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Speed limits in the cerebellum: constraints from myelinated and unmyelinated parallel fibers

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Abstract

Cerebellar parallel fibers are among the thinnest known vertebrate axons and represent an extreme anatomical adaptation. Until now a systematic examination of their properties across species has not been carried out. We used transmission electron microscopy and light microscopy to compare parallel fibers in mammals of different brain sizes. From mouse to macaque, the average unmyelinated parallel fiber diameter was 0.2–0.3 μm , consistent with the idea that they are evolutionarily selected for compactness. Average unmyelinated parallel fiber diameter scaled up slightly with brain size, and across species the estimated total conduction time is 5–10 ms. However, these conduction times can vary by milliseconds, and unmyelinated PFs consume large amounts of energy per action potential. These functional disadvantages are overcome in myelinated parallel fibers, which we found in the deep regions nearest the Purkinje cell layer in marmoset, cat and macaque. These axons were 0.4–1.1 μm wide, have expected conduction times of 0.5–1.0 ms, and may convey fast feedforward inhibition via basket cells to Purkinje cells.

Keywords

brain evolution; cerebellum; optimization; perceptron

Abbreviations

MPFs, myelinated parallel fibers; PFs, parallel fibers; TEM, transmission electron microscopy

Introduction

The layout and microstructure of cerebellar parallel fibers (PFs) may provide clues about their functional role. PFs are granule cell axons, which ascend through the Purkinje cell layer to the molecular layer, bifurcate, and pass through the dendritic trees of the Purkinje neurons (Fig. 1A). In the rat (Harvey & Napper, 1991) each granule cell forms hundreds of synapses on Purkinje neurons and interneurons, and each Purkinje cell receives approximately 200 000 PF synapses and inhibitory inputs. Axons from the Purkinje cells form the only efferent pathways from the cerebellum. This massive divergence and convergence motivated Marr (1969), Albus (1971) to propose that the cerebellar cortex acts as a pattern detector in which the number of Purkinje cell inputs is very high as part of a sparse coding scheme.

In this ‘perceptron’ model, maximizing the number of PFs would maximize combinatorial possibilities. Therefore PFs may be narrow in order to fit large numbers of axons into a limited

space. An axon's size determines its functional properties, so the size of PFs may be influenced not only by combinatorial needs, but also by selection pressure for speed and volume. Unmyelinated PFs conduct action potentials slowly, raising the possibility that PFs can also be used as time delay devices (Braitenberg, 1967), albeit somewhat variable ones (Gardner-Medwin, 1972; Llinás, 1982). However, unmyelinated axons have high capacitance and are energetically costly to operate per action potential.

These trade-offs might be better understood by comparing brains of different sizes, as variation in size may impose different functional constraints. Until now no systematic comparison of PFs has been carried out. We examined five species of mammals with brain sizes from 0.4 cm³ (mouse) to 90 cm³ (macaque). In addition to unmyelinated PFs, we characterized a subpopulation of myelinated PFs, which should have faster conduction velocity and less capacitance, thus offsetting disadvantages found in unmyelinated PFs.

Materials and methods

Electron microscopy

Cerebella were obtained from one mouse (*Mus musculus*, strain B6CBAF1/J, adult male, age 11 months), one rat (*Rattus norvegicus*, Sprague–Dawley, Taconic, young adult male, age 1.5 months), one marmoset (*Callithrix jacchus*, adult male, age 3.5 years), one cat (*Felis domesticus*, adult male, age 17 months), and one macaque monkey (*Macaca mulatta*, adult male, age unknown). Under deep anaesthesia, the animals were transcardially perfused manually (except the cat, which was gravity-perfused) with Dulbecco's modified Eagle medium at 37 °C followed by Karnovsky's fixative (1.5% glutaraldehyde, 2.0% paraformaldehyde, 2.0 mM CaCl₂ in 0.1 M sodium cacodylate buffer, pH 7.2) at 37 °C. Brains were removed immediately and postfixed in full-strength Karnovsky's at room temperature for 2 h, then overnight at 4 °C, and stored at 4 °C in 0.5% glutaraldehyde in 0.1 M sodium cacodylate with 5.0% sucrose. For each specimen, the cerebellum was sectioned at the midline and 1 × 2 × 3 mm blocks were dissected from the vermal region of lobule VI, left hemisphere.

Tissue blocks were osmicated on ice for 30 min with 2.0% OsO₄ in 0.1 M sodium cacodylate, reduced with 10 mg/mL potassium ferrocyanide, and rinsed with 0.1 M sodium cacodylate. Blocks were then serially dehydrated in ethanol followed by infiltration with propylene oxide and overnight incubation in 1:1 propylene oxide: Embed-812 resin, and finally embedded in Epon epoxy resin in sagittal orientation. Thick sections (0.5 µm) were stained with toluidine blue or Azure II stain and examined with a light microscope to select an area for ultrathin sectioning. Ultrathin sections (80 nm) were placed on 150 cc carbon grids and stained with 2% uranyl acetate and a modified Sato's lead solution. Grids were examined using a Zeiss 912 AB transmission electron microscope. The Purkinje cell layer was located, then serial transects of overlapping images, each approximately 9 µm × 9 µm (×3150 direct magnification) or 5.5 µm × 5.5 µm (×5000 direct magnification), were made with an AMT XR-100 2600-line CCD camera starting from the Purkinje cell layer and continuing upward through the molecular layer. Some transects started at the pia in order to explore the superficial molecular layer.

Electron micrograph analysis

Axon morphometry was performed using ImageJ (<http://rsb.info.nih.gov/ij/>). Axons containing mitochondria or more than three synaptic vesicles were excluded from analysis (Napper & Harvey, 1988; Sultan, 2000). PF profiles were traced using a tablet PC and fitted with ellipses. The diameter was taken as the average of the major and minor axes of the ellipse if the major axis to minor axis ratio was less than 1.5, and as the minor axis if the ratio was larger. Diameters were not corrected for shrinkage. For calculation of conduction velocities

and times, diameters were corrected assuming a tissue shrinkage estimate of 15% due to perfusion and tissue preparation.

Myelin staining and light microscopy

Using a Leica VT1000S microtome, 50- μ m coronal sections were taken from rat (all lobules), cat (lobules IV and V), and macaque (lobules III and V). Sections were stained for myelin using a modification of a previously described procedure (McNally & Peters, 1998). Sections were incubated in 0.5% Triton-X in Tris phosphate-buffered saline (TPBS; 100 mM Tris and 50 mM sodium phosphate, pH 7.6) for 36–48 h, rinsed twice in TPBS, reacted in 2.5% DAB for 15 min, rinsed in TPBS, incubated in 8.0% thioglycolic acid for 4 h, rinsed four times in 2.0% sodium acetate, developed for 10–15 min in a solution containing 2.5% sodium acetate, 1.0% ammonium nitrate, 1.0% silver nitrate, 5.0% tungstic acid, and 0.07% formaldehyde, incubated in 1.0% acetic acid for 5 min, reacted in 0.05% gold chloride for 15 min, rinsed in 2.0% sodium acetate, incubated in 3.0% sodium thiosulphate, and rinsed in 0.1 M sodium phosphate buffer. The sections were mounted onto coated slides, air-dried overnight, dehydrated, cleared, and coverslipped. Slides were examined using a Nikon Eclipse E800 microscope using a CCD camera and IPLab software.

Results

We used transmission electron microscopy (TEM) to examine sagittal sections of cerebellar samples taken from lobule VI of mouse, rat, marmoset, cat, and macaque (Fig. 1A). Sections were rich in unmyelinated PFs (Fig. 1B–D), which were identifiable by their microtubules, electron-light cytoplasm, and polygonal appearance in cross-section (Palay & Chan-Palay, 1974). Unmyelinated PF diameters were small (range 0.08–0.62 μ m) and increased weakly as a function of brain size (Fig. 1E; mouse $0.19 \pm 0.06 \mu$ m, $n = 179$; rat $0.17 \pm 0.04 \mu$ m, $n = 555$; marmoset $0.27 \pm 0.09 \mu$ m, $n = 381$; cat $0.23 \pm 0.09 \mu$ m, $n = 337$; and macaque $0.28 \pm 0.10 \mu$ m, $n = 132$; all measurements are mean $\pm$ SD; correlation coefficient of rank order with respect to rank order of brain size $\rho = 0.8$, $P = 0.1$).

Unmyelinated PFs have previously been reported to be wider in deep regions nearer to the Purkinje cell layer (Palkovits *et al.*, 1971; Palay & Chan-Palay, 1974; Sultan, 2000). We confirmed this in cat (Fig. 1F, rank order of average diameter by location correlated with rank order of distance from the Purkinje cell layer all the way to the pia, $\rho = 0.51$, 312 unmyelinated PFs in 14 locations, $P < 0.1$), mouse (Fig. 1G; up to 200 μ m away from Purkinje cell layer, $\rho = 0.96$, 179 unmyelinated PFs in seven locations, $P < 0.001$, two-tailed test), and rat (Fig. 1G; up to 70 μ m away, $\rho = 0.86$, 555 unmyelinated PFs in eight locations, $P < 0.01$), but not marmoset (Fig. 1G, up to 100 μ m away, $\rho = 0.30$, 316 unmyelinated PFs in eight locations, $P = 0.5$). Overall, the mean change in unmyelinated PF diameter relative to depth was weak (mouse, $0.05 \pm 0.01 \mu$ m increase in diameter per 100 μ m depth; rat, $0.03 \pm 0.01 \mu$ m per 100 μ m; cat, $0.03 \pm 0.01 \mu$ m per 100 μ m).

We also found a population of longitudinally oriented myelinated axons with near-circular profiles in marmoset, cat, and macaque (Fig. 2). These axons were found in the lower molecular layer and were 0.4–1.1 μ m wide (Fig. 2B; marmoset $0.75 \pm 0.16 \mu$ m, 63 axons; cat $0.64 \pm 0.15 \mu$ m, 25 axons; macaque $0.64 \pm 0.11 \mu$ m, five axons). They were less common in images taken from regions higher in the molecular layer (Fig. 2A) and were entirely absent in the upper molecular layer (Fig. 1D). In total we found 93 longitudinally oriented myelinated axons in individual micrographs (marmoset, 63 axons in eight 78- μ m² micrographs, cat, 25 axons in nine 32- μ m² micrographs, and macaque, five axons in four 32- μ m² micrographs) and more such axons in montages (macaque, Fig. 2G). Only one such axon was found in 13 mouse micrographs (32 μ m² per image) and none in eight rat electron micrographs (32 μ m² per image).

To further examine these myelinated fibers, we stained 50- μ m-thick sections of rat (all lobules), cat (lobules IV and V) and macaque (lobules III and V) cerebellar tissue for myelin and examined the sections with brightfield light microscopy. In cat and macaque tissue we found a dense band of myelinated fibers just above the Purkinje cell layer (Fig. 2D). In contrast, in rat tissue we observed only sparse staining (Fig. 2C), with no visible plexus of longitudinally oriented myelinated fibers in any lobule.

Myelinated axons (Fig. 2D and E) had key characteristics of parallel fibers. They ran parallel to the Purkinje cell layer along the longitudinal axis of the folium. Individual fibers ran for up to several hundred microns within the plane of each section and could often be traced back to a T-junction. Many ascending axons could be traced well into the granule cell layer (Fig. 2E). Fibers varied little in diameter and were myelinated all along their length. Unlike Lugaro cell axons, which are branched and can give rise to longitudinally running collaterals (Lainé & Axelrad, 1996, 2002), the axons we observed did not branch in the molecular layer. Taken together, these observations indicate that most of the myelinated fibers in the lower molecular layer are myelinated parallel fibers (mPFs).

We also found a second population of myelinated axons distinct from the mPFs. Like mPFs, these axons were found mainly in the lower molecular layer. However, they were less abundant, were several times thicker (1–2 μ m wide), and ran for only 15–50 μ m before turning and leaving the plane of section (Fig. 2C). These axons were visible in all three species examined by light microscopy and may be Lugaro cell axons.

Discussion

Parallel fibers (PFs) are often considered to be exclusively unmyelinated, and myelinated PFs (mPFs) are a neglected subject. A dense ‘supraganglionic plexus’ (Jakob, 1928) of myelinated fibers near the Purkinje cell layer has been observed in cats (Eccles *et al.*, 1967), macaques (Fox *et al.*, 1964) and humans (Jakob, 1928). At the time, these myelinated fibers were attributed to recurrent collaterals of Purkinje cell axons (Ramon y Cajal, 1911; Fox *et al.*, 1964; Eccles *et al.*, 1967). In contrast, Mugnaini (1972) reported that in cats a large fraction of the myelinated fibers in the lower molecular layer are PFs based on ultrastructure and the presence of ascending and bifurcating myelinated granule cell axons.

We find that the composition of the supraganglionic plexus varies by species. In rat it contains only a few longitudinally running myelinated fibers, in agreement with Palay and Chan-Palay (1974). These fibers are likely to be either recurrent Purkinje cell axon collaterals (Chan-Palay, 1971) or Lugaro cell axons (Lainé & Axelrad, 1996; Dieudonné & Dumoulin, 2000; Lainé & Axelrad, 2002). In the larger-brained animals these thick fibers are outnumbered by the thinner mPFs.

Myelinated PFs may arise from mechanisms for glial ensheathment triggered when axons pass a diameter threshold. Lange (1978) found that in kittens the supraganglionic plexus contains mainly recurrent Purkinje cell collaterals; he did find abundant mPFs but only in adults (Lange, 1976, 1978). In the central nervous system unmyelinated and myelinated axons are approximately separated by a diameter threshold (Hirano & Llana, 1995). For PFs our results indicate that this threshold is approximately 0.4 μ m. This raises the possibility that myelinated PFs may simply represent the endpoint of a gradient of PF diameters (see Fig. 1F and Sultan, 2000) without necessarily having a special function.

When mPFs fire is unknown. If these axons have the same length as the unmyelinated PFs, their conduction time would be approximately 0.5 ms (Fig. 3, dashed line). This is much shorter than measured cerebellar response latencies to auditory-evoked potentials; 8–18 ms in cat (Huang & Liu, 1985; Fig. 3, arrowhead) and 22 ms in macaque (Mortimer, 1975). These

physiological latency measurements reflect summed delays from brainstem synapses, conduction along mossy fibers to granule cells, and transmission through parallel fibers. The shortest latencies, 8 ms, can be accounted for if the total measured latency is dominated by conduction delays in unmyelinated PFs (Fig. 3, arrowhead).

What information might myelinated PFs convey? Myelinated PFs synapse predominantly onto basket cells (Mugnaini, 1972), thus providing a disynaptic pathway for feedforward inhibition to arrive at the soma and axon of Purkinje neurons faster than monosynaptic excitation via unmyelinated PFs. Such a pathway might not only allow myelinated PFs to counterbalance excitation (Bower, 2002), but arrive quickly enough to veto excitatory input from unmyelinated PFs. To our knowledge, such short-latency sensory inhibitory events have not been reported in large-brained mammals (though see measurements in rat in Korn & Axelrad, 1980; and in Mittmann *et al.*, 2004).

In the case of unmyelinated PFs, expected conduction velocities can be calculated from the diameter distributions using the relationship between unmyelinated PF diameter and conduction velocity (Hoffmeister *et al.*, 1991) and compared with physiological measurements. Calculation from the anatomical diameter distribution gives a range of 0.18–0.27 m/s for PF diameters of 0.2–0.3 μm , while the physiologically reported speed is somewhat higher, 0.3 m/s (Eccles *et al.*, 1967; their Figure 33). This small discrepancy could be due the use of hindlimb nerve axons by Hoffmeister *et al.*, (1991), which might have different membrane properties such as sodium channel density (Ritchie, 1995). Thus, calculations of conduction speed and time may be more useful for comparative purposes than as absolute values.

Selection pressure for conduction speed in large-brained mammals would not have been predicted from cerebellar anatomy alone. Unmyelinated PF lengths are not proportional to brain diameter (2.8 mm in mouse, Soha *et al.*, 1997; 4.5 mm in rat, Pichitpornchai *et al.*, 1994; 6.0 mm in cat, Brand *et al.*, 1976; 5.7 mm in macaque, Mugnaini, 1983). These values, halved to account for the fact that conduction begins at the T-junction, can be combined with our measurements and the relationship between unmyelinated PF diameter and conduction velocity (Hoffmeister *et al.*, 1991) to obtain estimates of total conduction time (Fig. 3, filled symbols). The resulting conduction times are largely in the 5–10-ms range in all species examined, and do not increase systematically with brain size. Selection pressure for speed may be imposed by other structures such as the neocortex, where long-distance conduction can reach hundreds of milliseconds (Swadlow & Waxman, 1976).

The maximal packing density of unmyelinated PFs creates a new problem – metabolic cost. Unmyelinated PFs are potentially metabolically expensive per unit volume because of their capacitance. However, the cerebellum does not use more energy than other brain regions (Sokoloff, 1983), suggesting that unmyelinated PFs fire sparsely and at low average rates, as suggested for neocortex (Lennie, 2003) and consistent with the low spontaneous firing rate of cerebellar granule cells *in vivo* under anaesthesia (Shambes *et al.*, 1978; Chadderton *et al.*, 2004). This would be consistent with the suggestion that sensory input triggers activity in a sparse population of PFs (Marr, 1969; Albus, 1971).

In summary, unmyelinated parallel fibers constitute a compact pathway that is well-suited to convey a sparse code. This may come at the expense of precise spike timing, indicating that millisecond or submillisecond processing operations would have to occur at earlier stages in the mossy fiber pathway. In contrast, myelinated fibers can conduct quickly and fire precisely. The appearance of these fibers in large-brained animals allows the possibility of an additional parallel fiber pathway in cerebellar cortex, one that rapidly inhibits Purkinje cells.

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Fig. 1. Unmyelinated and myelinated parallel fibers of the cerebellum. (A) Cerebellar anatomy (Ramon y Cajal, 1911). (B–D) Electron micrographs from marmoset cerebellum. All images are sagittal sections from lobule VI ($\times 3150$ direct magnification). Pc, Purkinje cell; Pcd, Purkinje cell dendrite; bc axon, basket cell axon; mpf, myelinated PF. (B) Lower molecular layer, adjacent to a Purkinje cell. (C) Middle molecular layer, approximately 90 μ m from Purkinje cell. (D) Upper molecular layer, approximately 40 μ m from the pial surface. (E) Unmyelinated PF diameters (average and SD) for five different species as a function of brain diameter. Brain diameters were obtained as described in Shultz *et al.* (2003), and were (in cm): mouse 0.9, rat 1.4, marmoset 2.4, cat 3.8, macaque 5.5. (F) Unmyelinated PF diameter as a

function of position in molecular layer in cat. (G) Unmyelinated PF diameter as a function of distance from the Purkinje cell layer in mouse, marmoset, and macaque. In (F) and (G), each point represents data from one TEM image.

Fig. 2.

Myelinated parallel fibers. (A) Number of myelinated PFs as a function of position in the molecular layer. Marmoset cerebellum, lobule VI. Individual counts were taken from 78 μm^2 TEM images ($\times 3150$ direct magnification, $\times 20\,000$ print magnification). (B) Myelinated PF diameter as a function of position in the molecular layer for marmoset, cat, macaque (lobule VI for all). Diameters were measured from $\times 20\,000$ and $\times 32\,000$ TEM prints ($\times 3150$ and $\times 5000$ direct magnification). (C) Brightfield image of rat cerebellum, coronal section from crus II ($\times 200$ direct magnification), stained for myelin. Pc, Purkinje cell; gcl, granule cell layer; ml, molecular layer; mf, myelinated fiber. Note the sparse plexus of myelinated fibers in the lower molecular layer, putatively corresponding to recurrent collaterals of Purkinje cell axons and/or Lugaro cell axons. (D and E) Brightfield images of coronal sections of macaque cerebellum lobule III stained for myelin taken at (D) $\times 200$ direct magnification and (E) $\times 1000$ direct magnification. Pc, Purkinje cell; gcl, granule cell layer; gc axon, granule cell axon; ml, molecular layer; mpf, myelinated parallel fiber. Note the many myelinated PFs coursing through the lower molecular layer above the Purkinje cell layer and the T-junction in E. (F) TEM micrograph of macaque cerebellum, lobule VI, $\times 5000$ direct magnification. Pc, Purkinje cell; mpf, myelinated PF. (G) Montage of multiple micrographs ($\times 5000$ direct magnification) of macaque lobule VI tissue in the lower molecular layer to show multiple myelinated PFs. The bottom of the montage begins approximately 5 μm from the top of the Purkinje cell layer.

Fig. 3.

Estimated parallel fiber conduction times. Estimated conduction times based on measured PF diameters corrected for shrinkage and literature values for PF lengths (mouse, Soha *et al.*, 1997; rat, Pichitpornchai *et al.*, 1994; cat, Brand *et al.*, 1976; macaque, Mugnaini, 1983), halved to give an estimate of the distance from the T-junction to the end of the PF. Conduction velocities were calculated as $v = 0.75 \times \text{diameter}$ for unmyelinated axons (Hoffmeister *et al.*, 1991), and as $v = 5.7 \times \text{diameter}$ for myelinated axons (Hursh, 1939) (diameters in μm , velocities in m/s). Arrowhead, range of auditory evoked potential latencies in Purkinje cell simple spikes in cat (Huang & Liu, 1985).