Published in final edited form as:

Anat Embryol (Berl). 2004 November ; 209(1): 49–58.

Quantitative analysis of granule cell axons and climbing fiber afferents in the turtle cerebellar cortex

D. L. Tolbert,

Francis and Doris Murphy Neuroanatomy Research Laboratory, Department of Surgery, School of Medicine, Saint Louis University, 1402 South Grand Blvd., St. Louis, MO 63104, USA

B. Conoyer, and

Francis and Doris Murphy Neuroanatomy Research Laboratory, Department of Surgery, School of Medicine, Saint Louis University, 1402 South Grand Blvd., St. Louis, MO 63104, USA

M. Ariel

Department of Pharmacological and Physiological Science, School of Medicine, Saint Louis University, St. Louis, MO 63104, USA

Abstract

The turtle cerebellar cortex is a single flat sheet of gray matter that greatly facilitates quantitative analysis of biotyninated dextran amine labeled granule cell and olivocerebellar axons and Nissl-stained granule and Purkinje neurons. On average, ascending granule cell axons are relatively thicker than their parallel fiber branches (mean±SD: 0.84 ± 0.17 vs 0.64 ± 0.12 μm , respectively). Numerous *en passant* swellings, the site of presynaptic contact, were present on both ascending and parallel fiber granule cell axons. The swellings on ascending axons (1.82 ± 0.34 μm , $n=52$) were slightly larger than on parallel fibers (1.43 ± 0.24 μm , $n=430$). In addition, per unit length (100 μm) there were more swellings on ascending axons (11.2 ± 4.2) than on parallel fibers (9.7 ± 4.2). Each parallel fiber branch from an ascending axon is approximately 1.5 mm long. Olivocerebellar climbing fiber axons followed the highly tortuous dendrites of Purkinje cells in the inner most 15–20% of the molecular layer. Climbing fibers displayed relatively fewer *en passant* swellings. The spatial perimeter of climbing fiber arbors (area) increased 72% from anteriorly (1797 μm^2) to posteriorly (3090 μm^2) and 104% from medially (1690 μm^2) to laterally (3450 μm^2). Differences in the size and spacing of *en passant* swellings on granule cell axons suggest that ascending axons may have a functionally more significant impact on the excitability of a limited number of radially overlying Purkinje cells than the single contacts by parallel fiber with multiple orthogonally aligned Purkinje cell dendrites. The spatially restricted distribution of climbing fibers to the inner most molecular layer, the paucity of *en passant* swellings, and different terminal arbor areas are enigmatic. Nevertheless, these findings provide important anatomical information for future optical imaging and electrophysiological experiments.

Keywords

Topography; Parallel fibers; Purkinje cells; Radial organization

Introduction

Our conceptualization of cerebellar cortical function is based on the twin tenets of spatial cytoarchitectonic homogeneity with synaptic interactions between cortical neurons across

cortical areas and topographically organized afferent systems synaptically contacting specific cortical neurons. Two principal afferent systems orchestrate the activity of Purkinje cells, the sole output neurons of the cerebellar cortex. Mossy fiber-type afferents activate granule cells whose axons ascend radially into the molecular layer before bifurcating into parallel fibers that travel orthogonally thereby collectively influencing many Purkinje cells. Olivocerebellar climbing fibers directly contact a single Purkinje cell at multiple sites. Interactions between parallel and climbing fiber inputs onto Purkinje cells have been posited to be the basis for plasticity of Purkinje cells in relationship to acquired motor behaviors (Marr 1969; Ito and Kano 1982; Gilbert 1993). More recently, the theoretical basis for parallel-climbing fiber interactions has been extended to include the ascending component of granule cell axons that synaptically contact radially overlying Purkinje cells (Mugnaini 1972; Llinás 1982; Llinás and Sugimori 1992; Cohen and Yarom 1998; Bower 2002).

The highly predictable cytoarchitectural organization of the cortex, the relatively limited and recognizable neuronal types, and the functional understanding of different synaptic relationships have facilitated the rigorous quantitative analysis of the cerebellar cortex (reviewed in Harvey and Napper 1991). Most quantitative morphological studies, however, generally focused on studying a single cortical element. For example, granule cell parallel fiber lengths (Pichitpornchai et al. 1994; Brand et al. 1976), spacing between parallel fiber en passant synaptic contacts (Pichitpornchai et al. 1994; Shepherd and Raastad 2003; Shepherd et al. 2002), ascending axon *en passant* contacts (Gundappa-Sulur et al. 1999), synaptic morphology (Pichitpornchai et al. 1994; Napper and Harvey 1988a; Napper and Harvey 1988b), synaptic strength (Barbour 1993), and numerical ratios between neurons (Lange 1982) have all been quantitatively analyzed. Olivocerebellar fiber afferents have also been extensively studied for their powerful synaptic activation of Purkinje cells and highly organized topography in the cortex (Sugihara et al. 1999, 2001). Furthermore, morphometric and electrophysiological analysis has largely focused on the highly foliated mammalian cerebellar cortex, thereby limiting the opportunity to make observations of widespread or even spatially contiguous cortical areas. The turtle cerebellum, however, offers a unique opportunity to quantify Purkinje cell input and function as it consists of a single, flat layer of cortex that can be isolated and maintained in vitro in a physiological condition due to the turtle's brain resistance to hypoxia (Ariel and Fan 1993; Fan et al. 1993).

The purpose for the present study was to investigate anatomical organization of granule cell and climbing fiber inputs onto Purkinje cells to provide anatomical basis for the functional imaging studies and further characterization of sensory afferent processing in the cortex (Ariel et al. 2002). Small ejections of tracer were made into multiple areas of the cortex or into the cerebellar peduncle. Labeled granule cell and climbing fiber axons were quantitatively analyzed. The ratio of granule cells/Purkinje neuron was semi-quantitatively estimated.

Methods

The turtle cerebellum can be isolated and maintained in a physiological state as an in vitro preparation allowing stabilized iontophoretic ejections and intraaxonal transport (Martin et al. 1998). Thirteen pond turtles (*Pseudemys scripta elegans*) were used for this study. Under an approved IACUC protocol the animals were anesthetized with thiopental (12.5 mg IM) and decapitated. Oxygenated Ringers solution was then perfused bilaterally through the internal jugular veins to exanguinate the brain. The cerebellum and brainstem were immediately removed, pinned to a Sylgard floor of a perfusion chamber, and bathed with oxygenated Ringers solution for the duration of the experiment. The cerebellar peduncle on one side was cut allowing the cerebellum to be flipped to an inverted position thereby exposing its ventricular surface. Ejections were made from the ventricular surface in an attempt to minimize spread of the tracer into molecular and Purkinje cell layers. Biotinylated dextran amine (10%, 3 kDa

BDA, Molecular Probes) was iontophoretically ejected (7 s on/off cycle, 15 μ Amps, 4 min total) from a glass micropipette into the granule cell layer 100 μ m deep to the ventricular surface or into the intact cerebellar peduncle. Ejections into the granule cell layer were made in a grid pattern of two anterior–posterior oriented medial and lateral columns of five evenly spaced ejection sites bilaterally.

Six to 8 h after the last ejection, the cerebellum was placed in phosphate buffered 4% paraformaldehyde (pH 7.4) for 24 h then cyroprotected in 30% sucrose/4% fixative solution for an additional 2 h. Serial 48- μ m-thick transverse or sagittal sections were cut with a freezing sliding microtome and immediately mounted directly onto gelatin-subbed slides. Slide mounted sections were processed for chromogenic demonstration of the BDA using a Elite ABC Kit (Vector Laboratories) and diaminobenzidine procedures with cobalt chloride intensification (Adams 1981; Veenman et al. 1982). Following the reaction, the sections were dehydrated, cleared, and coverslipped.

Labeled axons and terminals were quantitatively analyzed using Bioquant and Neurolucida image analysis software. All areas of the cerebellar cortex were analyzed (Fig. 1). The location of each measured element was standardized for all animals in the three axes. Anterior–posterior locations were expressed as the relative percent distance from the anterior end of the cerebellum. Medial–lateral locations were expressed as the relative percent distance between the midline raphe and the lateral edge of the cortex. Dorsal–ventral locations in the molecular layer were expressed as a relative percentage of the distance between the Purkinje cell layer and the pial surface of the cortex. Under high magnification (400x), Bioquant image analysis was used to measure individual axons and swellings by manually clicking on the opposing sides of the axons or varicosities at their widest extent. The medial/lateral and anterior/posterior location of each measured axon was recorded for each measured element. Intervaricosity distances were measured between centers of adjacent *en passant* swellings. The area measurements for climbing fibers arbors were calculated using measurement data of the arbor at its widest and tallest points. The raw data was imported into Microsoft Excel where the relevant data (length measurements and the products of area and percent distances) were grouped together for the corresponding fibers. Well-labeled granule cell axons and climbing fibers, which did not pass out of the section being analyzed, were manually traced and then reconstructed and displayed using Neurolucida image analysis software.

Granule cell nuclei fill almost the entire somata of these neurons (Palay and Chan-Palay 1974). The ratio of granule cells to Purkinje cells was calculated by measuring the area occupied by the basophilic stained nuclei of granule cells radially beneath a known number of serially adjacent Purkinje cells. The areas of 150 individual granule cell nuclei were measured and a mean area calculated (15.65 μ m²). The number of granule cells beneath a given number of Purkinje cells was then calculated by dividing the area occupied by basophilic-stained granule cell nuclei by the average size of granule cell nucleus (GC) and number of Purkinje cells (PCs) ($\text{Area}_{\text{gc}}/(\text{avg Area/GC} \times \text{no. of PCs})$). There were no corrections made in the data for shrinkage of the sections due to fixation or histological processing.

Results

The BDA ejections into the granule cell layer or the cerebellar peduncle labeled four principal elements in the molecular layer: granule cell axons; olivocerebellar climbing fiber axons; Purkinje cell dendrites; and processes of tanyocytes. Labeling of these different elements by BDA was somewhat capricious. Even though multiple iontophoretically ejections were made using consistent parameters, the size of the individual ejection sites were variable in size as were the number and types of neural elements labeled. Smaller ejection sites were characterized by solid labeling with BDA, whereas larger ejection sites consisted of an inner, vacuolated

core of damaged tissue, surrounded by densely labeled penumbra. In these larger sites the area of the lesion may have resulted from mechanical distortion by the tip of the microelectrode and/or from excess iontophoretic current. Similarly, small restricted ejection sites in the granule cell layer labeled many granule cell axons or at other sites relatively very few labeled axons were observed in the molecular layer. At other sites the ejections were localized to the molecular layer. When there was a small lesion in the molecular layer numerous labeled parallel fibers radiated laterally and medially from the ejection site.

The turtle cerebellum is approximately 8 mm wide (lateral edge to lateral edge) and 4.5 mm long (anterior–posterior). The cerebellum is 1300 μm thick with the molecular layer being 475 μm thick, the granule cell layer being on average 775 μm thick and the Purkinje cell layer 50 μm thick (Fig. 2). The white matter located beneath the granule cell layer and above the fourth ventricle varied in thickness with midline areas narrower than lateral parts adjacent to the peduncle.

Granule cell axons

The BDA ejections in the granule cell layer resulted in dense labeling of tanycytes and/or Purkinje cells and their processes in the molecular layer making it impossible to trace labeled ascending granule cell axons at and around the epicenter of the ejections sites. Around the periphery of the ejection sites, however, ascending granule cell axons could be followed in their radial ascent through the molecular layer (Fig. 3). Granule cell axons were analyzed in transverse sections and identified by their small diameter, ovoid-shaped *en passant* swellings, relatively straight radial ascent or orthogonal course, and distribution throughout the molecular layer (Fig. 3, Fig. 4). Rarely was a radially ascending axon followed from the Purkinje cell layer to its branch point in the molecular layer (Fig. 4B). Many ascending axons could be traced relatively short distances before either they passed out of the plane of the section or were obscured by other labeled elements and therefore not quantitatively analyzed. Ascending axons that could be traced through deep and intermediate thirds or intermediate and superficial thirds of the molecular layer were quantitatively analyzed. Ascending granule cell axons were 0.88 μm diameter (mean $\text{SD} \pm 0.17 \mu\text{m}$, $n=52$). Ascending granule cell axons did not vary significantly in diameter at anterior–posterior or medial–lateral locations (Fig. 5).

Labeled parallel fibers were observed traveling orthogonally in all depths of the molecular layer and from each ejection site were always more numerous than discernable ascending axons. The number of labeled parallel fibers depended on the location of the BDA ejection. Many fewer parallel fibers were labeled when the epicenter of the BDA ejections was localized to the granule cell layer compared with when the BDA ejection was in the molecular layer. Many labeled parallel fibers could only be traced a relatively short distance before passing from the plane of the section or, more frequently, becoming intermingled with and indistinguishable from other labeled axons. Individual labeled parallel fibers that could be only unequivocally traced for variable distances (13.05–291.9 μm , mean 64.1 μm) were quantitatively analyzed. Quantitative measurements of individual parallel fibers were only taken from axons labeled from ejections directly into the granule cell layer. In these cases parallel fibers were 0.64 μm in diameter ($\pm 0.19 \mu\text{m}$, $n=860$) being relatively constant in diameter in anterior–posterior and superficial-deep locations. There was a trend for parallel fibers to be smaller in diameter in the lateral cerebellum (data not shown).

The length of parallel fibers could only be semi-quantitatively analyzed by measuring the length of beams consisting of many labeled axons traveling together in loosely grouped, but distinct, bundles. Two experiments are especially noteworthy. In one case the ejection was centered in the cerebellar midline labeling many axons that traveled in bundles towards the sides of the cortex. The relative number of labeled parallel fibers declined in the bundles proximal-distally as both the size and intensity of labeling of the bundles became reduced

compared with more medial areas. In the superficial molecular layer the longest extending axons were between 1.3 and 1.6 mm in length. In another case where an ejection was localized to the ventricular half of the granule cell layer in intermediate medial–lateral levels, labeled axons in the deepest part of the molecular layer extended transversely at most approximately 1.4 mm.

En passant axonal swellings representing synaptic boutons were present on both the radial ascending and orthogonally oriented parallel fiber axons (Fig. 3, Fig. 4). Side branches from ascending axons were not observed and only rarely observed arising from parallel fibers. *En passant* swellings were ovoid-shaped with smooth contours. The diameter of the swellings on the ascending granule cell axons was on average $1.82\ \mu\text{m}$ (± 0.34 , $n=52$; Fig. 5). Swellings on proximal vs distal ascending segments averaged $1.85\ \mu\text{m}$ ($\pm 0.34\ \mu\text{m}$) and $1.78\ \mu\text{m}$ ($\pm 0.43\ \mu\text{m}$), respectively. The intervaricosity distance between centers of adjacent *en passant* swellings ($8.92\ \mu\text{m}$) was unremarkable in anterior–posterior and medial–lateral locations (Fig. 5). There was a slight trend for the *en passant* swellings to be slightly closer together in superficial vs deep parts of the molecular layer (data not shown).

Because labeled parallel fibers were far more numerous, the number of swellings analyzed was correspondingly larger. Swellings on parallel fibers were $1.43\ \mu\text{m}$ in diameter ($\pm 0.24\ \mu\text{m}$, $n=430$) and spaced $10.28\ \mu\text{m}$ ($\pm 4.6\ \mu\text{m}$) apart. There was a gradual increase in the average intervaricosity lengths posteriorly compared with anteriorly (Fig. 5). Nodal diameters did not vary significantly in different parts of the cortex (Fig. 5). In the thickness of the molecular layer the swellings were slightly wider deep in the cortex compared with superficial parts, but this trend was not significant (data not shown). Similarly, there was a trend for the swellings to be slightly smaller between posterior and anterior levels (8.33%) and between medial and lateral locations (11.67%).

Climbing fiber axons

Olivocerebellar climbing fiber axons were analyzed in sagittal sections and identified by their relatively larger diameters (compared with granule cell axons), multiple branching in the molecular layer, highly tortuous course along PC dendrites, irregular-shaped axonal swellings, and their restriction to the inner parts of the molecular layer (Fig. 6). One hundred fifty-seven climbing fiber axons labeled from BDA ejection sites in the cerebellar peduncle were analyzed. Climbing fibers appeared restricted in their distribution in the molecular layer in the turtle cerebellar cortex (Fig. 6). On average, climbing fibers occupied the inner 14.5% of the molecular layer near the midline but expanded to the lower 21.2% of the molecular layer near the lateral edge of the cortex (Fig. 7).

Labeled climbing fiber segments displayed few *en passant* swellings as they followed along the larger dendrites of Purkinje cell (Fig. 6). A relatively few climbing fibers appeared to be labeled in their entirety (Fig. 6B) confirming the paucity of *en passant* swellings and the planar orientation of the climbing fiber arbor on the dendrites of a single Purkinje cell (Fig. 6C). The average primary climbing fiber diameter was $1.13\ \mu\text{m}$ ($\pm 0.15\ \mu\text{m}$), with axons being slightly larger posteriorly and laterally compared with anteriorly and medially (Fig. 7). Unlike in rodents where climbing fibers form numerous *en passant* contacts on Purkinje cells (Palay and Chan-Palay 1974), only 24.2% of the climbing fibers analyzed had swellings on them. The intervaricosity distance between swellings decreased from $8.01\ \mu\text{m}$ at the midline to $6.69\ \mu\text{m}$ at the lateral edge of the cortex. The area occupied by individual climbing fibers in the molecular layer was measured by outlining the perimeter of terminal arbors in the parasagittal sections. Climbing fiber arbor area increased from $1797\ \mu\text{m}^2$ at the anterior area of the cerebellum to $3090\ \mu\text{m}^2$ at the posterior end reflecting a 72.0% increase in area. The average area also increased from $1690\ \mu\text{m}^2$ medially to $3450\ \mu\text{m}^2$ laterally reflecting a 104% increase (Fig. 7).

In one turtle a single BDA ejection was restricted to the cerebellar peduncle, which allowed us to determine if climbing fibers were distributed unilaterally or bilaterally in the cortex. In this case most labeled climbing fibers were localized to the ipsilateral hemi-cerebellum; however, some labeled climbing fibers also crossed the midline into the contralateral hemi-cerebellum. The most lateral of the crossed climbing fibers was located one-third the distance from the midline to the lateral edge of the cerebellum on that side. The limited number of labeled commissural climbing fibers ($n=8$) precluded quantitative analysis; however, there appeared to be one difference in these projections compared with the climbing fibers that remained ipsilateral in that the crossed projections appeared to extend higher into the molecular layer than on the opposite side.

Granule cells/Purkinje cell ratios

The number of granule cells potentially innervating a radially overlying Purkinje cell was estimated based on the measurements of the area of granule cell layer beneath Purkinje cell somata. This area was divided by an average area for a granule cell body to arrive at a granule cells per Purkinje neuron ratio. These measurements, however, also included the area occupied by Golgi neurons and glia and therefore probably overestimate slightly the number of granule cells. Nevertheless, based on these calculations there was an average of 426 granule cells beneath each Purkinje cell body. This value varied only slightly from medial (419) to lateral (435) and anterior (401) to posterior (451; Fig. 8).

Discussion

The simple structure of the turtle cerebellar cortex (Fig. 1) and this animal's resistance to hypoxia greatly facilitated the quantitative analysis of granule cell and climbing fiber projections to the molecular layer. *En passant* contacts were slightly closer together on ascending granule cell axons than on parallel fibers. Climbing fibers were restricted in their spatial distribution in the molecular layer, but appeared to maintain the 1:1 numerical relationship with Purkinje cells. Semi-quantitative estimates of the ratio of granule cells/Purkinje cell were high.

Qualitative and quantitative characterization of the cerebellar cortex suggests there are differences in the cytoarchitectonic organization within and across species (Harvey and Napper 1991). Quantitative differences may be either artifactitious due to diverse methods for histological processing or analytical sampling in difference studies (reviewed in Harvey and Napper 1991), or genuine due to foliation of the cortex. For example, the thickness of the granule cell layer and the number of Purkinje cells varies from its convex surface of a folium, through its relatively flat sides to its sharply concave base in the depth of the fissures (Braitenberg and Atwood 1958). These differences notwithstanding the rat cerebellar cortex has been the most quantitatively analyzed of mammalian cerebella and will be used for comparison with our findings in the turtle. As the turtle cerebellum is a single flat sheet of gray matter (Fig. 1), folding does not confound quantitative sampling or analysis.

Parallel fibers

Labeled parallel fibers did not follow an absolutely straight course in the molecular layer and frequently intermingled with other labeled fibers and therefore could not be followed with certainty over their entire length (Fig. 4). Based, however, on the distance groups or fascicles of labeled parallel fibers were seen in the cortex lateral to an ejection site, we estimate parallel fibers have a total length of about 3 mm in the turtle cortex. Granule cells in the midline would have parallel fiber branches of almost equal length and extending 1.5 mm toward the lateral edge of the cerebellum on each side. Conversely, granule cells near the lateral edge of the cortex would have parallel fibers of unequal lengths. We were unable to determine if the parallel

fiber branches directed medially were longer than the branches directed laterally. Parallel fiber length did not appear to vary from superficial to deep parts of the molecular layer. As the cortex on one side is about 4 mm wide and parallel fibers have a total length of about 3 mm, it is possible that an orthogonal beam of activated parallel fibers could affect a majority of Purkinje cells on one side of the cortex. In mammalian cerebella there seems to be some correspondence in the length of parallel fibers and the length of folia with the inference that parallel fibers may overall be longer in longitudinally longer folia (Brand et al. 1976). Rat parallel fibers average about 5 mm in length (Lauder 1978).

Parallel fiber diameters also appear to vary in different species. Turtle parallel fibers average 0.64 μm in diameter, irrespective of their position in the cerebellum. Rat's parallel fiber diameter varies from 0.15 to 0.7 μm and considerably in size from superficial to deep in the molecular layer and from the distance from the branch point distally (Pichitpornchai et al. 1994). There was also a trend for rat parallel fibers to be very small (0.1 μm) in the lateral cerebellum, which we were unable to confirm in the turtle. Turtle parallel fibers also were slightly thinner in the superficial molecular layer and thicker deeper in the molecular layer consistent with observations made in rat cerebellum (Pichitpornchai et al. 1994).

Parallel fibers varicosities represent synaptic contacts made with Purkinje cell dendritic spines (Palay and Chan-Palay 1974; Napper and Harvey 1988a; Harvey and Napper 1991; Pichitpornchai et al. 1994). Turtle varicosities varied in size, but not statistically, based on location with a decline from posterior to anterior (8.33%) and medial to lateral (11.67%). Conversely, the size of the varicosities in rats decreases significantly by 56% from medial to lateral (Pichitpornchai et al. 1994). The spacing between varicosities (intervaricosity length) represents the potential number of synapses made by an individual parallel fiber with a single Purkinje cell, with adjacent Purkinje cells or with serially spaced Purkinje cells. In rats the average intervaricosity length was 5.19 μm ($\pm 0.10 \mu\text{m}$), but varicosities consistently were closer together nearer the branch point (3.71 μm) than near the end of the parallel fiber (7.44 μm ; Pichitpornchai et al. 1994). Turtle varicosities were on average wider apart (10.28 μm) and with greater variability in distance between swellings (3.66–45.22 μm). The flat nature of the turtle cortex revealed a trend for the average intervaricosity lengths to be greater anteriorly than posteriorly. As the thickness of the Purkinje cell dendritic arbors were not measured, it was not possible to estimate the number of parallel fiber contacts with individual Purkinje cells; however, given the variation in spacing between some varicosities, it seems likely that some parallel fibers may not contact serially adjacent Purkinje cells.

Ascending granule cell axons

Quantitative analysis of the ascending part of granule cell axons has been much less intensively analyzed, because the functional significance of the ascending axons synapsing with radially overlying Purkinje cells was only recently considered. In rat and turtle cerebellum, and other species as well, all ascending axons display varicosities. Varicosities on rat ascending axons are on average 4.02 μm ($\pm 0.02 \mu\text{m}$) apart, whereas turtle varicosities are more widely separated (on average 8.9 μm apart). Varicosity size in turtles may be different on ascending and parallel fibers (Fig. 5). Quantitative analysis of *en passant* swellings on ascending axons was limited by our criteria for inclusion in that the ascending axon must extend at least two-thirds the radial distance of the molecular layer (Fig. 3), although many swellings were observed on shorter segments of ascending axons. The mean swelling diameter on ascending axons was 1.82 μm compared with an average of 1.44 μm for parallel fiber swellings. Interestingly, the size of ascending swellings and climbing fiber swellings were close and both about 8% larger than parallel fiber swellings. Ultrastructurally varicosities on ascending axons in rats contain more synaptic vesicles than parallel fiber varicosities, which may account for their slightly larger size (Gundappa-Sulur et al. 1999).

Climbing fibers

The highly contorted and tortuous course taken by climbing fibers in the molecular layer makes their quantitative analysis more difficult than that of granule cell axons. In rats, climbing fiber arbors are localized to the inner two-thirds or complete thickness of the molecular layer (Palay and Chan-Palay 1974; Sugihara et al. 1999). Occasional varicosities on the secondary branches of climbing fibers contact Purkinje cell dendrites, but it is mainly small delicate collaterals of these secondary and tertiary branches that contact Purkinje cell dendritic appendages that constitute the bulk of climbing fiber synaptic contacts with Purkinje cells (Palay and Chan-Palay 1974; Sugihara et al. 1999). Biotylinated labeled rat climbing fiber varicosities were mostly 1.2–1.6 μm in diameter and each climbing fiber had about 250 swellings (Sugihara et al. 1999). Similarly, BDA-labeled turtle climbing fibers were more restricted in their radial distribution in the molecular layer to the inner 15% (medially) and 21% (laterally), and slightly larger (1.83 μm) varicosities were fewer in number and present only on some climbing fibers (Fig. 7). Compared with the features of BDA-labeled rat climbing fibers (Sugihara et al. 1999), our observation in turtle for a paucity of labeled climbing fiber varicosities, fine collateral tendrils, and the limited radial distribution in the molecular layer requires us to seriously consider the possibility that our BDA labeling paradigm did not completely label climbing fiber terminal arbors. The consistency of labeling of small diameter parallel fibers was largely the result of orthograde transport from the granule cell body. The fact that the BDA injections were made into the cerebellar peduncle to label climbing fibers of passage may account for the latter's incomplete filling. From this it follows that our measurements of the spatial spread of climbing fiber arbors probably is an underestimate of their total areas.

Granule/Purkinje cell ratios

The ratio of granule cells to Purkinje cells has been calculated/estimated using a number of different measurement strategies. Not unexpectedly, ratios differ between studies in the same species (Harvey and Napper 1988), between closely related species (Lange 1982), and between disparate species (Lange 1982). These differences also do not take into consideration changing numerical relationships between neurons as the result of the normal aging process (Sturrock 1990). In a single study, which looked at granule cell/Purkinje cell ratios in monkey and rat using the same methodology, the number of granule cells per Purkinje cells was significantly higher in the primate (437) than in the rodent (274; Harvey and Napper 1991). Undoubtedly, differences may also reflect disparate numbers of cells in regions of multifoliate cortex where the numerical relationship between granule and Purkinje cells differs by regions where the thickness of the granule cell layer varies from its greatest width at the apex on folia to its narrowest width of the folia at the base of the fissures separating folia. The flattened, unfoliated natures of the turtle cerebellum results in relatively uniform thickness of the cortex where we calculated there are 426 granule cells for each Purkinje neuron (Fig. 8). Our estimate of a 426:1 granule cell/Purkinje cell ratio certainly is an overestimation of the number of granule cells as the areal measurements of the granule cell layer includes Golgi and glial nuclei.

Functional correlation

The classical view of how climbing fibers directly and mossy fibers indirectly via granule cells affect Purkinje cell function is undergoing revision. Perhaps the greatest change is how Purkinje cells respond to mossy fiber inputs. It was initially posited that mossy fibers activated Purkinje cells along a longitudinal beam by parallel fibers; however this view started to change when electrophysiological findings first (Kassel et al. 1984; Shambes et al. 1978), and anatomical evidence later, demonstrated that mossy fiber afferents terminated in patch-like manner in the granule cell layer (Tolbert et al. 1993; Tolbert and Gutting 1997; Alisky and Tolbert 1997). This patch pattern of organization of mossy fiber afferents is then transmitted through the

ascending axons of granule cell to a spatially congruous patch of radially overlying Purkinje cells (Bower and Woolston 1983).

More recently, optical imaging studies in the cerebellar cortex of afferent activation have confirmed and extended these above observations (Cohen and Yarom 1998; Bower and Devor 2003). Stimulation of mossy fiber afferents evoked a non-propagating activation of patches of Purkinje neurons. Orthogonal beams of Purkinje cells occur only after direct electrical stimulation of parallel fibers (Cohen and Yarom 1998). Collectively, these data support the tenet that functional modules in the cortex are organized based on the types of endings of two major afferent inputs: mossy fiber–granule cell–Purkinje cell modules are organized in patchy mosaics which are modified by parallel fiber connections, whereas climbing fiber–Purkinje cell compartments are organized parasagittally (Ebner and Chen 1995).

The quantitative data reported in this study in turtle indicates that ascending granule cell axons potentially exert a strong influence on radially overlying Purkinje cells. As there are an estimated 426 granule cell ascending axons with *en passant* swellings with an average inter-varicosity separation of 8.9 μm , if all these axons spanned the entire thickness of the molecular layer (475 μm), about 20,000 *en passant* synaptic contacts would be possible. However, as parallel fibers are laminated in the molecular layer based on their origin from granule cells in different depths of the layer, ascending axons of deeper granule cells would only ascend a short distance in the molecular layer before branching. If one considers that an average height of ascending granule cell axon would travel half the thickness of the molecular layer, then approximately 10,000 *en passant* contacts by ascending axons are possible.

The findings reported in this article provide important anatomical data for ongoing and future neuroanatomical, electrophysiological, and optical imaging studies. These data confirm that the turtle cerebellar cortex is an ideal model for quantifying integration of diverse afferent systems.

Acknowledgements

The authors thank J. Martin for his assistance with the Neurolucida reconstructions of labeled granule cell axons and climbing fibers. This study was partially supported by NINDS Grant NS-46499

References

- Adams JC. Heavy metal intensification of DAB-based HRP reaction product. *J Histochem Cytochem* 1981;29:775. [PubMed: 7252134]
- Alisky JM, Tolbert DL. Quantitative analysis of converging spinal and cuneate mossy fibre afferent projections to the rat cerebellar anterior lobe. *Neuroscience* 1997;80:373–388. [PubMed: 9284341]
- Ariel M, Fan TX. Electrophysiological evidence for a bisynaptic retinocerebellar pathway. *J Neurophysiol* 1993;69:1323–1330. [PubMed: 8492166]
- Ariel M, Lu H, Robbins KA, Bower JM, Senseman DM. Comparison of cerebellar activation by afferent and direct electrical stimulation using high-speed VSD imaging. *Soc Neurosci Abstr* 2002;26:360.2.
- Barbour B. Synaptic currents evoked in Purkinje cells by stimulating individual granule cells. *Neuron* 1993;11:759–769. [PubMed: 8398158]
- Bower JM. The organization of cerebellar cortical circuitry revisited: implications for function. *Ann N Y Acad Sci* 2002;978:135–155. [PubMed: 12582048]
- Bower JM, Devor A. Parallel fiber beams produce a strong excitatory effect on Purkinje cells along the beam only under direct electrical stimulation. *Soc Neurosci Abstr* 2003;27:75.3.
- Bower JM, Woolston DC. Congruence of spatial organization of tactile projections to granule cell and Purkinje cell layers of cerebellar hemispheres of the albino rat: vertical organization of cerebellar cortex. *J Neurophysiol* 1983;49:745–766. [PubMed: 6300353]

- Braitenberg V, Atwood RP. Morphological observations on the cerebellar cortex. *J Comp Neurol* 1958;109:1–33. [PubMed: 13563670]
- Brand S, Dahl AL, Mugnaini E. The length of parallel fibers in the cat cerebellar cortex. An experimental light and electron microscopic study. *Exp Brain Res* 1976;26:39–58. [PubMed: 61126]
- Cohen D, Yarom Y. Patches of synchronized activity in the cerebellar cortex evoked by mossy-fiber stimulation: questioning the role of parallel fibers. *Proc Natl Acad Sci USA* 1998;95:15032–15036. [PubMed: 9844010]
- Ebner TJ, Chen G. Use of voltage-sensitive dyes and optical recordings in the central nervous system. *Prog Neurobiol* 1995;46:463–506. [PubMed: 8532849]
- Fan TX, Rosenberg AF, Ariel M. Visual-response properties of units in the turtle cerebellar granular layer in vitro. *J Neurophysiol* 1993;69:1314–1322. [PubMed: 8492165]
- Gilbert PF. Theories of motor learning by the cerebellum. *Trends Neurosci* 1993;16:177–178. [PubMed: 7685939]
- Gundappa-Sulur G, De Schutter E, Bower JM. Ascending granule cell axon: an important component of cerebellar cortical circuitry. *J Comp Neurol* 1999;408:580–596. [PubMed: 10340507]
- Harvey RJ, Napper RMA. Quantitative studies of granule and Purkinje cells in the cerebellar cortex of the rat. *J Comp Neurol* 1988;274:151–157. [PubMed: 3209738]
- Harvey RJ, Napper RM. Quantitative studies on the mammalian cerebellum. *Prog Neurobiol* 1991;36:437–463. [PubMed: 1947173]
- Ito M, Kano M. Long-lasting depression of parallel fiber-Purkinje cell transmission induced by conjunctive stimulation of parallel fibers and climbing fibers in the cerebellar cortex. *Neurosci Lett* 1982;33:253–258. [PubMed: 6298664]
- Kassel J, Shambes GM, Welker W. Fractured cutaneous projections to the granule cell layer of the posterior cerebellar hemisphere of the domestic cat. *J Comp Neurol* 1984;225:458–468. [PubMed: 6725654]
- Lange, W. Regional differences in the cytoarchitecture of the cerebellar cortex. In: Palay, SL.; Chan-Palay, V., editors. *The cerebellum: new vistas*. Berlin Heidelberg New York: Springer; 1982. p. 93-105.
- Lauder JM. Effects of early hypo- and hyperthyroidism on development of rat cerebellar cortex. IV. The parallel fibers. *Brain Res* 1978;142:25–39. [PubMed: 626917]
- Llinás, R. General discussion: radial connectivity in the cerebellar cortex: a novel view regarding the functional organization of the molecular layer. In: Palay, SL.; Chan-Palay, V., editors. *The cerebellum: new vistas*. Berlin Heidelberg New York: Springer; 1982. p. 189-192.
- Llinás, R.; Sugimori, M. The cerebellum revisited. Llinás, R.; Sotelo, C., editors. Berlin Heidelberg New York: Springer; 1992. p. 167-181.
- Marr D. A theory of cerebellar cortex. *J Physiol* 1969;202:437–470. [PubMed: 5784296]
- Martin J, Kogo N, Ariel M. Morphology of basal optic tract terminals in the turtle, *Pseudemys Scripta Elegans*. *J Comp Neurol* 1998;393:267–283. [PubMed: 9548549]
- Mugnaini, E. The histology and cytology of the cerebellar cortex. In: Larsell, O.; Jansen, J., editors. *The comparative anatomy and histology of the cerebellum: the human cerebellum, cerebellar connections and cerebellar cortex*. Minneapolis: University of Minnesota Press; 1972. p. 201-262.
- Napper RM, Harvey RJ. Quantitative study of the Purkinje cell dendritic spines in the rat cerebellum. *J Comp Neurol* 1988a;274:158–167. [PubMed: 3209739]
- Napper RM, Harvey RJ. Number of parallel fiber synapses on an individual Purkinje cell in the cerebellum of the rat. *J Comp Neurol* 1988b;274:168–177. [PubMed: 3209740]
- Palay, SL.; Chan-Palay, V. *Cerebellar cortex: cytology and organization*. Berlin Heidelberg New York: Springer; 1974.
- Pichitpornchai C, Rawson JA, Rees S. Morphology of parallel fibres in the cerebellar cortex of the rat: an experimental light and electron microscopic study with biocytin. *J Comp Neurol* 1994;342:206–220. [PubMed: 8201032]
- Shambes GM, Gibson JM, Welker W. Fractured somatotopy in granule cell tactile areas of rat cerebellar hemispheres revealed by micromapping. *Brain Behav Evol* 1978;15:94–140. [PubMed: 638731]

- Shepherd GM, Raastad M. Axonal varicosity distributions along parallel fibers: a new angle on a cerebellar circuit. *Cerebellum* 2003;2:110–113. [PubMed: 12880178]
- Shepherd GM, Raastad M, Andersen P. General and variable features of varicosity spacing along unmyelinated axons in the hippocampus and cerebellum. *Proc Natl Acad Sci USA* 2002;99:6340–6345. [PubMed: 11972022]
- Sturrock RR. A comparison of quantitative histological changes in different regions of the ageing mouse cerebellum. *J Hirnforsch* 1990;31:481–486. [PubMed: 2254657]
- Sugihara I, Wu H-S, Shinoda Y. Morphology of single olivocerebellar axons labeled with biotinylated dextran amine in the rat. *J Comp Neurol* 1999;414:131–148. [PubMed: 10516588]
- Sugihara I, Wu H-S, Shinoda Y. The entire trajectories of single olivocerebellar axons in the cerebellar cortex and their contribution to cerebellar compartmentalization. *J Neurosci* 2001;21:7715–7723. [PubMed: 11567061]
- Tolbert DL, Gutting JC. Quantitative analysis of cuneo-cerebellar projections in rats: differential topography in the anterior and posterior lobes. *Neuroscience* 1997;80:359–371. [PubMed: 9284340]
- Tolbert DL, Alisky JM, Clark BR. Lower thoracic upper lumbar spinocerebellar projections in rats: a complex topography revealed in computer reconstructions of the unfolded anterior lobe. *Neuroscience* 1993;55:755–774. [PubMed: 7692349]
- Veenman CL, Reiner A, Honig MG. Biotinylated dextran amine as an anterograde tracer for single- and double-labeling studies. *J Neurosci Methods* 1992;41:239–254. [PubMed: 1381034]

Fig. 1.

Two-dimensional plots of the sampling sites used for quantitative analysis of the ascending axons, parallel fibers, climbing fibers, and granule cell/Purkinje cell ratios. The sampling sites and the quantitative data were normalized by expressing all measurements as a percentage of total distance in the medial/lateral and anterior/posterior dimensions. The midline and the anterior end of the cerebellum are represented at 0 and the lateral edge and posterior end of cerebellum is at 1

Fig. 2.

Low-power photomicrograph of a cerebellar section from a turtle with a peduncular ejection illustrating labeled Purkinje cell dendrites in the molecular layer (*ML*), the granule cell (*GCL*) layer and labeled axons in the white matter (*WM*). Labeled Purkinje cell somata are located at the junction of the molecular and granule cell layers. The *scale bar* represents 250 μm . The *inset* to the figure is a photograph of the dorsolateral aspect of the turtle cerebellum and brain stem with the peduncle indicated by an *arrow*

Fig. 3. Labeled granule cell axons ascended radially into the molecular layer grouped together (**A**, **B**). **A** illustrates four ascending axons and two ascend together in **B**. Ascending axons have numerous *en passant* swellings throughout their ascent in the molecular layer. The axon indicated by the *arrow* in **B** is reconstructed in **C** and rotated 90° to illustrate its relatively straight ascent in the molecular layer. The *scale bars* represent 25 µm in **A** and 10 µm in **B**

Fig. 4. Labeled parallel fibers followed an irregular orthogonal course in the molecular layer (**A**). **B** A photomontage of a bifurcating ascending axon and its parallel fiber branches. The number and spatial distribution of *en passant* swellings varied considerably along different parallel fibers (compare **A** with **B**). The labeled axon in **B** was reconstructed and rotated in **C** to illustrate that, while parallel fibers may wander into the superficial to deep plane of the molecular layer, their course laterally is restricted to a narrow orthogonal plane. The *scale bars* represent 25 μm in both **A** and **B**

GRANULE CELL AXONS

Fig. 5.

The intervaricosity distances between adjacent *en passant* swellings and swelling diameters on ascending granule cell axons and parallel fibers plotted by their medial/lateral and anterior/posterior locations. There were not significant differences in these data within a group or between groups with respect to cerebellar position

Fig. 6. Climbing fibers were labeled from biotinylated dextran amine ejection sites in the cerebellar peduncle. Climbing fibers were restricted in their terminal distribution to the inner most one-fourth of the molecular layer (see Fig. 7). **A** is a photomontage of a climbing fiber with several *en passant* swellings. **B** is a bifurcated climbing fiber with its branches following the labeled secondary dendrites of a Purkinje cell. The climbing fiber in **B** is reconstructed and rotated in **C** to illustrate the relative paucity of *en passant* swellings and planar orientation of the axonal branches corresponding to the orientation of Purkinje cell dendrites. The *scale bars* represent 25 μm in both **A** and **B**

CLIMBING FIBERS

Fig. 7.

Different quantitative data on climbing fibers. There was a trend for the axonal diameters to increase in size in lateral and posterior areas of the cortex compared with anterior medially. The intervaricosity distance between adjacent *en passant* swellings did not vary by location. Climbing fibers that extended into more superficial parts of the molecular layer were located in the lateral parts of the cortex. The spatial distribution of climbing fiber arbors, as reflected by the area occupied by branches, increased progressively in posterior and lateral parts of the cortex.

Granule Cell / Purkinje Cell Ratios

Fig. 8.

The area of basophilic stained nuclei beneath the soma of individual Purkinje cells was used to calculate granule cell/Purkinje cell ratios at different anterior/posterior and medial/lateral locations. The numerical relationship between granule and Purkinje cells did not vary by location with an average of 426 granule cells/Purkinje neuron. The actual number of granule cells/Purkinje cells is probably overrepresented for reasons detailed in the discussion