parallel fibers. The granule cells interact with the Purkinje cells during this period and differentiate in response to positional cues that are in register with the Purkinje cell/mossy fiber lattice. It has not been demonstrated that granule cells acquire positional information at this stage, although there is evidence that Purkinje cells can influence the differentiation of granule cells and *vice versa*.

Stage 3: Mossy fiber afferent growth cones are organized into parasagittal terminal fields by the early Purkinje cell clusters. When the granule cells begin to descend from the EGL to the granular layer the mossy fiber growth cones leave the Purkinje cells and synapse on the granule cell dendrites. In this way, mossy fiber and Purkinje cell topographies are kept in register.

Stage 4: Once in the granular layer, granule cell dendrites receive synaptic input from the growth cones of the awaiting mossy fibers. From the early development of the granular layer, molecular signs of the topography of the granular layer can be seen (e.g., NADPHd/NOS). Finally, as cerebellar function begins to develop, a new set of *dynamic* criteria emerge to refine the topography of the granular layer: between the parasagittal bands, inappropriate mossy fiber projections are eliminated (targeting errors); within the parasagittal bands, mossy fibers with similar firing patterns become concentrated together (through the twin processes of sprouting and collateral elimination) thereby subdividing the parasagittal bands into patches. This stage may involve activity-dependent refinement, similar to that seen in the development of ocular dominance columns or whisker barrels in the isocortex.

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