Wiley InterScience
DISCOVER SOMETHING GREAT

Journal of Neuroscience Research 85:2080–2086 (2007)

NeuroTechnique

Morphometric Analysis of Oligodendrocytes in the Adult Mouse Frontal Cortex

Joshua C. Murtie,[1] **Wendy B. Macklin,**[2] **and Gabriel Corfas**[1*]

[1]Division of Neuroscience, Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts
[2]Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio

Oligodendrocytes (OLs), the myelinating cells of the central nervous system, have specialized morphologies that subserve their function. Numerous qualitative studies suggest that OLs in different brain regions can differ in their morphological characteristics, including number of branches and internodes, internode length, etc. However, progress in identifying and characterizing the diverse types of OLs and their distribution in the brain has been made difficult by several technical constraints. Here we report a new strategy to analyze OL morphology with a high degree of quantitative power and throughput. We used confocal microscopy and three-dimensional cell tracing software to study OLs in the frontal cortex of mice expressing enhanced green fluorescent protein (eGFP) under the control of the proteolipid protein (*Plp*) gene promoter. Three-dimensional reconstructions were then used to analyze and quantify cell morphology, including total process length, total process surface area, total internode length, number of primary processes, number of branch points, and number of internodes. In addition, these reconstructions were subjected to Sholl analysis, which allows for the quantitative measure of OL arbor complexity. By using this approach, we identified and characterized a previously undescribed population of small OLs with a compact but complex morphology that includes numerous branching processes and a large number of short internodes. Our data suggest that other populations of OLs remain to be identified and characterized and that the tools we have developed could help in the process of characterizing them. **© 2007 Wiley-Liss, Inc.**

Key words: oligodendroglia; morphology; myelin internode

Oligodendrocytes (OLs), the myelinating cells of the central nervous system (CNS), have very specialized morphology that subserves their biological function, allowing the efficient transmission of electrical impulses and support and maintenance of myelinated axons (Edgar and Garbern, 2004). Though sharing many characteristics, OLs are quite diverse, both at the biochemical and at the morphological levels. For example, myelin composition differs between small- and large-diameter axons (Sternberger et al., 1979; Hartman et al., 1982; Norton, 1984; Hildebrand et al., 1993), an observation that led to the speculation that these biochemical variations could underlie distinct classes of OLs (Norton, 1984). Likewise, OLs in different CNS regions can have significantly distinct morphologies (Ogawa et al., 1985; Friedman et al., 1989; Butt and Ransom, 1993; Bjartmar et al., 1994; Butt et al., 1994; Weruaga-Prieto et al., 1996; Kettenmann and Ransom, 2005). Thus, it is possible that OLs with different morphologies may have specific influences on their associated neurons. These observations also raise the possibility that alterations in OL morphology could be implicated in neurological or neuropsychiatric disorders (Sullivan and Pfefferbaum, 2003; Hajek et al., 2005; Cannistraro et al., 2006; Walterfang et al., 2006; Whiteside et al., 2006). However, identification and characterization of the different OL populations and their alterations in disease states has lagged for technical reasons, such as the complexity of quantifying the intricate morphological characteristics of OLs.

Here we show that mice expressing enhanced green fluorescent protein (eGFP) under the control of the proteolipid protein (*Plp*) promoter (Mallon et al., 2002) can be used to study the morphology of large numbers of OLs in the brain with great quantitative

Contract grant sponsor: NINDS; Contract grant number: R01 NS35884; Contract grant sponsor: National Multiple Sclerosis Society; Contract grant number: RG 3786; Contract grant sponsor: National Multiple Sclerosis Society Postdoctoral Fellowship.

*Correspondence to: Gabriel Corfas, PhD, Division of Neuroscience, Children's Hospital, 300 Longwood Ave., Boston MA 02115.
E-mail: gabriel.corfas@childrens.harvard.edu

Received 23 January 2007; Revised 23 February 2007; Accepted 26 February 2007

Published online 10 May 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21339

© 2007 Wiley-Liss, Inc.

power. With the combination of confocal microscopy and a computerized cell tracing system, we three-dimensionally reconstructed OLs in the frontal cortex of adult mice and thoroughly measured and analyzed morphological parameters for which this was previously untenable, including total process length, total process surface area, total internode length, number of primary processes, number of branch points, number of internodes, and maximal branch order per cell. By using this technique, we identified a previously unrecognized population of OLs in the frontal cortex that is different from previously characterized OLs in having numerous highly branched processes and large numbers of relatively short myelin internodes. Our results suggest that specific populations of OLs remain to be identified and that careful analysis of their morphology may lead to the identification of functional differences between OL populations.

MATERIALS AND METHODS

Plp-eGFP Transgenic Mice

Previously characterized mice expressing eGFP under the control of the *Plp* gene promoter were used to identify OL-lineage cells in the frontal cortex of adult mice (Mallon et al., 2002). The Animal Care and Use Committee of Children's Hospital Boston approved the use of these animals.

Tissue Collection and Confocal Imaging

Eight-week-old male mice were perfused intracardially with 4% paraformaldehyde in 0.1 mM phosphate buffer. After perfusions, tissue was dissected, and brains were postfixed in 4% paraformaldehyde overnight at 4°C. Brains were transferred to phosphate-buffered saline (PBS) and stored at 4°C until vibratome sectioning. Coronal vibratome sections were cut at 150 μm and mounted on Superfrost plus slides using Gel-Mount. Tissue compression was avoided by building up the slide around the edges with nail polish prior to placing the coverslip over the tissue. Mature OLs were randomly selected and identified morphologically by the presence of myelin internodes. After OLs were selected, a laser scanning confocal microscope was used to acquire z-stacks of whole tissue at optical slices of 0.75 μm with a ×63 objective (1.4 numerical aperture).

Three-Dimensional Cell Tracing

Confocal z-stacks were used to trace 15 individual OLs with the Neurolucida three-dimensional cell tracing system (MBF Biosciences, Williston, VT). The optical slice at the center of the soma was used to trace the circumference of the soma in two dimensions. Next, each primary process was traced as an individual dendritic arbor (Neurolucida requires the labeling of each process as either an axon or dendrite) until the entire branched process with myelin internodes was traced. Myelin internodes were identified based on the presence of nodes of Ranvier, followed by another internode at their ends. Some internodes were associated with visible nodes of Ranvier only at one extreme. These were infrequent and most probably represent internodes closest to the cell body or the axonal terminal. Incomplete cells were excluded from analysis if, during tracing, a process was observed to extend beyond the volume of the z-stack in any axis (x, y, z). Analysis of tracings, including Sholl analysis, was performed in Neuroexplorer software (MBF Biosciences, Williston, VT).

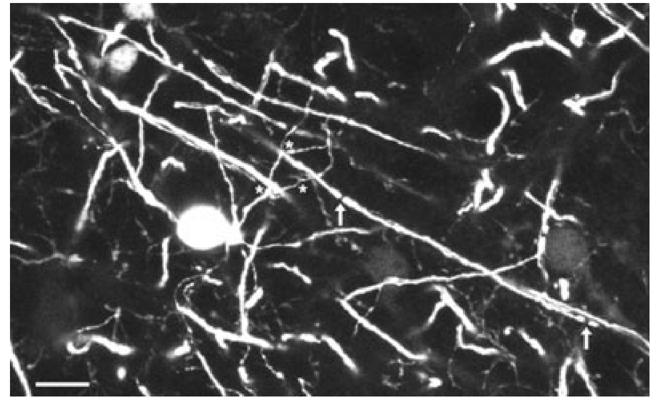


Fig. 1. Oligodendrocytes in the frontal cortex possess many branching processes and maintain numerous short internodes. A portion of a representative oligodendrocyte is shown here as a maximum projection with a single processes containing at least three branch points (asterisks). A short internode measuring approximately 45 μm in length is also shown (arrows). Scale bar = 10 μm.

RESULTS

Identification and Tracing of OLs in the Frontal Cortex

Randomly chosen eGFP-expressing cells in the frontal cortex were selected for in-depth analysis based on their morphological characteristics, for example, the presence of at least one process forming a myelin internode. Examination of these cells under confocal fluorescence microscopy resulted in some surprising qualitative observations. First, the eGFP$^+$ cells in this region had multiple branched processes extending from their soma, each having numerous myelin internodes (Fig. 1). The branching pattern of OLs we analyzed appeared to be more complex than the patterns described in previous studies (Ogawa et al., 1985; Butt et al., 1994; Weruaga-Prieto et al., 1996). Second, myelin internodes appeared much shorter than expected based on previous reports on optic nerve (Butt et al., 1994).

After OLs were identified and confocal z-stacks acquired, cells were traced in the Neurolucida software (Fig. 2). Because Neurolucida is designed for tracing neuronal morphology, we had to make several adaptations for OL analysis. For example, each OL primary process was treated as an individual dendritic arbor, or tree, in order to acquire all necessary measurements. Neurons do not posses myelin internodes, so internodes were traced as extensions of cellular processes, and measurements specific to internodes were gathered post hoc. As shown in Figure 2, the three-dimensional cell tracing

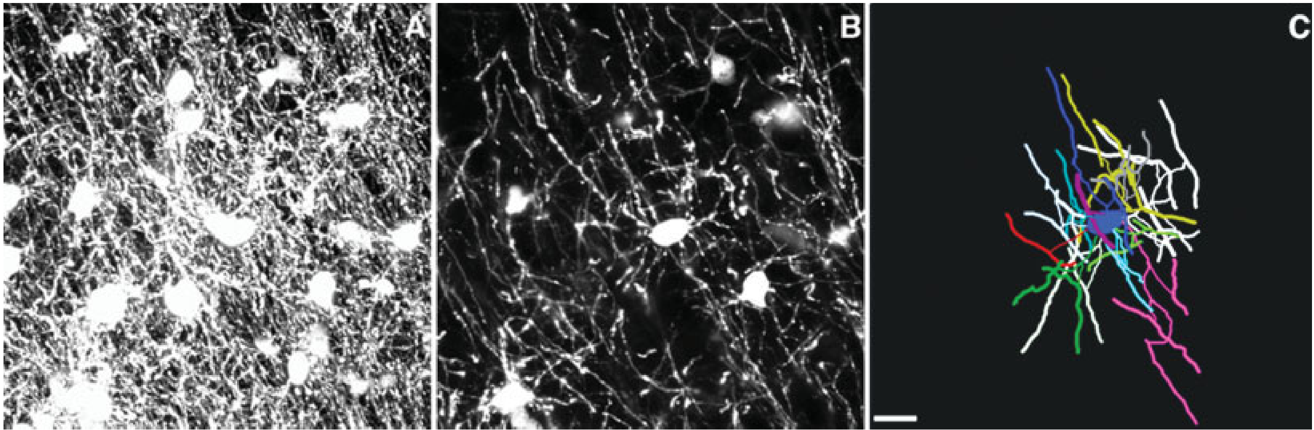


Fig. 2. The Neurolucida three-dimensional cell tracing system generates a detailed representation of oligodendrocytes in vivo. **A:** Maximum projection image of the entire z-stack acquired via confocal microscopy depicts the dense network of eGFP$^+$ OL processes in slices of frontal cortex. **B:** Maximum projection of a portion of the z-stack shown in A demonstrates the level of detail that can be observed during cell tracing. **C:** Two-dimensional representation of a three-dimensional tracing of a cell shown in A and B illustrates the complexity of oligodendrocyte morphology in vivo; each color represents an individual primary process and subsequent branches and internodes. Scale bar = 20 μm.

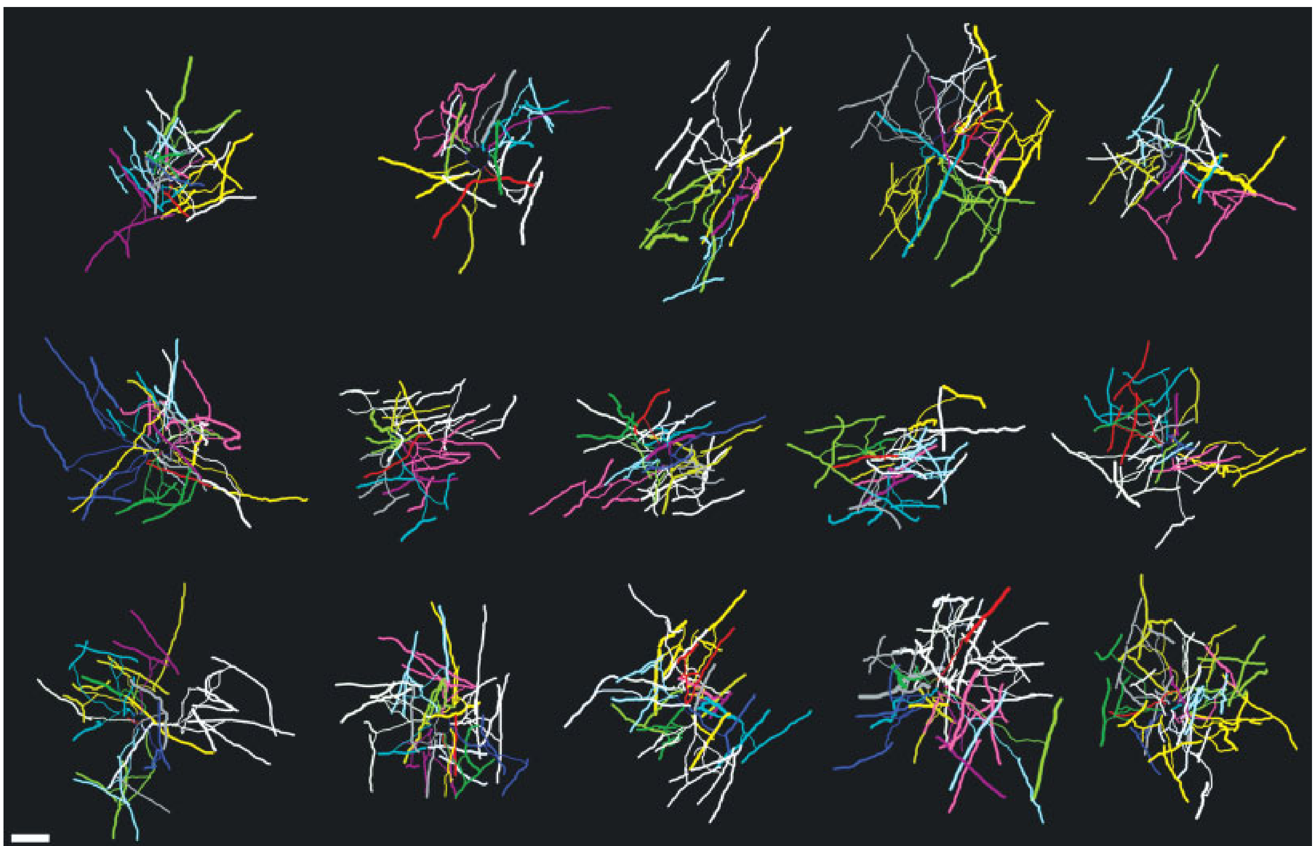
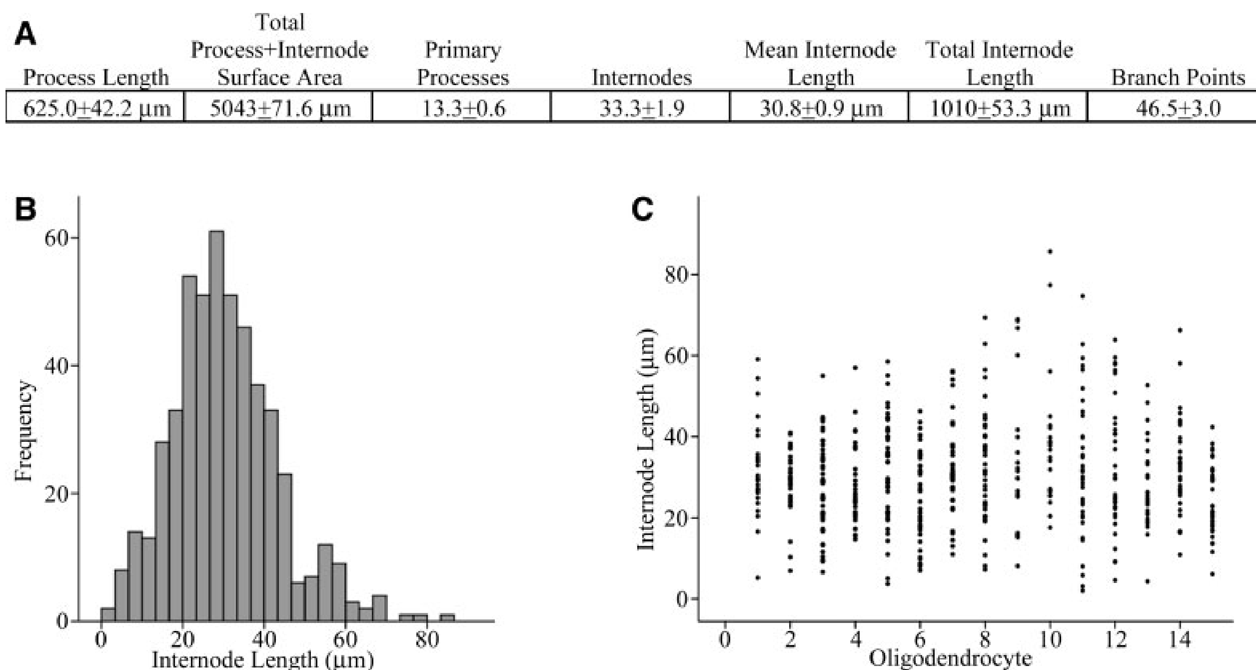


Fig. 3. Two-dimensional representations of three-dimensional tracings for the 15 cells analyzed in this study illustrate the similarity of cells within this population. All cells appear to be compact, with many branching processes, and maintain numerous internodes. As expected, internodes do not appear to be oriented in parallel arrays as they would be in major white matter tracts. Scale bar = 20 μm.



Process Length	Total Process+Internode Surface Area	Primary Processes	Internodes	Mean Internode Length	Total Internode Length	Branch Points
625.0±42.2 μm	5043±71.6 μm	13.3±0.6	33.3±1.9	30.8±0.9 μm	1010±53.3 μm	46.5±3.0

Fig. 4. The population of oligodendrocytes analyzed is highly homogenous. **A:** Morphometric profile of oligodendrocytes analyzed in this study demonstrates the similarity of measurements within the population (N = 15). Each measure analyzed also had a normal distribution, suggesting that the group of cells is an individual population and not a portion of a larger population (Kolmogorov-Smirnov test for normality, $P \geq 0.38$, N = 15). **B:** Frequency histogram of internode lengths for the entire population of cells demonstrates the normality of internode length distribution (N = 500). **C:** Scatter diagram of internode lengths per cell illustrates the similarity of internode length distribution between cells.

system generates a clear and detailed representation of the cell in vivo despite the large amount of fluorescence present in the volume of tissue acquired in the z-stack.

Morphometric Analysis of OLs in the Frontal Cortex

The population of traced cells appeared grossly homogeneous at the qualitative level (Fig. 3), and quantitative analysis showed a low degree of variability in all morphological parameters (Fig. 4). For example, the mean process circumference (total process surface area including internodes/total process length including internode length) was quite constant at 3.11 ± 0.06 μm. As expected from the qualitative observations, internode lengths were quite similar within each cell and between cells, and they were normally distributed (Fig. 4). Similarly, the number of internodes, primary processes, and branch points per cell did not vary significantly between the cells (Fig. 4A), and the maximal branch order, or number of branch points on the single most complex cell process, for this population of OLs was 5.47 ± 0.26. Together, these data suggest that this is a highly homogeneous population of OLs with distinct morphological characteristics.

Sholl Analysis of OLs

Sholl analysis is a method for quantitative study of radial distribution of arborization patterns around the cell's perikaryon and has been used primarily for analysis of dendritic morphology and complexity. Here we used this method to analyze OL morphology. Traced cells were analyzed by centering nested spheres around the cell body with each sphere spaced 10 μm apart. To measure the extent to which processes grow out from the cell body, we identified the outermost sphere that each cell intersected and determined that the mean maximal shell radius for cells in this population was 68.0 ± 2.0 μm. To define further the morphological characteristics of these cells, we used Sholl analysis to determine the distribution of cell processes, branch points, and myelin internodes around the cell body. Approximately 80% of branch points were located between 10 and 40 μm away from the cell (Fig. 5A). Similarly, about 80% of internode endings were located between 10 and 50 μm away from the cell (Fig. 5B), and 80% of total process length, regardless of branch order, was located between 10 and 40 μm away from the cell (Fig. 5C). Together these data indicate that cells in this population of OLs are small, compact cells that do not myelinate axons far from their cell bodies.

DISCUSSION

Developing tools to quantify OL morphology is an important step toward understanding the processes of OL development, myelination, and remyelination after injury.

Here we define a method by which OL three-dimensional morphology can be analyzed and quantified in vivo in great detail and with high throughput. We have done this with transgenic mice in which all OLs express eGFP under control of the *Plp* promoter. In contrast, most previous studies of OL morphology have used one of three approaches: modified Golgi or dicyanoargentate stain (Râio-Hortega, 1928; Ogawa et al., 1985), electron microscopy (Remahl and Hildebrand, 1990; Berger and Frotscher, 1994; Bjartmar et al., 1994), or labeling individual cells using cellular injections or viral infection (Ransom et al., 1991; Butt et al., 1994; Weruaga-Prieto et al., 1996; McIver et al., 2005). Although these methods have been useful, they have significant drawbacks. The modified Golgi stain is a stochastic method that labels some but not all OLs and can often result in incomplete staining of cellular processes and little or no staining of the myelin sheath. Furthermore, this staining technique also labels other glial cells, making the identification of cells more difficult. Although EM can give the highest resolution imaging, three-dimensional reconstruction of cells as complex as OLs is a very labor-intensive approach not conducive to the high throughput necessary for analysis of cell populations. Cellular injections of dyes and viral infections carry the risk of altering cell morphology. Thus, eGFP-expressing mice provide an effective tool for studying OL morphology. These mice can be subjected to treatments that affect OLs or be crossed with genetically modified mice to study OL morphology under different conditions.

Most prior studies of OL morphology have been limited to two-dimensional analysis (using camera lucida). Here we show that a cell tracing system originally designed to quantify neuronal morphology can be use to study OL morphology in great detail. This cell tracing system requires only that the processes and internodes of the cell being traced be easily identified after confocal imaging. Therefore, any method by which individual OLs are visualized, dye filling of cells, RIP monoclonal antibody staining, or infection by virus, is compatible with the cell tracing system, and future studies using these methods are likely to affect our understanding of OL function in the CNS.

The original classification of OL morphology, using a modified Golgi method (Penfield, 1932; Râio-Hortega, 1928), subdivided OLs into four types: type I OLs had small cell bodies and five to 10 processes; type II cells had larger cell bodies and three to five processes; type III and IV OLs had large cell bodies, were associated with large-diameter axons, and had only one or two primary processes. More recent studies using a variety of techniques for

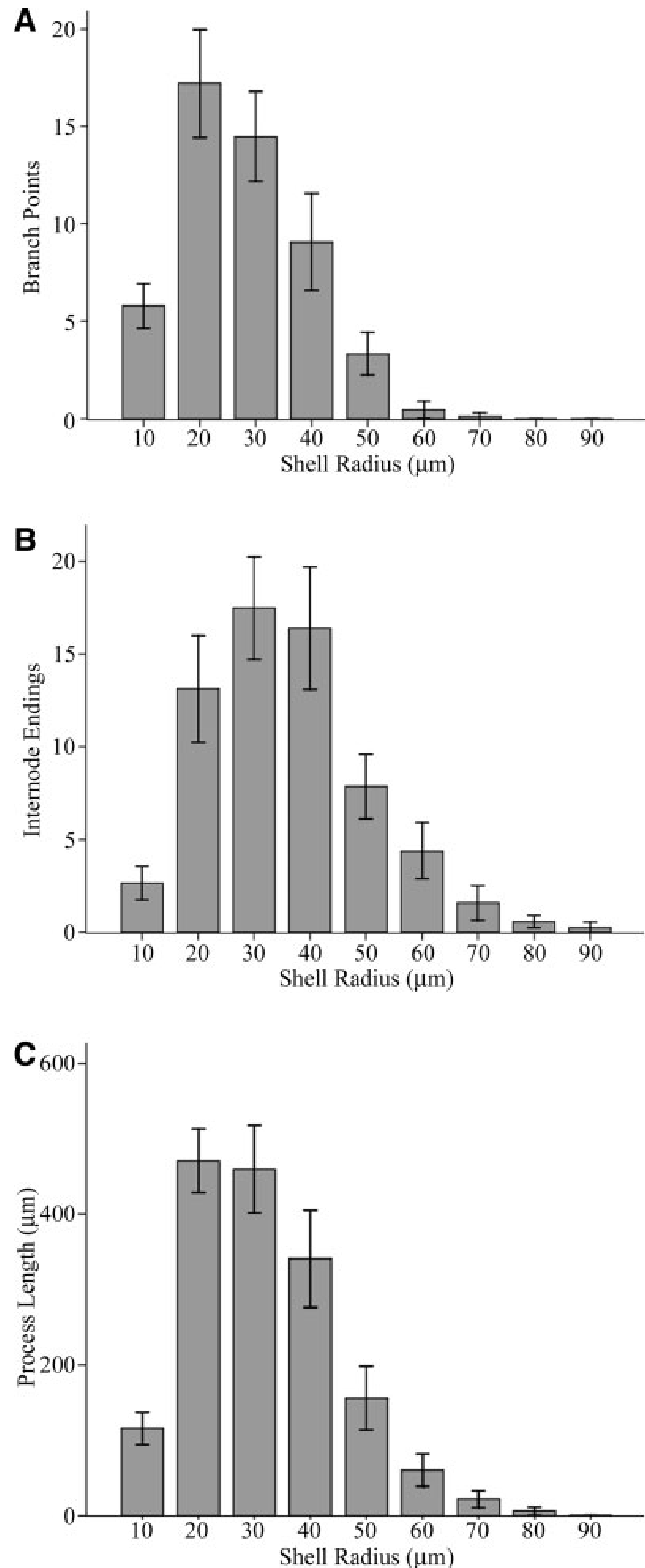


Fig. 5. Sholl analysis of oligodendrocytes in the frontal cortex indicates that cells in this population are highly compact. That distances from the cell body for branch points (**A**), internode endings (**B**), and process length (**C**) occur independently of the previous shell indicates that the vast majority of the network of processes and internodes is found very close to the soma and that oligodendrocytes in this population do not myelinate targets far from the cell body. Error bars represent SEM.

visualizing OLs have described a broader range of possible OL morphologies that did not strictly adhere to the original classification system (Ogawa et al., 1985; Butt and Ransom, 1989, 1993; Friedman et al., 1989; Remahl and Hildebrand, 1990; Jhaveri et al., 1992; Bjartmar et al., 1994; Butt et al., 1994; Weruaga-Prieto et al., 1996), suggesting that more than the original four types of OLs might exist. By using new methods, we have now identified a population of OLs in the adult frontal cortex with unique morphological characteristics. The OL population we characterized appears remarkably homogeneous in all morphological parameters, much more so than reported in previous studies (Râio-Hortega, 1928; Ogawa et al., 1985; Berger and Frotscher, 1994; Butt et al., 1994). These cells are also different from previously described OLs in several ways. For example, the OLs described herein are at the upper end of the distribution of the number of primary processes and internodes found in previously characterized OLs (Butt et al., 1994; Weruaga-Prieto et al., 1996). Furthermore, the average internode length of the OLs described herein is considerably less than that in previous reports, in which internode length ranged from approximately 30 μm to 1 mm. Furthermore, many of the internodes in this OL population were smaller than 20 μm. It is interesting that OLs that have long internodes appear to have fewer of them. Thus, our findings suggest that there may be a trend toward an inverse relationship between the number of processes and the number of internodes, as well as support previous speculation on a similar inverse relationship between the number of internodes per cell and the average internode length (Butt et al., 1994). Although the cells described in this study were selected randomly, some cells in the frontal cortex were excluded from analysis because part of the arbors extended beyond the volume contained in the z-stack. It is possible that populations of OLs with other morphological characteristics exist within the adult frontal cortex. Nevertheless, the fact that all morphological parameters of the OLs that we have analyzed have normal distribution indicates that this is truly an individual and particular population. It is likely that differences in OL morphology result from differences in the neurons that they myelinate. Whether differences in the morphological characteristics of these neurons, for example, axonal diameter, or differences in their molecular make-up are responsible for the variations in OL morphology remains to be determined. Further analysis of OL morphology in different brain regions as well as comparisons of OL morphology in mouse mutants with defects in molecules that are expressed by neurons and are known to regulate OLs should provide insights into these questions.

In summary, we developed a high-throughput technique that allows for the analysis of OL morphology to a degree that was previously unattainable. Using this method, we have identified and characterized a unique population of small and compact OLs in the frontal cortex of adult mice that have an unusually high number of processes and many short internodes. With the tools described herein, it becomes possible to study how OL morphology is altered under different conditions, e.g., mutations in specific signaling pathways, exposure to drugs or injury, or experience. Furthermore, this method should facilitate studies investigating how OL morphology impacts the function of associated neurons and how morphology influences OL function during development and disease.

REFERENCES

Berger T, Frotscher M. 1994. Distribution and morphological characteristics of oligodendrocytes in the rat hippocampus in situ and in vitro: an immunocytochemical study with the monoclonal Rip antibody. J Neurocytol 23:61–74.

Bjartmar C, Hildebrand C, Loinder K. 1994. Morphological heterogeneity of rat oligodendrocytes: electron microscopic studies on serial sections. Glia 11:235–244.

Butt AM, Ransom BR. 1989. Visualization of oligodendrocytes and astrocytes in the intact rat optic nerve by intracellular injection of lucifer yellow and horseradish peroxidase. Glia 2:470–475.

Butt AM, Ransom BR. 1993. Morphology of astrocytes and oligodendrocytes during development in the intact rat optic nerve. J Comp Neurol 338:141–158.

Butt AM, Colquhoun K, Tutton M, Berry M. 1994. Three-dimensional morphology of astrocytes and oligodendrocytes in the intact mouse optic nerve. J Neurocytol 23:469–485.

Cannistraro PA, Makris N, Howard JD, Wedig MM, Hodge SM, Wilhelm S, Kennedy DN, Rauch SL. 2006. A diffusion tensor imaging study of white matter in obsessive-compulsive disorder. Depress Anxiety (in press).

Edgar JM, Garbern J. 2004. The myelinated axon is dependent on the myelinating cell for support and maintenance: molecules involved. J Neurosci Res 76:593–598.

Friedman B, Hockfield S, Black JA, Woodruff KA, Waxman SG. 1989. In situ demonstration of mature oligodendrocytes and their processes: an immunocytochemical study with a new monoclonal antibody, rip. Glia 2:380–390.

Hajek T, Carrey N, Alda M. 2005. Neuroanatomical abnormalities as risk factors for bipolar disorder. Bipolar Disord 7:393–403.

Hartman BK, Agrawal HC, Agrawal D, Kalmbach S. 1982. Development and maturation of central nervous system myelin: comparison of immunohistochemical localization of proteolipid protein and basic protein in myelin and oligodendrocytes. Proc Natl Acad Sci U S A 79:4217–4220.

Hildebrand C, Remahl S, Persson H, Bjartmar C. 1993. Myelinated nerve fibres in the CNS. Prog Neurobiol 40:319–384.

Jhaveri S, Erzurumlu RS, Friedman B, Schneider GE. 1992. Oligodendrocytes and myelin formation along the optic tract of the developing hamster: an immunohistochemical study using the Rip antibody. Glia 6:138–148.

Kettenmann H, Ransom BR. 2005. Neuroglia. New York: Oxford University Press. p 601.

Mallon BS, Shick HE, Kidd GJ, Macklin WB. 2002. Proteolipid promoter activity distinguishes two populations of NG2-positive cells throughout neonatal cortical development. J Neurosci 22:876–885.

McIver SR, Lee CS, Lee JM, Green SH, Sands MS, Snider BJ, Goldberg MP. 2005. Lentiviral transduction of murine oligodendrocytes in vivo. J Neurosci Res 82:397–403.

Norton WC. 1984. Myelin. Morell P, editor. New York: Plenum Press. p 147–195.

Ogawa Y, Eins S, Wolff JR. 1985. Oligodendrocytes in the pons and middle cerebellar peduncle of the cat. Topographical relations to neurons and transverse axon bundles. Cell Tissue Res 240:541–552.

Penfield W. 1932. Cytology and cellular pathology of the nervous system, vol 3. New York: P.B. Hoeber, Inc.

Râio-Hortega Pd. 1928. Tercera aportaciâon al conocimiento morfolâogico e interpretaciâon funcional de la oligodendroglâia. Madrid, 122 p.
Ransom BR, Butt AM, Black JA. 1991. Ultrastructural identification of HRP-injected oligodendrocytes in the intact rat optic nerve. Glia 4:37–45.
Remahl S, Hildebrand C. 1990. Relations between axons and oligodendroglial cells during initial myelination. II. The individual axon. J Neurocytol 19:883–898.
Sternberger NH, Quarles RH, Itoyama Y, Webster HD. 1979. Myelin-associated glycoprotein demonstrated immunocytochemically in myelin and myelin-forming cells of developing rat. Proc Natl Acad Sci U S A 76:1510–1514.
Sullivan EV, Pfefferbaum A. 2003. Diffusion tensor imaging in normal aging and neuropsychiatric disorders. Eur J Radiol 45:244–255.
Walterfang M, Wood SJ, Velakoulis D, Pantelis C. 2006. Neuropathological, neurogenetic and neuroimaging evidence for white matter pathology in schizophrenia. Neurosci Biobehav Rev 30:918–948.
Weruaga-Prieto E, Eggli P, Celio MR. 1996. Topographic variations in rat brain oligodendrocyte morphology elucidated by injection of Lucifer Yellow in fixed tissue slices. J Neurocytol 25:19–31.
Whiteside SP, Port JD, Deacon BJ, Abramowitz JS. 2006. A magnetic resonance spectroscopy investigation of obsessive-compulsive disorder and anxiety. Psychiatry Res 146:137–147.