Ascending Granule Cell Axon: An Important Component of Cerebellar Cortical Circuitry

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ABSTRACT

Physiologic evidence suggests that local activation of the cerebellar granule cell layer produces a much more restricted spatial activation of overlying Purkinje cells than would be expected from the parallel fiber system. These results have led to the suggestion that synapses associated with the ascending granule cell axon may provide a large, direct, excitatory input to Purkinje cells, whereas parallel fiber synapses may be more modulatory in nature. In the current experiments, serial electron microscopy was used to reconstruct synapses associated with these two segments of the granule cell axons in the cerebellar cortex of albino rats. The results indicate that there are significantly more presynaptic vesicles in ascending segment synapses than in parallel fiber synapses. Furthermore, a first-order linear regression analysis revealed positive correlations between all measures of pre- and postsynaptic morphology for parallel fibers, but not for ascending segment synapses. Perhaps most surprisingly, serial reconstructions of postsynaptic spines and their associated dendrites demonstrated that spines contacted by ascending segment synapses are located exclusively on the smallest diameter distal regions of the Purkinje cell dendrites, whereas parallel fiber synapses are found exclusively on intermediate- and large-diameter regions of the spiny branchlets. Based on two independent calculations, we estimate that 20% of the granule cell synapses onto a Purkinje cell are actually made by the ascending segment. By using computer simulations of a single Purkinje cell dendrite, we have also demonstrated that synchronous activation of these distal ascending segment inputs could produce a substantial somatic response. Taken together, these results suggest that the two different regions of granule cell axons may play very different physiologic roles in cerebellar cortex. J. Comp. Neurol. 408:580–596, 1999.
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Indexing terms: cerebellum; Purkinje cells; synapses; electron microscopy; parallel fibers

The cerebellar cortex is often described as the best anatomically and physiologically understood circuit in the mammalian central nervous system. Starting with the work of Ramon y Cajal (1911) at the turn of the century, its nearly crystalline structure has allowed highly detailed descriptions of cerebellar network architecture to be obtained (Palkovits et al., 1971a–c, 1972; Mugnaini, 1972; Palay and Chan-Palay, 1974; Schild, 1980; Bloedel and Courville, 1981; Lainè and Axelrad, 1994; Mugnairi and Floris, 1994). The resulting description of basic cerebellar anatomy and physiology has had a strong influence on theories and models of cerebellar function (Eccles et al., 1967; Marr, 1969; Albus, 1971; Fujita, 1982; Szenthagothai, 1983; Ito, 1984; Kawato and Gomi, 1992; Killeen and Fetterman, 1993). In turn, several of these theories, such as the Marr/Albus model of cerebellar plasticity and motor control (Marr, 1969; Albus, 1971), have strongly influenced modern physiologic, anatomic, and behavioral experiments (Thompson, 1988; Ito, 1989, 1996; Karachot et al., 1994; De Schutter and Maex, 1996).

The feature of cerebellar circuitry that has most influenced speculations on cerebellar function is the stereo-

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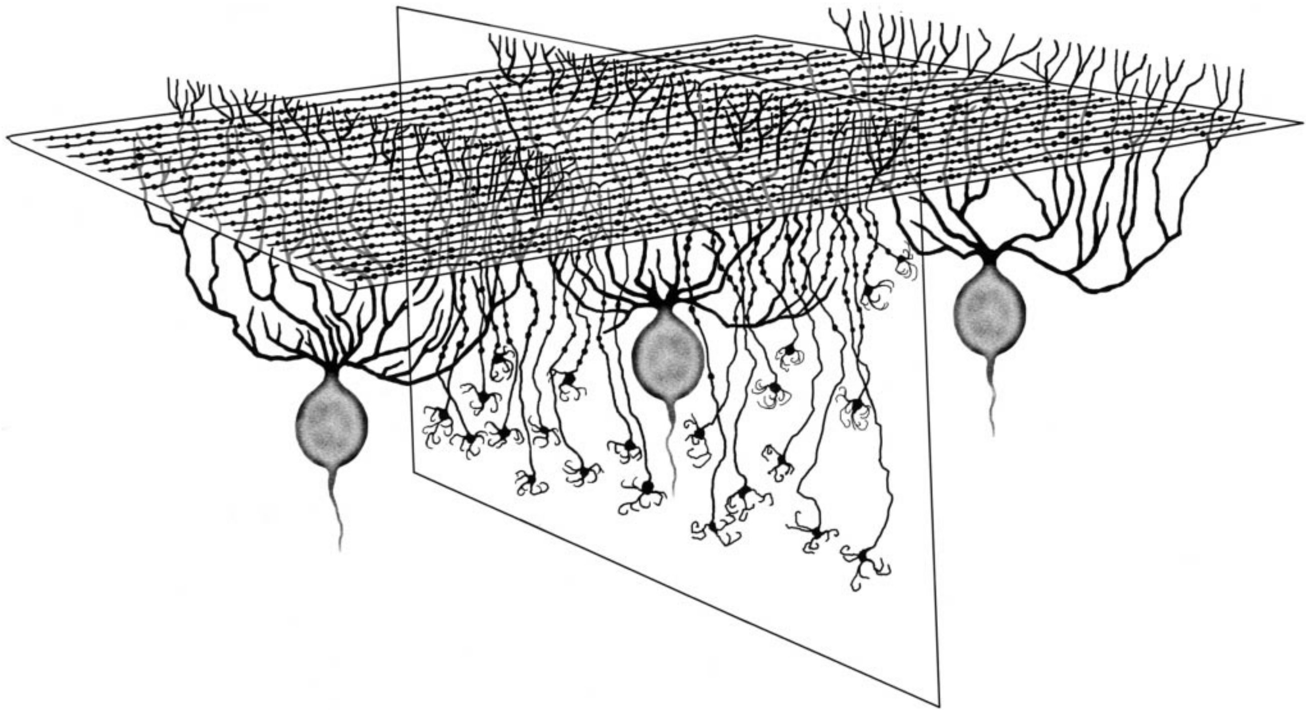

Fig. 1. This schematic drawing shows the two planes of section used to distinguish between ascending and parallel fiber synapses. The horizontal plane of section was used to characterize synapses associated with parallel fibers, whereas the parasagittal plane of section was used to characterize synapses associated with the ascending segment. Although only one horizontal plane is shown, data were obtained from two planes: one high in the molecular layer and one low, as described in Materials and Methods.

typed synaptic relation between the parallel fiber axons of the granule cells and the dendrites of the Purkinje cells (Braitenberg and Atwood, 1958; Eccles et al., 1967; Marr, 1969; Albus, 1971; Sabah, 1971; Eccles, 1973; Mittenthal, 1974; Gilbert, 1975; Fujita, 1982; Ito, 1984; Lisberger, 1988; Kawato and Gomi, 1992; Killeen and Fetterman, 1993; Keifer and Houk, 1994; Heck, 1995). Very large numbers of parallel fibers run through the molecular layer at right angles to the isoplanar Purkinje cells (Fig. 1), where, collectively, they make up to 150,000 synaptic contacts on each Purkinje cell in the rat cerebellum (Harvey and Napper, 1991). It was demonstrated many years ago that direct molecular layer stimulation of these parallel fibers results in a sequentially activated "beam" of cerebellar Purkinje cells (Eccles et al., 1966). The resulting "beam hypothesis" (Braitenberg and Atwood, 1958; Eccles et al., 1967) has been a dominant component of many, if not most, theories of cerebellar cortical function (for recent examples, see Gabbiani et al., 1994; Braitenberg et al., 1997), including the influential Marr/Albus hypothesis (Albus, 1971; Marr, 1969).

Although the physiologic basis for the beam hypothesis is based on electrical stimulation experiments (Eccles et al., 1966), it was a natural extension of this hypothesis to assume that any focal activation of the granule cell layer also would set up a "beam" of activated Purkinje cells up and down the folium (Eccles et al., 1967). However, the few Purkinje cell recording experiments that have been conducted using natural peripheral stimuli have failed to demonstrate beams of activation (Eccles et al., 1972; Bower and Woolston, 1983; Bell and Grimm, 1969). Instead, peripheral stimuli produce "clumps" or "patches" of activated Purkinje cells (Eccles et al., 1972). Although previous authors have assumed that the clumps and patches were a result of a complex interaction of parallel fiber beams (Eccles et al., 1972), experiments we conducted a number of years ago (Bower and Woolston, 1983) suggested that this patchy pattern of Purkinje cell response directly reflected the patchy pattern of tactile peripheral inputs to the granule cell layer (Shambes et al., 1978; Welker, 1987; Bower and Kassel, 1990). In other words, a comparison between the spatial extent of granule cell layer and Purkinje cell responses to tactile stimuli suggested that there was a previously unsuspected vertical influence of granule cells on Purkinje cells (Bower and Woolston, 1983). More recent in vivo and in vitro intracellular experiments in our laboratory have confirmed that granule cells do have a profound physiologic influence on immediately overlying Purkinje cells (Jaeger and Bower, 1994).

Shortly after our original description of the vertical organization of cerebellar cortex (Bower et al., 1980), Llinas (1982) proposed that the restricted activation of Purkinje cells might reflect synapses made by granule cell axons as they ascend into the molecular layer past the Purkinje cell dendrite. Although Mugnaini (1972) previously described these synapses anatomically, several subsequent anatomic studies have questioned their importance (Napper and Harvey, 1988a,b). To date, the presence of synapses on the ascending segments of the granule cell axon is not included in most descriptions of cerebellar circuitry (Ito, 1984; Kandel et al., 1991), nor is it taken into account in theories of cerebellar function (c.f. Gabbiani et al., 1994; Braitenberg et al., 1997). Instead, synapses

associated with the granule cell parallel fibers are still considered to provide the primary excitatory drive on Purkinje cells.

In the experiments described here, we used serial electron microscopic reconstruction techniques to directly compare the ultramorphology of ascending and parallel fiber segment synapses in the cerebellar cortex of the albino rat. We examined the structure of the pre- and postsynaptic components of both types of synapses as well as the regions of Purkinje cell dendrites they contact. The results reveal clear differences in the ultramorphology of these two types of synapses as well as differences in the locations of their synaptic terminations on the Purkinje cell dendrite. The data suggest that the ascending segment synapses constitute a much larger fraction of granule cell input to Purkinje cells than was thought previously (cf. Napper and Harvey, 1988a,b). When these results are incorporated into a computer simulation of the cerebellar Purkinje cell (De Schutter and Bower, 1994a), simulations of ascending segment synaptic activation produce Purkinje cell responses very similar to our previous physiologic results with tactile peripheral stimuli (Bower and Woolston, 1983; De Schutter and Bower, 1994c). Taken together, these results suggest that the ascending and parallel fiber segments of granule cell axons subserve distinctly different physiologic functions within cerebellar cortical circuitry and that the ascending segment synapses constitute an important feature of cerebellar cortical circuitry. These results were published previously in abstract form (Gundappa-Sulur and Bower, 1990).

MATERIALS AND METHODS

Electron microscopy procedures

Animal preparation. Two adult female Sprague-Dawley rats were deeply anesthetized with ketamine hydrochloride (50 mg/kg) and sodium pentobarbital (12 mg/kg) and then perfused transcardially with 0.1 M phosphate buffer at physiological pH, followed by 2% paraformaldehyde, 2% glutaraldehyde, and 2 mM CaCl in 0.1 cacodylate buffer, pH 7.35. The brains were left undisturbed in the cranium for 1 hour, and then the cerebellum was removed. The intact cerebellum was postfixed for 2 hours in the same aldehyde mixture followed by an overnight soak in buffer. All research reported here was carried out in accordance with the guidelines for animal use established by the National Institutes of Health and with approval of the Caltech animal use committee.

Tissue sectioning. The tissue staining procedures used were generally similar to those used previously by Harris and colleagues to study the dendritic spines and synapses of cerebellar Purkinje cells (Harris and Stevens, 1988). Prior to sectioning of the tissue, several small areas of the cerebellar vermis were removed from the fixed brain and cut either sagittally or horizontally, immersed for 1 hour in 1% OsO_4, and then rinsed in cacodylate and acetate buffers (these two different sectioning angles, as described in more detail below, aided in distinguishing between synapses associated with the parallel fiber and ascending segments of the granule cell axon). After these rinses and dehydration through graded alcohols and propylene oxide, the tissue was embedded in Epon. Serial sections were then cut from these thin sections to be between pale gold and silver on an LKB ultramicrotome (LKB Ultrascan XL, Bromma, Sweden), following the same sagittal or horizontal planes of section described above. These interference colors are consistent with an average section thickness of 0.07 µm.

Serial reconstructions. Stained and mounted tissue was viewed by using a Phillips 201 electron microscope (Phillips, Mahwah, NJ). All sets of serial sections were mounted on Formar-coated slot grids (Ted Pella Inc., Redding, CA.) and stained for 1 minute with 1% uranyl acetate followed by 30 seconds in lead citrate. Each grid of each series was mounted in a grid cassette (Stevens and Trogadis, 1984) and stored in a numbered gelatin capsule. The series identification numbers reflected the sequential number assigned to each series. A mark was placed on each grid to serve as a reference with which to align particular sections at each use. Photomicrographic negatives were exposed for each section in each series. The photomicrographs used for the figures were obtained by scanning positive photographic prints with a 600 × 1,200 dots per inch (DPI) resolution scanner. Slight retouching of the digital images was performed when needed for the sole purpose of removing imperfections resulting from the photographic process.

Analysis procedures

Distinguishing ascending and parallel fiber synapses. Synapses made by the ascending and parallel fiber segments of the granule cell axon were distinguished by making use of the well-known geometry of the granule cell axon in the cerebellar cortex (Palay and Chan-Palay, 1974). Specifically, parallel fiber synapses were identified in brain tissue cut in a horizontal plane of section, whereas ascending segment synapses were identified in sections cut with a sagittal orientation (see Fig. 1). In horizontal sections, all granule cell axons running in the plane of the section are associated with the parallel fibers. On the other hand, granule cell axons running in the plane of sagittal sections are associated with the ascending segment.

Synaptic ultramorphology. The ultramorphology of each type of synapse was quantified by tracing the outlines of synaptic features through successive serial sections with computer-aided reconstruction software of our own design. Structures quantified in each section included the pre- and postsynaptic terminal regions, the location of postsynaptic densities, and the position and numbers of presynaptic vesicles. Pre- and postsynaptic regions were included in the measures of area and volume if the section being scored contained presynaptic vesicles. The outlines of terminal regions, the locations of individual presynaptic vesicles, and the location of the postsynaptic density were digitized for each section by using electron microscopic photomicrographs and a custom-mounted Science Accessories Corp. (Norwalk, CT) GP-8 sonic digitizer connected to a Silicon Graphics work station (Iris, Mountainview, CA). The total number of presynaptic vesicles, the volume of presynaptic and postsynaptic regions, and the area of the postsynaptic densities were determined for each synapse by using custom-designed software. The volumes of the pre- and postsynaptic terminal regions were calculated by using procedures similar to those described by Harris and Stevens (1988).

Dendritic origins of synaptic spines. The diameter of the Purkinje cell dendrite of origin for spines contacting either the ascending segment (from sagittal sections) or the parallel fiber (from horizontal sections) segments of the granule cell axon was determined by following the contacting spine through consecutive serial sections to the

parent dendrite. This dendrite was then reconstructed from serial sections until a round dendritic profile could be reconstructed. This procedure was necessary to ensure that the true dendritic diameter was determined independent of the orientation of the dendrite with respect to the plane of section. Cross-sectional areas were measured directly, omitting any contribution by the spine necks. The measured dendrite cross sections were then converted into diameters under the simplifying assumption that the cross section was perfectly circular. All diameters were increased by 10% to compensate for tissue shrinkage (Rapp et al., 1994).

Distributions of different dendritic diameters. In addition to the serial reconstructions, a series of experiments was also conducted to determine the orientation and distribution of Purkinje cell dendrites of different cross-sectional areas in different section planes and depths within the molecular layer. To collect these data, 250 dendritic cross sections were sampled randomly in sections cut in both the sagittal and the horizontal planes. In horizontal sections, data were collected for both superficial (50 μm below the surface) and deep (200 μm below the surface) regions of the molecular layer. In all cases, round dendritic profiles were digitally traced and used to calculate the distribution of dendritic diameters. Again, all final diameters were adjusted by 10% to compensate for tissue shrinkage (Rapp et al., 1994).

Purkinje cell simulations

To determine the possible physiologic significance of the results presented here, we performed several computer simulations by using a previously described Purkinje cell compartmental model (De Schutter and Bower, 1994a). Space does not permit a complete description of this model, but full details can be found in the referenced publications (De Schutter and Bower, 1994a–c; Jaeger and Bower, 1997). For the results described here, the only change in published model parameters involved modifying the distribution of synaptic inputs over the dendrite in a way consistent with the results of the current study. Specifically, modeled parallel fiber synapses were made to contact the 909 compartments with a diameter between 1.3 μm and 3.2 μm, whereas modeled ascending segment synapses contacted the 563 compartments with diameters smaller than 1.3 μm (see Results). Similar to previous simulations (De Schutter and Bower, 1994b,c), the primary synaptic activation pattern of the parallel fiber system was asynchronous at a mean frequency of 46 Hz. Also similar to previous simulations, asynchronous inhibition representing stellate cell firing was provided at 1.0 Hz (De Schutter and Bower, 1994b,c). Together, these asynchronous excitatory and inhibitory inputs resulted in a background, irregular Purkinje cell firing frequency of 78.6 Hz ± 4.5 Hz. To assess the physiologic consequences of the distal dendritic location of ascending segment synaptic contacts (see Results), we contrasted the response of the model to synchronous input with more distal and proximal dendrites. The model has suggested previously that the Purkinje cell dendrite specifically amplifies such synchronous inputs (De Schutter and Bower, 1994c). The response of the Purkinje cell model, as found previously, was quantified as the number of spikes in the 10-msec interval following the synchronous input. All simulations were performed with GENESIS software (Bower and Beeman, 1995).

RESULTS

Electron microscopy

The organization of granule cell axons. The axon of the granule cell axon emerges from the cell body in the granule cell layer and then courses vertically through the molecular layer until it bifurcates into its parallel fiber segment (Palay and Chan-Palay, 1974). In the molecular layer, multiple ascending segments often course together in axonal bundles (Mugnaini, 1972; Palay and Chan-Palay, 1974). Parallel fibers course in a plane orthogonal to the ascending granule cell axons. The regular vertical course of the ascending segment axons made them relatively easy to identify and track in prepared tissue.

Differences in synaptic ultrastructure. Figure 2 compares single electron photomicrographic images of typical synaptic contacts made by the ascending segments (Fig. 2A) and the parallel fibers (Fig. 2B). The image in Figure 2A, as described in Materials and Methods, was taken from tissue cut in the sagittal plane, whereas that in Figure 2B was taken from a horizontal plane of section. Note the overall similarity in appearance between these two types of synapses, except for the larger number of vesicles associated with the ascending segment. This comparison is quantified in Figure 3 based on pooled measurements of the presynaptic volumes, the number of presynaptic vesicles, the postsynaptic volumes, and the area of the postsynaptic densities for 20 serially reconstructed synapses of each type. The data show that, in fact, the only statistically significant difference between the pooled synaptic values for these synapses was in the larger number of presynaptic vesicles associated with the ascending segment (standard t test; $P < 0.001$). Although it was harder to quantify, the only other ultramorphologic difference we noted in this tissue was a tendency for the presynaptic vesicles of the ascending segment to stain more darkly than those of the parallel fiber system.

Differences in correlation between synaptic components. Beyond comparing the mean values of pre- and postsynaptic components (Fig. 3), we were also interested in examining correlations between these values in individual synapses. Table 1 shows the results of a linear regression analysis made between all measured values of synaptic structure. The data demonstrate that there are positive correlations in all measures for synapses associated with the parallel fibers, with r^2 values ranging from 0.294 to 0.536. In contrast, the r^2 value for the ascending segment synapses are below 0.01, except for a relatively weak value of 0.115 obtained when comparing the presynaptic volume with the number of presynaptic vesicles. Figure 4A,B shows that this same comparison resulted in the largest r^2 value (0.536) for the parallel fiber synapses. Figure 4C,D illustrates the correlation between the area of the postsynaptic density and the number of presynaptic vesicles in each synapse. This correlation, as discussed below, may be particularly important to synaptic function, because the number of presynaptic vesicles presumably reflects available transmitter for release (Murthy et al., 1997), and the area of the postsynaptic density reflects the location of postsynaptic transmitter receptors (Korn and Faber, 1991). For this comparison, the parallel fiber data showed a highly significant correlation ($r^2 = 0.481$), whereas there was no correlation whatsoever for the ascending segment synapses ($r^2 = 0.000$).

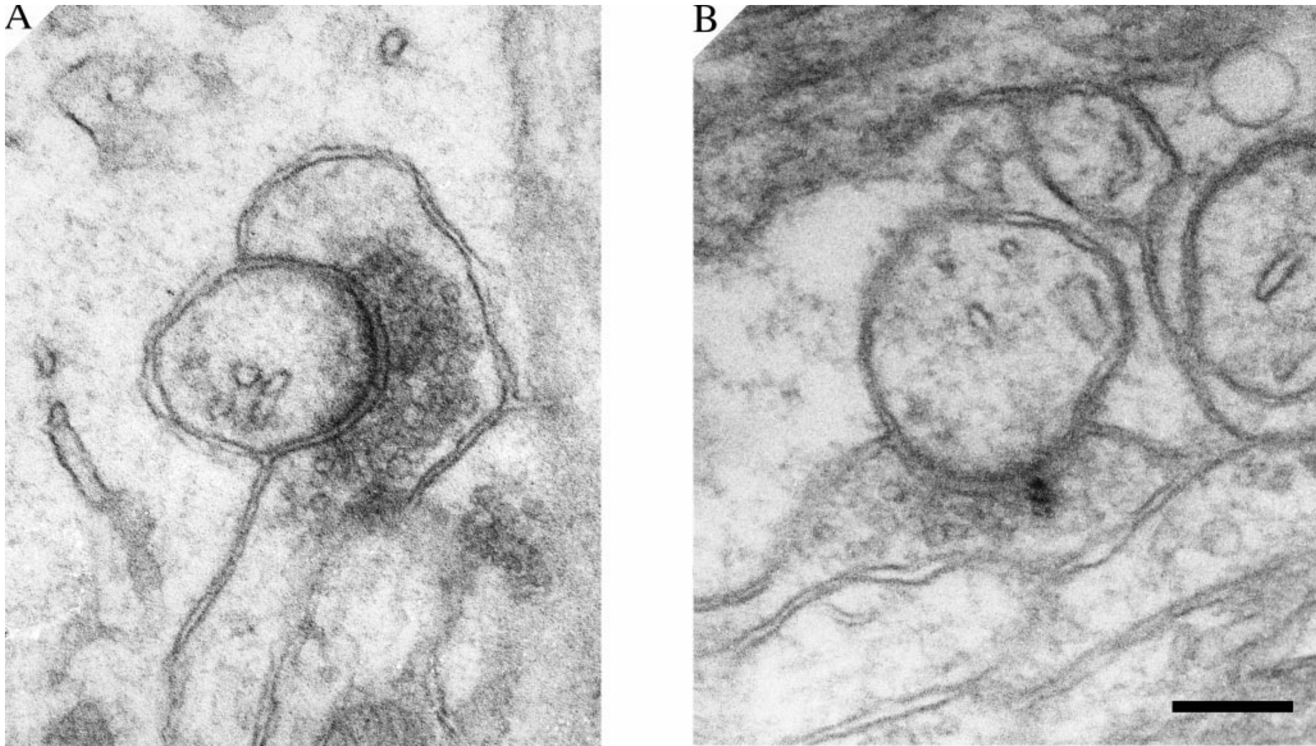

Fig. 2. High-power electron photomicrographs of two Purkinje cell spines characteristic of those associated with synapses of either the ascending segment or the parallel fiber segments of the granule cell axon. **A:** Image from a sagittal section showing a Purkinje cell dendrite in cross section and a synapse associated with an ascending segment axon. **B:** Image from a horizontal section showing a synapse associated with a parallel fiber segment axon. Serial reconstruction, as described in Materials and Methods, confirmed that the axons associated with the indicated synapses were coursing in each plane of section. Note the larger number of presynaptic vesicles associated with the ascending segment synapse. Scale bar = 0.5 μm.

Dendritic origins of spines contacting ascending and parallel fiber synapses. In these studies, we examined the dendritic sites of termination for the two segments of the granule cell axons on Purkinje cells. To do this, serial sections were used to trace contacted postsynaptic spines back to their parent dendrites. The cross-sectional areas of these dendrites were then estimated, as described in Materials and Methods. The results were compiled from 32 ascending segment synaptic contacts and from 60 parallel fiber synapses. To ensure that the horizontal plane of section did not bias the parallel fiber data, 30 synapses were analyzed from sections obtained from the first 50 μm of the surface of the molecular layer, and 30 synapses were analyzed from sections obtained at depths below 150 μm. Ascending segment synapses were sampled throughout the vertical extent of the molecular layer.

Figure 5 presents two electron photomicrographs taken from this analysis. Figure 5A shows two ascending segment synapses that were made on spines associated with a single Purkinje cell dendrite. Figure 5B shows the same arrangement between two parallel fibers and a different Purkinje cell dendrite. Keeping in mind that the scale of the photomicrograph of the ascending segment synapses (Fig. 5A) is twice that for the parallel fibers (Fig. 5B), it is clear that these synapses are made on dendritic shafts of very different sizes. The cross-sectional areas of the dendrites associated with all of the reconstructed synapses are shown in Figure 6. This graph indicates that there is a clear difference in the location of termination of these two types of synapses. More specifically, ascending segments were found to terminate only on Purkinje cell dendrites <1.3 μm in diameter (<1.2 μm^2 cross-sectional area), whereas parallel fiber synapses were found only on dendrites of 1.5–5.0 μm diameter (>1.2 μm^2 cross-sectional area). The figure also shows that no molecular layer depth-related differences were seen between dendrites associated with either type of synapse. In our data, there is no evidence for any overlap between these two termination zones. The dendritic profiles shown in the electron photomicrographs of Figure 5 are close to the mean for the data presented in Figure 6.

Depth distributions of dendrites with small diameters. The result that ascending and parallel segments terminate on different regions of the Purkinje cell dendrite was surprising and unexpected. Although these data were obtained from serial analysis of a relatively large number of synapses ($n = 90$), we felt it prudent to test this result by using a second, independent measure. Given the known geometry of granule cell axons, if ascending segment synapses terminate exclusively on Purkinje cell dendrites <1.3 μm in diameter, then one would expect Purkinje cell dendrites in deeper regions of the molecular layer to have a larger percentage of these very small, spiny branchlets than dendrites in superficial layers. This prediction is based on the assumptions that the ascending segments bifurcate into parallel fibers in roughly equal proportion at all horizontal levels of the molecular layer (Palay and Chan-Palay, 1974) and that ascending segment axons

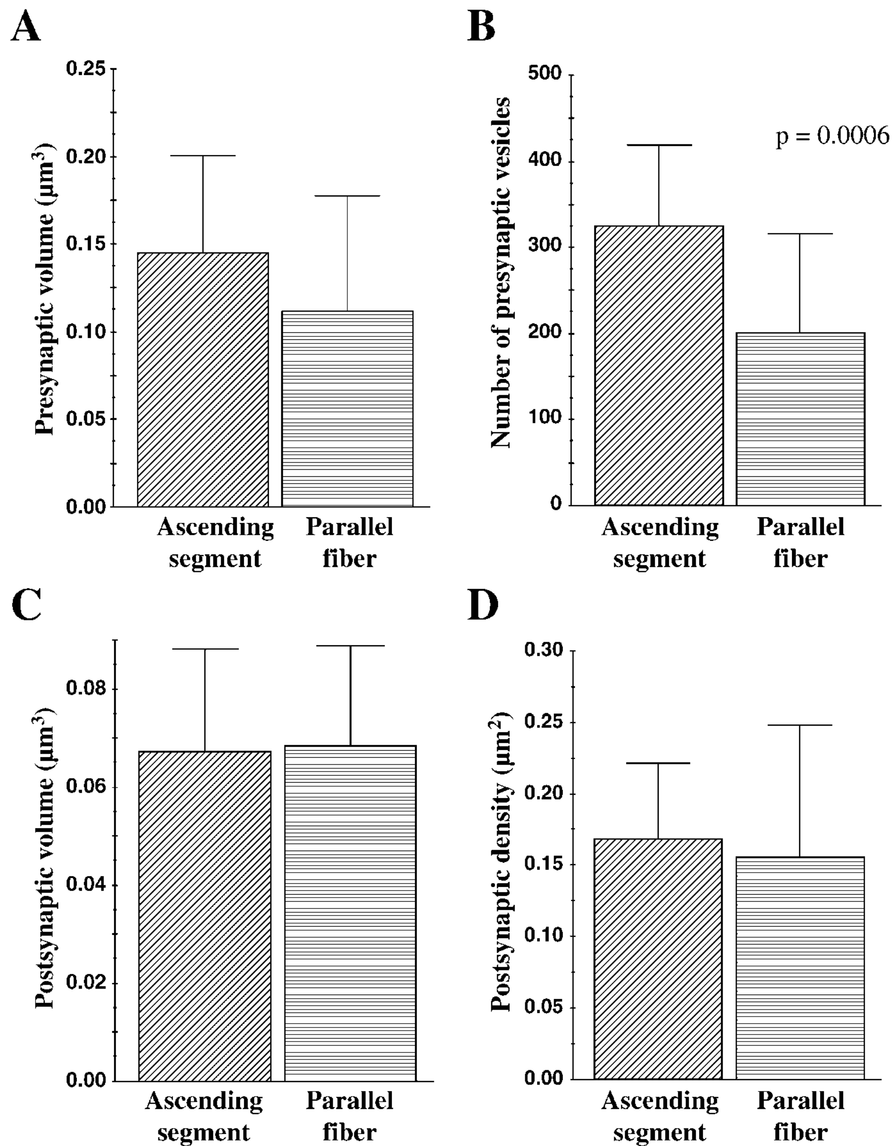

Fig. 3. Quantitative comparison of the ultrastructural features of ascending segment and parallel fiber synapses based on serial reconstructions of entire synapses. This figure compares presynaptic volumes (**A**), the number of presynaptic vesicles (**B**), the postsynaptic volumes (**C**), and the area of the postsynaptic densities (**D**) for each type of synapse. The only significant difference between these measures (as indicated in B) was in the number of presynaptic vesicles (t-test; $P = 0.0006$). All other measures were statistically identical.

TABLE 1. Regression Analysis of Synaptic Morphology[1]

Correlation	r^2, Ascending segment	r^2, Parallel fibers
Presynaptic volume, number of presynaptic vesicles	0.115	0.536
Presynaptic volume, postsynaptic volume	0.000	0.314
Presynaptic volume, postsynaptic density	0.006	0.423
Postsynaptic volume, number of presynaptic vesicles	0.001	0.411
Postsynaptic volume, postsynaptic density	0.000	0.294
Postsynaptic density, number of presynaptic vesicles	0.000	0.481

[1]Linear regression analysis made between the pre- and postsynaptic volumes, the area of the postsynaptic densities, and the number of presynaptic vesicles for ascending and parallel fiber synapses.

make an equal number of synapses along their entire vertical length prior to branching (Pichitpornchai et al., 1994). If both of these assumptions are correct, then there should be many more ascending segment synapses and, thus, more Purkinje cell dendrites <1.3 μm in diameter in deeper than superficial regions of the molecular layer.

To test this conjecture, we measured cross-sectional areas for a large number of randomly selected, round-profile Purkinje cell dendrites taken at different depths of the molecular layer (see Materials and Methods). Only round dendritic profiles were used in this comparison on the assumption that round profiles are more likely to have been sectioned perpendicular to their long axis. Figure 7A directly compares the distribution of round dendritic profiles at depths of 50 μm and 200 μm below the pia. Figure 7B,C shows the same data in the form of pie charts for easier comparison of the percentage of dendritic sizes at each depth. All three graphs clearly demonstrate that there are substantially larger numbers of dendrites <1.3

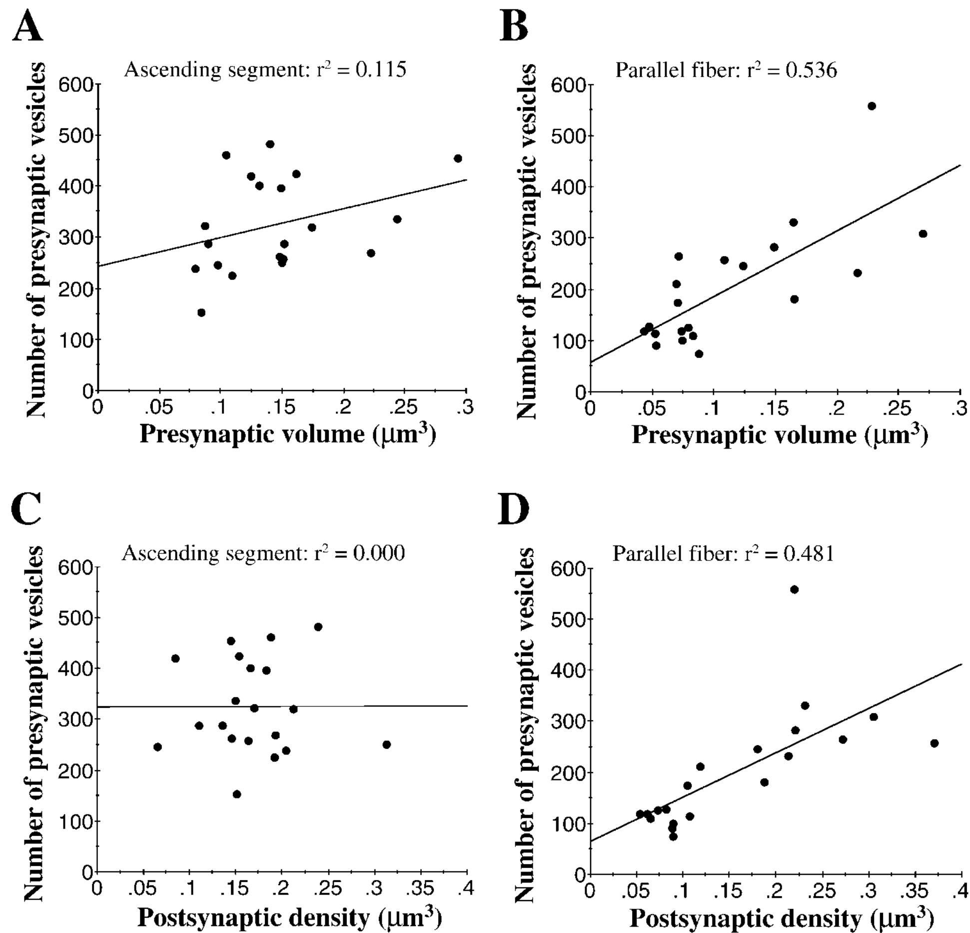

Fig. 4. Two sets of regression comparisons between ultrastructural features of ascending segment and parallel fiber synapses. **A,B:** Comparison of presynaptic volume and the number of presynaptic vesicles for ascending and parallel fiber synapses, respectively. **C,D:** Comparison of the area of the postsynaptic density with the number of presynaptic vesicles in each synapse. Full analysis of the data for all combinations of comparisons is shown in Table 1.

µm in cross-sectional area at deeper levels of the molecular layer. In our random sample, dendrites in this size range constituted 27% of the dendrites 200 µm below the surface and only 8% in the upper region. The reader should note that these differences are matched inversely to the percentage of dendrites of intermediate size, which are more prevalent in the higher regions of the molecular layer. It should also be noted that this result is the opposite of what would be expected in most neurons, in which dendritic size usually decreases with increased distance from the soma. In the case of the Purkinje cell, the small-diameter dendrites at the base of the molecular layer arise from those secondary dendrites that first branch off of the main dendritic stem. Thus, the actual distance along the dendrite from the soma to these small, deep dendrites is rather short. The important point here, however, is that these results are completely consistent with our serial electron microscopic reconstructions showing that ascending segments synapse on the smallest diameter dendrites.

Orientation of small-diameter dendrites. Finally, as a second independent check of the serial reconstruction data, we were interested in determining whether the smallest diameter Purkinje cell dendrites might have a particular spatial orientation within the molecular layer. Specifically, consistent with previous reports (Mugnaini, 1972), we have found that ascending segment axons pursue an essentially straight vertical course through the molecular layer, usually in bundles of several axons. Given the isoplanar orientation of the Purkinje cell dendrite and the density of dendritic branches within this plane (Palay and Chan-Palay, 1974), it seemed reasonable to predict that the small-diameter dendrites contacting the ascending axons might extend horizontally outward, toward the ascending segment axons. Comparing the number of round (i.e., perpendicular), small Purkinje cell dendritic profiles in sagittal sections with those found in horizontal sections tested this prediction. Figure 8 shows that 56% of the round dendritic profiles traced in sagittal sections were <1.3 µm in diameter. In contrast, only 27% of the profiles found in horizontal sections were this small. These results suggest that the smallest diameter dendrites tend to be oriented perpendicular to the sagittal plane of the Purkinje cell dendrite and into the plane of the ascending granule cell axon bundles.

Comparison of electron and light microscopic diameter distributions. The results described above suggest that the restriction of ascending segment synapses to the smallest diameter Purkinje cell dendrites results in an

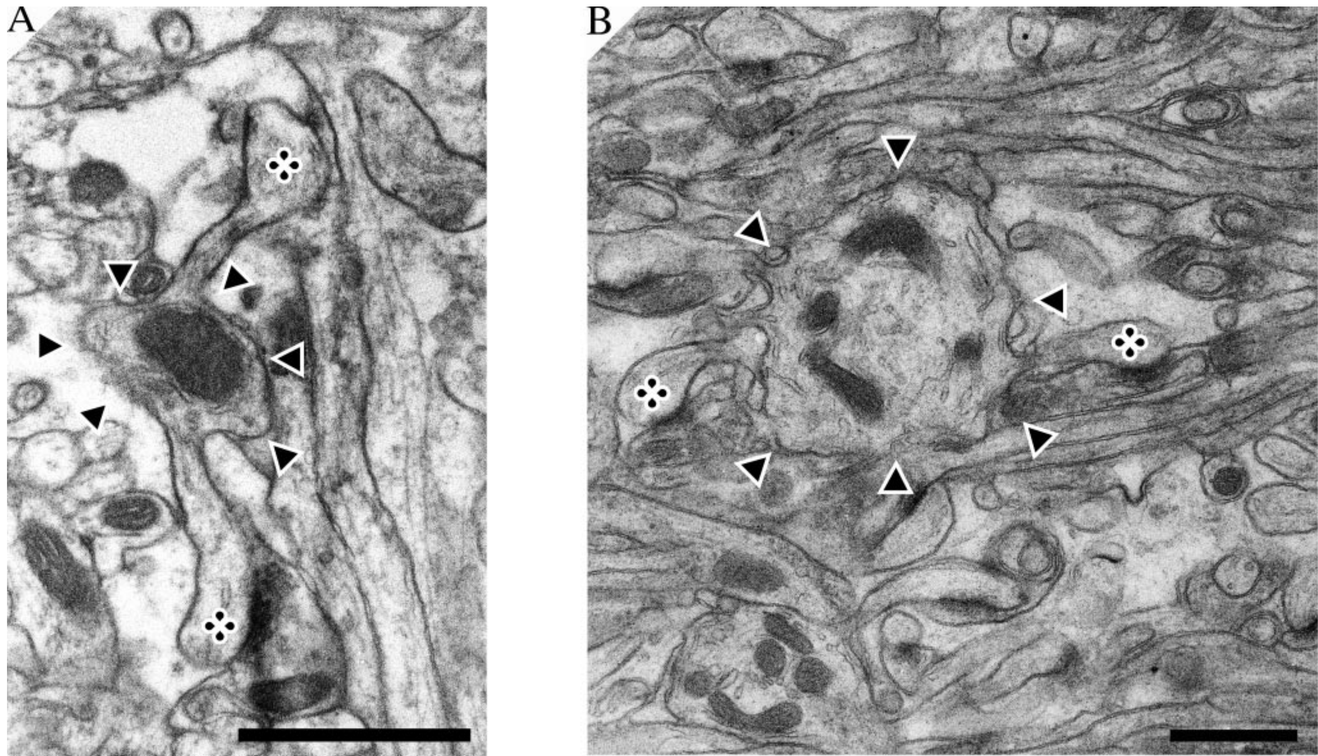

Fig. 5. Direct comparison of two electron photomicrographs of synapses made by ascending (**A**) and parallel fiber (**B**) segments of granule cell axons. Arrowheads highlight the Purkinje cell dendrite in each case. Asterisks indicate the spines associated with each synapse. Note the characteristic appearance of mitochondria in the dendrite of the Purkinje cell as well as the similar shape of the Purkinje cell spines in each case. The scale bar in each figure corresponds to a length of 1.2 μm, indicating an almost twofold difference in magnification between the two photographs. Taking this scale difference into account, the size of the parent dendrite contacting the ascending segment is much smaller. Also note the appearance of several other ascending segment axons in a "bundle" with the axon whose synapse is highlighted. Scale bars = 1.2 μm.

increase in small-diameter dendrites in the deeper regions of the molecular layer (Fig. 7). However, this conclusion is based on a random sample of specifically round dendritic profiles in our electron microscopic material. If this conclusion is correct, then this differential distribution also should be reflected in morphology of single Purkinje cell dendrites. To determine whether this is the case, we calculated the distribution of dendritic diameters in previously published (Rapp et al., 1994) light microscopic reconstructions of guinea pig Purkinje cells (data kindly provided by M. Rapp, Y. Yarom, and I. Segev). Specifically, we divided the reconstructed Purkinje cell dendrites into upper and lower halves and then calculated the frequency of dendritic diameters as the ratio of the sum of the lengths of all segments with a particular diameter divided by the sum of the lengths of all segments. The results of this analysis are shown in Figure 9A. Like the electron microscopic data (Fig. 7), we found many more small-diameter dendrites in the lower half of the dendrite than in the upper half. A second comparison, which is shown in Figure 9B, indicates that, overall, the dendritic diameters of the light data closely match those in the electron microscopic data. The frequency distributions from both samples were statistically similar (paired t test; $P < 0.10$). Thus, the results of the electron microscopic analysis are in complete agreement with a completely independent data set obtained from light microscopic reconstruction in a separate laboratory.

Simulation of Purkinje cell responses to ascending segment inputs

Finally, as described in Materials and Methods, we used realistic computer simulations to begin to explore the functional consequences of the distal termination sites of the ascending segment synapses. These simulations are based on a realistic single-cell model we used previously used to study Purkinje cell responses to granule cell inputs (De Schutter and Bower, 1994b,c). One unexpected result of these initial simulations was that even a relatively small number of synchronously activated granule cell inputs results in a somatic action potential due to a calcium-dependent amplification mechanism (De Schutter and Bower, 1994c). We previously proposed that the ascending granule cell axon is the most likely source of this synchronous activation of granule cell synapses (Jaeger and Bower, 1994; Bower, 1997a–c) and that this synchronous activation might account for the vertical organization of cerebellar cortex (Bower and Woolston, 1983). However, in models of other neuronal cell types, the passive properties of a neuron can reduce substantially the effectiveness of synaptic inputs on small-diameter distal dendrites (Rall, 1964; Jack et al., 1975). Although Purkinje cell dendrites are clearly active, we were still surprised to find the ascending segment synapses on the distal most regions of the Purkinje cell dendritic tree, given the apparent potency of their inputs (Jaeger and Bower, 1994).

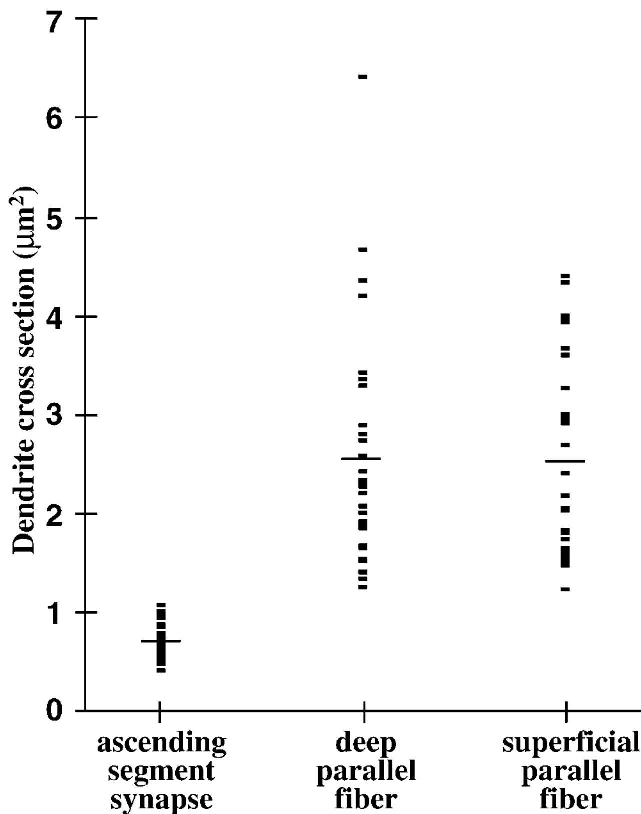

Fig. 6. Graph showing the diameters of the parent dendrites for spines associated with ascending segment and parallel fiber synapses. For the parallel fibers, the analysis was performed at two different depths of the molecular layer. Thirty measurements were made in horizontal sections 50 μm below the pial surface, and 30 measurements were made at a depth of 150 μm. The 32 measurements for the ascending segment synapses were made throughout the molecular layer. The horizontal bar in each data set indicates the mean value of dendritic cross section in each case.

We used our Purkinje cell model to specifically compare somatic responses to small synchronous inputs on larger diameter and smaller diameter dendritic branches. For these simulations, the balance of asynchronous excitatory and inhibitory input was adjusted to produce a mean firing frequency of 78 Hz, which is within the physiologic range for this cell (Murphy and Sabah, 1970). Previous modeling results have shown that, under these conditions, synchronous granule cell inputs produce somatic spiking responses that are very similar to those recorded experimentally (De Schutter and Bower, 1994c). This amplification mechanism is characterized by a near-linear relation between response amplitude and the number of synchronously activated inputs (De Schutter and Bower, 1994c). Figure 10 compares this relation for synchronously activated synapses on the distal (Fig. 10, open circles) and more proximal (Fig. 10, solid circles) Purkinje cell dendrites. The simulations demonstrate that there is very little if any difference in the amplification mechanism in each case. Statistically, the somatic responses to distal and proximal activation were identical (paired t-test; $P < 0.04$).

DISCUSSION

We have described and contrasted the ultramorphologic properties of synapses associated with the ascending and the parallel fiber segments of the cerebellar granule cell axon. To our knowledge, this is the first time that these two synaptic populations have been compared specifically. Harris and Stevens (1988) previously described the ultramorphology of parallel fiber synapses on Purkinje cells, and their calculated values for the number of presynaptic vesicles (485 ± 246) and the area of the postsynaptic density (0.15 ± 0.08) are comparable to data presented here for granule cell synapses in general. Their finding that different measured pre- and postsynaptic features of the reconstructed parallel fiber synapses were correlated positively is also consistent with our results for parallel fibers (Table 1). However, those authors did not specifically identify or describe the synapses made by the ascending granule cell axons.

The fact that the ascending segment was not taken into account in earlier electron microscopic studies is not surprising, because these synapses were not considered previously to be an important component of the cerebellar cortical circuit (Eccles et al., 1967; Ito, 1984; Napper and Harvey, 1988a,b). However, physiologic studies in our laboratory have suggested that the ascending segment synapses may have a substantial and more obvious physiologic effect on Purkinje cells than the parallel fibers (Bower and Woolston, 1983; Jaeger and Bower, 1994). It is our view that the distinct morphologic differences between these two types of synapses reported here also suggest that these synapses are functionally distinct.

Accuracy of anatomic procedures

Before considering the possible functional implications of our anatomical data, it is necessary to consider whether the anatomic techniques used in this study could have biased our basic results. There are several important methodological issues that must be addressed.

Are we certain that reconstructed synapses belonged to granule cell axons? All measured synapses were confirmed to be associated with granule cell axons by using serial electron microscopic reconstruction techniques. The characteristic asymmetric morphology of excitatory granule cell synapses (Figs. 2, 5) clearly distinguishes them from the symmetric profiles made by inhibitory synapses associated with other cerebellar interneurons (Palay and Chan-Palay, 1974; Sultan et al., 1995). Excitatory granule cell synapses also are clearly distinguishable from the excitatory contacts made by climbing fibers on Purkinje cells, because granule cell synapses occur on spines associated with the spiny branchlets, whereas climbing fiber synapses occur on spines with a very different morphology (e.g., with a spine stem) occurring on the much larger diameter "smooth" Purkinje cell dendrites (Palay and Chan-Palay, 1974).

Can we really distinguish between ascending and parallel fiber synapses? These experiments used different planes of section to distinguish between synapses associated with the ascending and parallel fiber segments. This approach relies on the fact that the general geometry of the granule cell axon is both regular and consistent throughout the cerebellar cortex (Palay and Chan-Palay, 1974). Although parallel fibers generally run in a horizon-

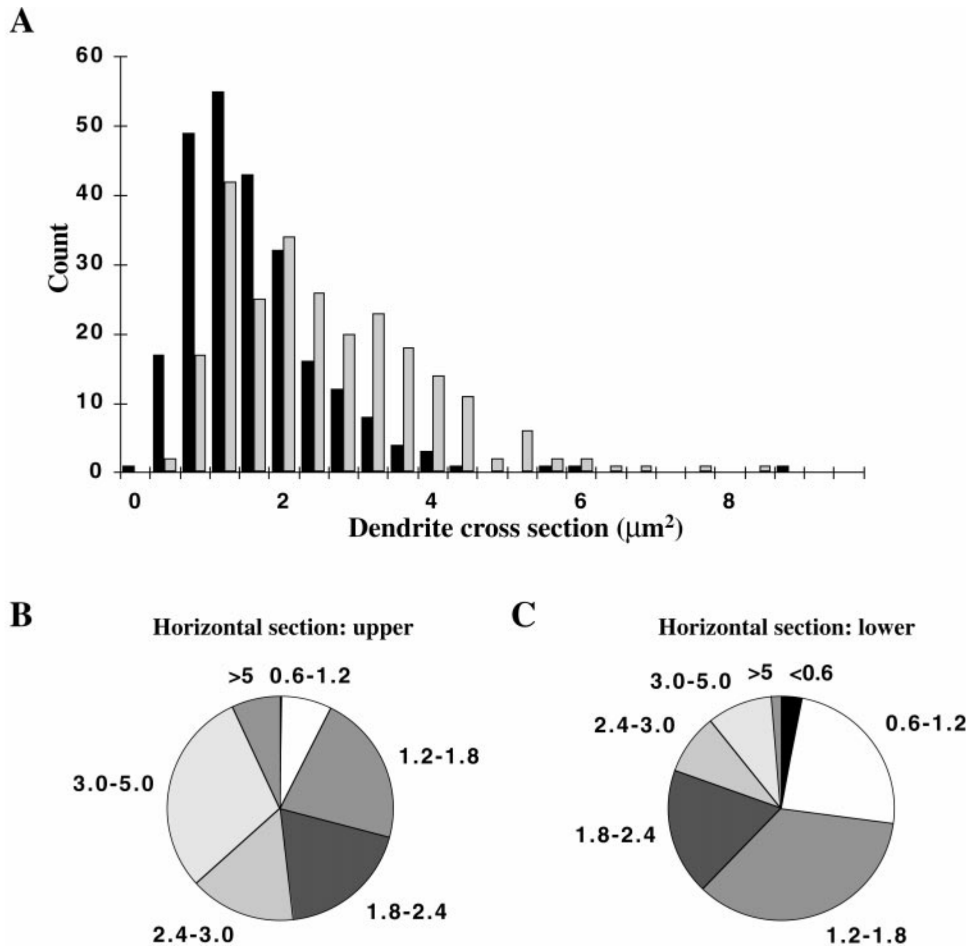

Fig. 7. Comparison of the distribution of 250 round dendritic cross sections for randomly sampled dendrites in horizontal sections at each of two depths in the molecular layer. **A:** Comparison of the total number of profiles of different diameter for each horizontal level. The striped bars correspond to sampled dendritic cross sections at a depth of 50 μm, and the solid bars represent randomly sampled dendritic cross sections found 200 μm below the pial surface. **B,C:** Charts showing the percentage of dendrites of each diameter at each level.

tal plane, and ascending segments run in a vertical (sagittal) plane (Fig. 1), the trajectories of individual granule cell axons are never as straight as they are represented schematically. However, in our analysis, serial reconstructions were used to assure that the axon associated with each measured synapse actually ran in the correct plane of section. In the case of ascending segment synapses, this was aided by the fact that these axons tend to run in vertical bundles containing several axons.

Are we certain that all measured synaptic terminations are on Purkinje cell dendrites? Another possible source of error in our measurements involves the specific identification of granule cell synapses on Purkinje cell dendrites. In fact, it has been demonstrated previously that ascending segment axons contact Golgi cell dendrites in the molecular layer (Hamori, 1981). We recently showed that ascending segment synapses also contact molecular layer interneurons (Sultan et al., 1995). Nevertheless, we are confident that the data presented here reflect synaptic contacts only with Purkinje cells. First, Purkinje cell dendrites can be distinguished unambiguously from other dendrites in the molecular layer on the basis of their characteristic dendritic spines (Figs. 2, 5). None of the other neurons with dendrites in the molecular layer have spines of this type (Palay and Chan-Palay, 1974). Second, Purkinje cell dendrites are well known to contain extensive amounts of endoplasmic reticulum (cf. Fig. 5). This is true even for dendrites of the smallest diameter (Martone et al., 1993). The existence of both spines and endoplasmic reticulum have been used by other neuroanatomists to identify synaptic contacts on Purkinje cells (Harris and Stevens, 1988). However, in this study, we further tested our electron microscopic level identification of Purkinje cell dendrites by comparing the measured dendritic diameters in the electron microscopic material with diameters obtained by using single cell light microscopic reconstruction techniques (Rapp et al., 1994). Despite the fact that this reconstruction was performed in a separate laboratory and under different histologic conditions, the diameter profiles in the light and electron microscopic data were virtually the same (Fig. 9B). Given the known differences between Purkinje cell dendrites and the dendrites of other neurons in the molecular layer (Palay and Chan-Palay, 1974), this correspondence strongly suggests that our

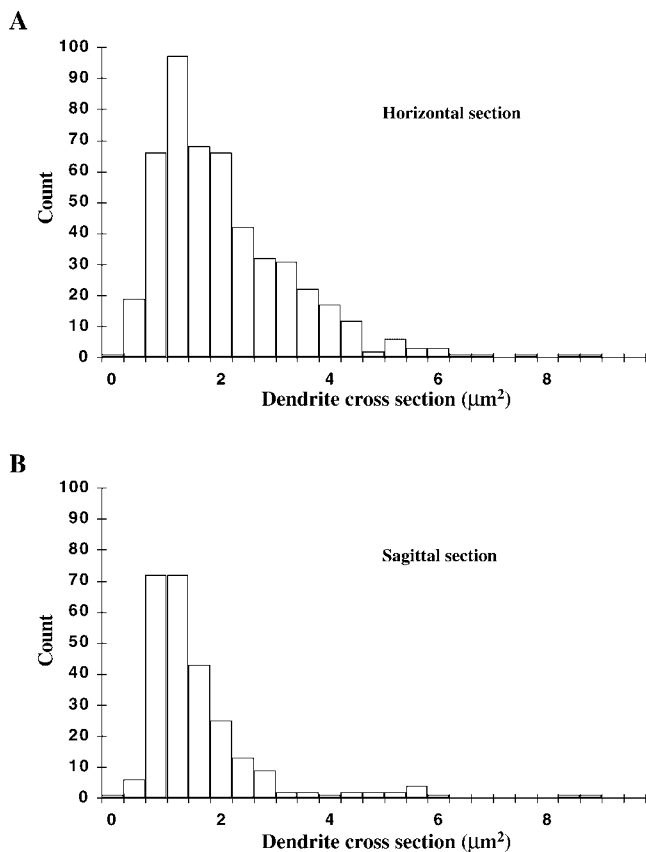

Fig. 8. Distributions of randomly sampled cross sectional areas of Purkinje cell dendrites. **A:** Dendritic profiles in horizontal sections sampled at random with respect to depth. **B:** Dendritic profiles in sagittal sections sampled at depths of both 50 μm and 200 μm in the molecular layer.

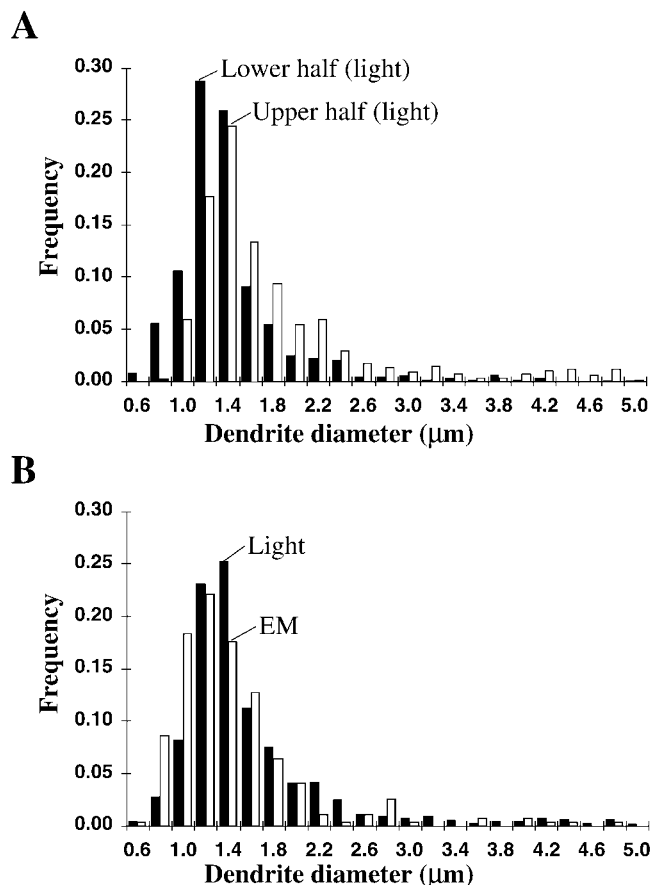

Fig. 9. Comparison of frequency distributions of dendritic diameters in light microscopic (Light) and electron microscopic (EM) reconstructions. **A:** Frequency distribution of dendritic diameters in the lower and upper halves of the light microscopy reconstructed Purkinje cell 1 of Rapp et al. (1994) are compared. **B:** Dendritic diameters in a light microscopic reconstructed Purkinje cell (also cell 1 of Rapp et al., 1994) are compared with the frequency of randomly sampled profiles in sagittal electron microscopic sections. Because the dendritic dimensions available for the light microscopic reconstructed Purkinje cell were lengths of segments with constant diameters, by using the electron and light microscopic data, we have converted them into relative frequencies of specific dendritic diameters under the assumption of random sampling.

electron microscopic analysis was restricted to Purkinje cell dendrites.

Is there any significant plane-of-section-related sampling bias? Because our method of distinguishing between ascending segment and parallel fiber synapses relied on comparing data from different planes of section, the differential distribution and orientation of the smallest dendrites we reported (Fig. 8) requires that we consider any possible effect of the plane of section on the results. Although we cannot rule out such an effect completely, it cannot account for our basic results. First, our primary evidence that ascending segment synapses terminate on small-diameter dendrites is based on tracing synapses through several serial sections and, thus, is not dependent on any one plane of section. Second, whereas the increased number of small-diameter dendrites in the deeper layers of the molecular layer could result in some sampling bias toward smaller dendrites at those depths, this bias should apply uniformly to reconstructions of both ascending and parallel fiber synapses. The fact that we saw no overlap in the distribution of contacted dendrites between these two types of synapses (Fig. 6) makes it unlikely that our results are due to such a bias. Furthermore, Figure 6 also shows that the cross-sectional distribution of dendrites contacted by parallel fiber synapses is virtually identical at two different horizontal depths with very different percentile distributions of dendritic size (see Fig. 7). This similarity would not be expected if there were systematic sampling biases with depth. Finally, our analysis of random dendritic profiles in sagittal sections shows that more than half of the round profile dendrites are >1.3 μm^2; however, no contacts were found between ascending segments and these larger diameter dendrites in these same sections.

Functional significance

With confidence that our anatomic results are accurate, the next important question concerns the possible functional significance of our data. This is an especially important question given the substantial influence that the anatomic organization of cerebellar circuitry has had historically on theories of cerebellar function. Unlike most other regions of the brain, cerebellar theorists have generally taken the anatomic structure of the cerebellum into account in their models and theories (cf. Eccles et al., 1967;

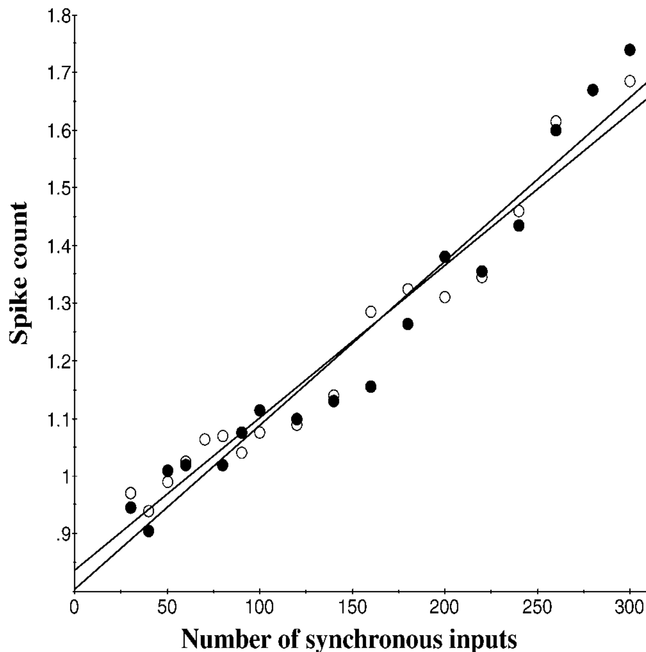

Fig. 10. Comparison of the response of the Purkinje cell model to synchronous activation of different numbers of synapses on regions of the dendrite that our data show are occupied by parallel fiber (open circles) and ascending segment (solid circles) inputs. The number of simple spikes in a 10-msec interval after the synchronous input is plotted against the number of inputs activated synchronously. Each data point represents the average response of the model to 200 stimulus trials. Background asynchronous input frequency for these simulations was 46 Hz for parallel fiber inputs and 1 Hz for stellate cell inputs.

TABLE 2. Estimates of the Number of Ascending Segment Synapses[1]

Purkinje cell	Total length spiny dendrite (mm)	Percent <1.3 μm diameter segments	Estimated number of ascending synapse spines (density; 8/μm)	Estimated total number of spines (density; 13/μm)
1	11.11	37.4	33,222	144,456
2	8.27	69.1	45,690	107,473
3	7.92	29.6	18,190	102,985
Average	9.10	45.4	32,367	118,305

[1]Estimates of the number of spines on three morphologically reconstructed guinea pig Purkinje cells. See Rapp et al. (1994) for drawings of the cells. The estimates of the number of ascending segment synapses are based on the assumption that all dendritic branches with a diameter smaller than 1.3 μm receive such inputs and that the density of spines is constant and identical for all cells.

Marr, 1969; Albus, 1971; Fujita, 1982; Ito, 1984; Kawato and Gomi, 1992; Killeen and Fetterman, 1993). For this reason, any substantial change in our understanding of the anatomic organization of this circuit will have a substantial effect on existing theories.

How many ascending segment synapses are there? These anatomic investigations were motivated by the suggestion that our previous physiologic results showing a vertical organization in cerebellar cortical circuitry (Bower et al., 1980; Bower and Woolston, 1983) might reflect the synaptic influence of the ascending segment of the granule cell axon (Llinas, 1982). The predominance of the vertical effects of granule cell layer activation on Purkinje cells was proposed at that time to result from the multiple synapses likely to be made by the ascending segments on single Purkinje cell dendrites (Llinas, 1982). However, since this original proposal was made, there has been a general reluctance to consider the importance of the ascending segment in cerebellar physiology. One possible reason for this lack of interest is the calculation published by Harvey and Napper (1988a) purporting to demonstrate that the ascending segment synapses represent at most 3% of the total granule cell synaptic contacts with Purkinje cells. Although those authors did note subsequent to the first preliminary report of the results in this paper that any change in the assumptions used in their indirect calculations would require a modification of this estimate (Harvey and Napper, 1991), no efforts have been made to recalculate these numbers or explore this issue. By using the anatomic data from the current experiments, as described below, we estimate that the ascending segment synapses may actually constitute up to 20% of the total granule cell synaptic input to Purkinje cells.

Estimates based on granule cell counts. Harvey and Napper's (1988a) 3% estimate for the percentage of ascending segment synapses was calculated indirectly by multiplying the estimated number of granule cells underneath each Purkinje cell (274) by the average length of ascending axon segments (125 μm), and then dividing by the number of synapses per unit length of the parallel fiber segment of the axon (one synapse every 7.3 μm). However, several of these values have been altered by more recent anatomic data. For example, it has been reported recently (Pichitpornchai et al., 1994) that, on average, there is one ascending segment synapse for every 4.0 μm of axon length, which is considerably higher than the rate of one every 5.2 μm for parallel fibers near the axon bifurcation point. This number drops to one synapse per 7.4 μm of axon length farther along the parallel fiber. These revised measurements immediately raise the estimated number of ascending segment synapses to 5.5% of the total. Second, Korbo et al. (1993) have reported a value of 434 granule cells per Purkinje cell, which is between the Harvey and Napper estimate of 274 and the much higher earlier estimate of 897 by Lange (1975). If one also considers that between 11% (Pichitpornchai et al., 1994) and 19% (Harris and Stevens, 1988) of granule cell axon varicosities actually make two synaptic contacts, then these values estimate that the numbers of ascending segment synapses range between 10,000 and 35,000 per Purkinje cell. Assuming that there are between 100,000 and 150,000 granule cell synapses per each Purkinje cell (Napper and Harvey, 1988b), these indirect calculations give us a percentage of ascending segment synapses ranging anywhere between 7% and 24% of the total. Either number is substantially higher than previous estimates.

Estimates based on dendritic profiles. Our finding that the ascending segment synapses contact only the smallest diameter Purkinje cell dendrites allows us to make a much more direct estimate of the number of ascending segment synapses per Purkinje cell. Specifically, the total length of dendrites <1.3 μm in diameter on a particular cell multiplied by the frequency of dendritic spines per length of dendrite should provide a reasonable estimate of the number of ascending segment synapses. Such an estimate is shown in Table 2 for the three Purkinje cells that were reconstructed completely by Rapp et al. (1994) using light microscopic techniques. The first column in Table 2 shows the total length of the spinous dendrite for each neuron,

and the second column contains the percentage of each cell's dendrite <1.3 μm in diameter. Somewhat surprisingly, dendrites of this diameter average 45% of the total dendrite. Converting this percentage into an estimate for the number of ascending segment synapses requires only an accurate estimate of the density of spines per unit length of the dendrite. However, the previously published value of 13 spines per 1.0 μm probably applies only to the parallel fibers (Harris and Stevens, 1988). Given the smaller size of the dendrites receiving ascending projections, we have calculated an adjustment factor for the published density estimates based on comparing the number of spines on 30 dendritic profiles contacted by the ascending segment with the number of spines on 30 profiles contacted by the parallel fibers. We found an average of 2.5 ± 1.3 spines per profile for ascending segments and 3.9 ± 1.4 spines per profile for parallel fibers. Given this ratio, we estimate that the dendrites receiving ascending segment synapses have a spine density of 8 spines per 1.0 μm length. In Table 2, the third column shows that, when even this conservative estimate is multiplied by the total length of the small dendrites for these three reconstructed cells, we calculate an average of 32,000 ascending segment synapses per Purkinje cell. Despite the fact that this number is calculated from entirely different numbers than the modified Harvey and Napper estimate discussed above, it still falls within the range of the previous calculation. In addition, when the average reported spine density is multiplied by the calculated total length of the dendrite for each neuron (Table 2, second column), we obtain a range of values from 100,000 to l50,000 for the total number of granule cell synapse per Purkinje cell (Table 2, last column). These numbers are well within the range usually reported for Purkinje cells in the rat (Napper and Harvey, 1988b). Because the overall number of synapses that we calculate fits with previous estimates, we have increased confidence in our new method of estimating the number of ascending segment synapses. Accordingly, we suggest that, on average, ascending segment synapses are likely to contribute at least 20% of the granule cell synapses on each Purkinje cell.

What are the functional implications of differences in synaptic morphology?

Presynaptic physiology. Beyond the apparently large numbers of contacts made by the ascending segment synapses, the results reported here also demonstrate potentially important differences in synaptic morphology. For example, our data show that the average ascending segment synapse contains significantly more presynaptic vesicles per synapse than the average parallel fiber synapse. In several other systems, the number of presynaptic vesicles per synapse has been correlated directly with the probability of transmitter release (Murthy et al., 1997). Although there is no evidence to date that ascending segment synapses may be more potent than those of parallel fibers, our data suggest that this might be a worthy subject for further investigation.

Although it is harder to quantify, we have also consistently observed that the staining density of vesicles in the ascending segment is much darker than for the parallel fibers. This is not likely to be an effect of fixation or tissue preparation, because the same blocks of tissue were used for analysis of the two types of synapses, and the processing procedures were identical in all cases. In other systems, differences in vesicle staining density have been taken as indications of possible differences in the pharmacology of synaptic transmission (Schmidle et al., 1991). Obviously, any difference in the transmitter pharmacology of these two synaptic populations could have important physiologic consequences.

Correlation in pre- and postsynaptic features. Another significant difference between ascending and parallel fiber synapses involves correlations between the values obtained for the pre- and postsynaptic features of single synapses. All measures made of pre- and postsynaptic ultramorphology were correlated highly positively in parallel fibers, whereas only the volume of the presynaptic terminal and the number of presynaptic vesicles in the ascending segment synapses showed any tendency to be correlated. In their previous electron microscopic analysis of granule cell synapses on Purkinje cell spiny branchlets, Harris and Stevens (1988) also noted the correlation in pre- and postsynaptic features of parallel fiber synapses and speculated that this correlation might reflect the presence of a mechanism responsible for regulating synaptic efficacy. The same authors found similar pre- and postsynaptic ultramorphologic correlations in the CA1 region of the hippocampus (Harris and Stevens, 1989) in which mechanisms responsible for long-term synaptic potentiation (LTP) are now generally considered to require coordination between the pre- and postsynaptic terminals (Gustafsson et al., 1987; Markam and Tsodyks, 1996). In the cerebellum, parallel fiber synapses have been shown to undergo a form of long-term synaptic depression (LTD) under certain stimulus conditions (Ito, 1989; Linden, 1994; De Schutter 1995a; De Schutter and Maex, 1996). Although initial descriptions of LTD emphasized postsynaptic effects (De Schutter, 1995a), more recent studies suggest that LTD may also involve molecular interactions between pre- and postsynaptic terminals (Lev-Ram et al., 1997). The correlation in pre- and postsynaptic ultramorphology shown here for the parallel fibers is consistent with these results.

Whatever the relationship may be between the morphologic correlations described here for the parallel fiber system and LTD, the lack of any pre- or postsynaptic correlation in the synapses of the ascending segments suggests that pre- and postsynaptic morphologic properties are not coordinated in these synapses. Consistent with this idea, De Schutter (1995a) has recently proposed that LTD specifically may regulate parallel fiber synaptic strength in relation to the overall level of stellate cell inhibition. Our recent modeling studies, as described in more detail below, suggest that maintaining a balance between these two types of synaptic input may be important in controlling the overall level of Purkinje cell dendritic excitability (De Schutter and Bower, 1994b; Jaeger and Bower, 1997; Jaeger and Bower, 1996). In fact, we have proposed that this is the primary role of the parallel fiber inputs within cerebellar cortex (Bower, 1997a,b). We believe that it is the ascending segment synapses that provide the more classical excitatory synaptic drive on the Purkinje cell soma (Bower and Woolston, 1983; Jaeger and Bower, 1994).

Differences in dendritic location of synaptic terminations. Perhaps the most surprising finding of this study is that ascending and parallel fiber synapses appear to terminate on completely separate regions of the Purkinje cell dendrite. It has been known for a long time that granule cell synapses in general are restricted to the

smaller diameter Purkinje cell “spiny branchlets” rather than the large central dendritic shafts (Palay and Chan-Palay, 1974). These “smooth” regions of the Purkinje cell dendrite are reserved for the other major excitatory input to the Purkinje cell, the climbing fiber (Eccles et al., 1967). However, the current results indicate a further suborganization of the spiny branchlets into those associated with ascending segment and parallel fiber synapses. This results in a spatially organized sequence of excitatory synaptic connections to any particular branch of a Purkinje cell dendrite that starts with the climbing fiber, progresses through parallel fiber inputs, and terminates with synapses from the ascending segment of the granule cell axon.

One interesting aspect of this spatial pattern of synaptic organization is its possible correlation to the development of the Purkinje cell dendrite itself (for review, see Altman and Bayer, 1997). Specifically, the first contact made on the newly developing Purkinje cell dendrite is from the climbing fiber, the synapses of which in the adult are found on the large central dendritic shafts. The unusual developmental pattern of the cerebellum, however, makes it very likely that the second axonal contact made on the developing Purkinje cell dendrite is from the parallel fiber and not from not the ascending segment. The reason for this is that the granule cells, which, in the adult, are located beneath the Purkinje cells, actually are generated by mitotically active precursors located above the developing Purkinje cells. After these precursor cells divide, the neophyte granule cell first elaborates its parallel fiber axon and then migrates past the Purkinje cells into the internal granule cell layer. For this reason, elaborating parallel fibers are likely to contact newly forming intermediate dendrites (Landis, 1987), whereas newly formed ascending segments are found in regions of the molecular layer that already contain parallel fibers. Although it is important to note that granule cell axons do not appear to make adult form synapses until the granule cell contacts mossy fibers in the granule cell layer at the end of their development (Altman and Bayer, 1997), they do form morphologically distinct contacts at earlier stages. When considered in the context of the data reported in this paper, it is likely that the ascending segment is available to make contacts with the Purkinje cell dendrite as its last dendritic extensions are developing. In this way, the climbing fiber-parallel fiber-ascending segment innervation sequence in the adult dendrite may mirror directly the temporal sequence of development of the Purkinje cell dendrite and its innervation.

What are the implications for the physiologic organization of cerebellar cortical circuits? These anatomic experiments were motivated by our previous physiologic findings suggesting that ascending segment synapses have a powerful excitatory effect on overlying Purkinje cells (Bower and Woolston, 1983; Jaeger and Bower, 1994). The finding that ascending segment synapses are numerous and contain large numbers of presynaptic vesicles is consistent with their having a strong influence on overlying Purkinje cells. However, the lack of “beams” reported in previous physiologic experiments using peripheral stimulation also imply that parallel fibers are much less potent than previously suspected (Eccles et al., 1972; Bower and Woolston, 1983; Bell and Grimm, 1969). One possibility raised by our data is that differences in the dendritic location of these two types of synapses might account for this difference in synaptic effectiveness. In addition, we have already discussed the likelihood that the molecular or cellular properties of this synapses might differ, given the differences in the structural correlations between pre- and postsynaptic morphologies. In this regard, there is considerable evidence that synaptic segregation on dendrites can be associated with biophysical or molecular specializations in either pre- or postsynaptic membranes (cf. Hasselmo and Bower, 1993). Within the cerebellum, some preliminary results suggest that metabotrophic glutamate receptors may be associated with parallel fiber synapses and not with those of the ascending segments (T. Knöpfel, personal communication). Given the possible importance of these receptors in mechanisms of synaptic modification like LTD (De Schutter, 1995a), this finding is consistent with our previous speculation that LTD may not occur in ascending segments.

It is our hope that molecular and cellular biologists interested in the cerebellum will make a point in future experiments to distinguish between synapses on ascending and parallel fiber axonal segments in their studies. However, it is also worth considering whether either differential dendritic location or differential activation due to the normal operation of cerebellar cortical circuitry also might account for the apparent differences in the physiologic effects of these two synaptic populations. In this regard, it has been known for many years that the relative position of synapses on the dendrite of a cell can directly effect their contribution to action potential generation. However, in the current case, the apparent physiologic effects of the two populations of synapses are the reverse of what classically would be expected from their spatial positions. Specifically, if Purkinje cell dendrites acted as simple electrical cables, then one would expect that the parallel fiber synapses closer to the cell body would have a greater effect on the soma than the more distal ascending synapses (Rall, 1964). The reverse appears to be the case. Accordingly, something more complicated apparently is going on.

Synaptic effects of parallel fibers in the Purkinje cell dendrite. There is abundant evidence, of course, that Purkinje cell dendrites are not simple cables but, instead, contain a complex combination of voltage- and calcium-gated conductances (Llinas and Sugimori, 1980, 1992). Over the last several years, we have developed a detailed single-cell model of a cerebellar Purkinje cell to provide a tool to unravel this complexity (De Schutter and Bower, 1994a–c; Jaeger and Bower, 1996). These simulations have produced several unexpected results relevant to the synaptic effects of ascending and parallel fiber synapses.

First, the model has predicted that the large, intrinsic, dendritic and somatic voltage- and calcium-gated conductances primarily control the timing of somatic spikes, not the relatively small conductances associated with the excitatory parallel fiber inputs (Jaeger and Bower, 1996). Although a complete discussion of the underlying mechanisms is well beyond the scope of the current paper, the model suggests that parallel fiber synaptic effects on somatic spiking are indirect at best and, instead, that this input provides one half of a partial voltage clamp of local regions of the Purkinje cell dendrite (Jaeger and Bower, 1996). The other half of the local voltage clamp is provided by the inhibitory synaptic effects of molecular layer interneurons. In the model, these inhibitory neurons, working in opposition to the parallel fibers, modulate local dendritic voltage levels and, thus, have a large effect on the

state of activation of the intrinsic voltage-dependent dendritic conductances. Recent electrophysiologic experiments support these modeling predictions (Jaeger and Bower, 1996), as do our current network simulations, which demonstrate that stellate cell-mediated, feed-forward inhibition effectively blocks beam-like activation of Purkinje cells by parallel fibers (Santamaria and Bower, 1997). Thus, the lack of parallel fiber beams would appear to be a consequence of the biophysical properties of the Purkinje cell dendrite combined with the anatomic and physiologic correlations within the cerebellar cortical circuitry itself. Given the probable functional importance of the correlation between the synapses of molecular layer interneurons and the parallel fibers, it would be very interesting to know whether inhibitory synapses also were found selectively on the intermediate-sized Purkinje cell spiny branchlets.

Synaptic effects of ascending synapses on Purkinje cells. The second relevant result from our modeling efforts relates to the possible effect on single Purkinje cells of the simultaneous activation of a large number of ascending synapses. The data presented in this paper suggest that activation of the granule cell layer will result in activation of a large number of excitatory synapses on overlying Purkinje cells. In addition, at least some of these synaptic inputs will be nearly synchronous, especially if single ascending segments make multiple contacts on the same Purkinje cell. Recent multisite recording experiments in our laboratory have demonstrated that the granule cell layer is activated synchronously during exploratory behavior in rats (Hartmann and Bower, 1998). In our Purkinje cell model, this type of synaptic input evokes a subthreshold dendritic calcium activation, resulting in a substantial somatic depolarization and, usually, an action potential (De Schutter and Bower, 1994c). Therefore, it appears that the Purkinje cell dendrite has a mechanism that specifically detects the presence of ascending segment inputs. Of course, given the enormous numbers of parallel fiber synapses per Purkinje cell, it is reasonable to expect that parallel fibers also would generate synchronous inputs under the right conditions. At least one recent model of cerebellar cortical function, in fact, relies on such a mechanism (Braitenberg, et al., 1997). However, as discussed above, cortical circuitry would seem to assure that any parallel fiber activity is counterbalanced by inhibition (Santamaria and Bower, 1997). The ascending synapses, in contrast, probably influence the Purkinje cell dendrite before inhibition can be established (Santamaria and Bower, 1997). Thus, again, the unique response of the Purkinje cell to ascending synapses also appears to rely on a combination of the biophysical properties of the Purkinje cell dendrite and the neuronal connections within the cerebellar cortex.

Functional correlations between ascending and parallel fiber synaptic inputs. Finally, it also is clearly important to determine how the two components of the granule cell influence on Purkinje cells interact. Originally, we proposed (Bower and Woolston, 1983) that the parallel fibers might be more modulatory in function. By using our models, in fact, we have found that the level and timing of background parallel fiber excitatory and molecular layer inhibitory synaptic inputs can modulate Purkinje cell responses to synchronous ascending segment inputs (De Schutter, 1995b). Again, it is not possible here to describe the detailed mechanisms underlying this modulation; however, it is very likely to be mediated by the large calcium-related conductances in the Purkinje cell dendrite. Such a modulatory effect also is consistent with the data presented here showing that parallel fibers synapse in a position between the ascending branch input on the distal dendrites and the large proximal dendrites and the Purkinje cell somata. In this way, parallel fiber synapses are in a position between the ascending synapses and the somata and, the model suggests, modulate the very calcium conductances responsible for the subthreshold activation that drives somatic responses to synchronous ascending inputs (De Schutter and Bower, 1994c). Thus, the anatomic position of these two different types of synapses very well may have an important influence on their apparent differential physiologic effects.

CONCLUSIONS

It is our view that the anatomic results reported here demonstrate that the cerebellar cortical neuronal circuit, long believed to be the best understood in the mammalian brain, actually includes a previously under-considered component. This component, the ascending segment of the granule cell axon, actually may have a dominant influence on the response properties of the cerebellar Purkinje cell. These experiments also have uncovered an additional structural complexity in the synaptic organization of the Purkinje cell dendrite. We propose that there are actually three distinctly different afferent excitatory influences on Purkinje cells: climbing fibers, parallel fibers, and the ascending segment of the granule cell axon. Each influences its own region of the Purkinje cell dendrite, and each has its own physiologic consequences. Although numerous authors have recognized that any such change in our understanding of the cerebellar cortex has important consequences for interpretations of Purkinje cell function (cf. Epema et al., 1985; Sharp and Gonzales, 1985; Armstrong, 1986; Campbell and Hesslow, 1986; Edgley and Lidierth, 1987; Boegman et al., 1988; Theunnissen et al., 1989), all existing theories of cerebellar function except our own (Bower, 1997a,b) continue to assume that parallel fibers provide the primary excitatory drive on cerebellar Purkinje cells.

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