The Distribution of Mitochondrial Activity in Relation to Optic Nerve Structure

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Background: The observation of a buildup of mitochondria at the level of the lamina cribrosa in the optic nerve head has traditionally been attributed to axoplasmic stasis. However, this region is also the transition zone for myelination, resulting in differing energy requirements.

Objective: To investigate the relationship between myelination and mitochondrial activity in optic nerve tissue.

Methods: Histological, histochemical, and immunocytochemical techniques were used to demonstrate the distribution of myelin, cytochrome-\text{c} oxidase activity, and laminar structure in human optic nerve tissue. A study of rabbit optic nerve and retina and unmyelinated human pituitary stalk was also performed. Cytochrome-\text{c} oxidase activity in the human optic nerve tissue was measured using microphotometry.

Results: There was a striking inverse relationship between myelination and mitochondrial distribution in all tissue studied. Statistical analysis of microphotometric data showed this distribution to be highly significant.

Conclusion: We caution against the previous inference of a process of axoplasmic stasis and suggest that, instead, the distribution of mitochondria reflects the functional requirement of different regions of the ganglion cell axon.

Clinical Relevance: Optic neuropathy is associated with several inherited disorders of mitochondria. We suggest that a fine balance exists between energy demand and tissue function in the optic nerve, which may explain why optic nerve pathological features are seen in those with mitochondrial disease.

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The optic nerve is an extension of the central nervous system with unique structural features. Unmyelinated nerve fibers exit the eye through the lamina cribrosa, becoming myelinated at the posterior border. At the ultrastructural level, the concentration of mitochondria decreases dramatically at the level of the lamina. The finding of increased numbers of mitochondria in the prelaminar and laminar regions of the optic nerve has traditionally been attributed to mechanical constriction or axoplasmic stasis at the lamina.\footnote{This, in turn, has influenced our investigation and understanding of optic neuropathies, such as Leber hereditary optic neuropathy and glaucoma, but leaves many unanswered questions.} We suggest that mitochondrial distribution has little to do with the laminar structure, but rather reflects a functional requirement of this highly specialized tissue, as myelinated and unmyelinated fibers have different bioenergetic properties.

In this study, we sought to relate the differences in mitochondrial enzyme activity to the distribution of myelination. In addition to normal human optic nerve, we studied pig optic nerve, which is structurally similar to the human, and rabbit optic nerve, which has important differences. In contrast to human and pig optic nerve, the rabbit has no well-ordered laminar structure and the entire optic nerve is myelinated.

Myelination continues onto the retina in a horizontal band.\footnote{This provides an opportunity not only to look at mitochondrial enzyme activity in the myelinated optic nerve but also to observe what happens at the myelination/demyelination interface on the retina. We also studied human pituitary stalk nerve fibers, as these are one of few examples of other unmyelinated tissue within the central nervous system.}

We confirmed that there is a striking inverse relationship between cytochrome-\text{c} oxidase activity and myelination following observation of longitudinal (Figure 1) and transverse (Figure 2) sections of the
Materials and Methods

Materials and Preparation

Following approval by the local research ethics committee, postmortem human optic nerve tissue was obtained from 8 corneal donors, aged 22 to 84 years, with no history of ocular or mitochondrial disease. Once the corneal scleral disc was removed and placed in transport medium (Optisol GS; Chiron Intraoptics, Irvine, Calif) for transfer to one of the national eye banks, the optic nerve was removed from the globe with a small surrounding collar of retina. Specimens were mounted on gelatin blocks and rapidly frozen in isopentane and liquid nitrogen before storing at −80°C.

Pig optic nerve tissue was prepared in a similar fashion, while rabbit globes were frozen whole before dissection to maintain the fragile retinal nerve fiber layer. All animal tissue was harvested postmortem, and care and housing conformed to codes of practice under the Prevention of Cruelty to Animals Act 1986 (part 3, scientific procedures).

The postmortem time for human optic nerve ranged from 3 to 22 hours; for pig, 3 to 6 hours; and for rabbit, 20 to 60 minutes. A sample of pituitary stalk tissue (35 hours post mortem) had been snap frozen and stored at −80°C.

Sections (10 µm thick) were cut using a cryostat microtome (model 2800N Frigocut; Reichert Ophthalmic Instruments, Depew, NY). Longitudinal and transverse sections of the optic nerve were studied.

Histological Methods

Sudan black B fat staining was used to demonstrate myelination, and hematoxylin-eosin staining was used to confirm good tissue preservation. Histochemical localization of cytochrome-c oxidase activity was performed by incubating sections at 38°C for 1 hour in a combination of 4mM 3,3′-diaminobenzidine hydrochloride and 100µM cytochrome c in 0.1M phosphate buffer (pH 7.0). A mouse monoclonal antibody to cytochrome-c oxidase subunit II was used in a 1:500 dilution to confirm the distribution of cytochrome-c oxidase components in human tissue. The technique used has been previously described. The lamina cribrosa structure was demonstrated using mouse monoclonal laminin antibodies α5 (1 in 100), α2 (1 in 100), γ1 (1 in 100), and β1 (1 in 1000) (Chemicon International, Inc, Temecula, Calif). Sections were air dried for 1 hour before application of the primary antibody and incubation for 1 hour at room temperature. Sections were then washed in 0.0125M phosphate-buffered saline for 30 minutes and incubated for 1 hour with rabbit anti–mouse peroxidase conjugate (1 in 100; Dako, Glostrup, Denmark). Following further washes in phosphate-buffered saline for 30 minutes, the sections were developed in 0.05% 3,3′-diaminobenzidine and 0.01% hydrogen peroxide for 15 minutes. The neurofilament antibody SMI 31 (1 in 200; Affiniti Research Products Ltd, Exeter, England) was used to confirm the presence of nerve fiber tissue in sections of pituitary stalk. The same secondary antibody labeling method as previously described was used.

Sections of normal striate muscle were used for controls. Retinal tissue surrounding the optic nerve in all sections also served as an internal control for cytochrome-c oxidase studies, because the outer segment of photoreceptors has high cytochrome-c oxidase activity.

Quantification

Seven 10-µm longitudinal sections of human optic nerve, taken from 2 individuals, were used. Following histochemical demonstration of cytochrome-c oxidase activity, the images were divided into columns of 20 points, separated by approximately 100 µm. A total of 1195 points were sampled using a microphotometer (Universal Microphometer System, model 30; Carl Zeiss, Göttingen, Germany) with a computer-controlled scanning stage.
oxidase subunit II antibody conforms to the same pattern, showing that the high cytochrome-c oxidase activity in unmyelinated regions is a result of an increased concentration of respiratory chain components.

We quantified the variation in cytochrome-c oxidase activity across the human optic nerve head. Microphotometric analysis shows that the difference in cytochrome-c oxidase activity between prelaminar and laminar regions when compared with the postlaminar optic nerve is highly statistically significant in all sections studied. A t test comparing the cytochrome-c oxidase activity in prelaminar vs postlaminar regions in patient A was significant ($P<.001$, $t_{29} = 26.93$). For patient B, the comparison was also significant ($P<.001$, $t_{19} = 29.36$; Figure 3).

The pig optic nerve has a similar arrangement to the human, although the laminar structure is placed in a more anterior position. In the sections studied, myelination corresponded closely to the posterior lamina. As in the human optic nerve, cytochrome-c oxidase activity was inversely related to myelination.

The rabbit optic nerve has a different structure. Sudan black B fat staining confirmed that the optic nerve head in these animals is myelinated throughout. Immunocytochemistry using laminin antibodies also confirmed that there is no organized laminar structure.

When compared with pig and human tissue, cytochrome-c oxidase activity in the rabbit was low and uniform throughout the optic nerve head. Serial sections of

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**Figure 2.** Serial sections of the human optic nerve in cross section. A, Sudan black B fat staining shows myelination. B, Immunocytochemical labeling for laminin to demonstrate the posterior laminar position. C, Cytochrome-c oxidase histochemistry demonstrating relatively high levels of activity in unmyelinated regions. D, Immunocytochemical labeling of the cytochrome-c oxidase subunit II monoclonal antibody showing a similar distribution to that shown by cytochrome-c oxidase histochemistry. Original magnification ×40 for all illustrations.

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**Figure 3.** Relative cytochrome-c oxidase (COX) activity across regions of the optic nerve head in patients A and B.
the retina at the myelination interface show low cytochrome-c oxidase activity in the myelinated nerve fibers, similar to that seen in the optic nerve head, and higher cytochrome-c oxidase activity in the unmyelinated nerve fibers. These features are demonstrated in Figure 4.

Immunocytochemical labeling with antibody SMI 31 confirmed that sections of pituitary stalk contained the neurohypophysial tract. Sudan black B fat staining also demonstrated that these nerve fibers were unmyelinated. There was a stark contrast between the low cytochrome-c oxidase activity of surrounding pituitary tissue and the reaction seen in the unmyelinated neurohypophysial fibers, which was comparable to that of an unmyelinated human optic nerve head of a similar age despite a considerably longer postmortem delay. These features are demonstrated in Figure 5.

The concept of constriction at the lamina cribrosa of the optic nerve originates from the work of Weiss and Hiscoe on large myelinated fibers, published in 1948. They simulated compression with arterial sleeves and observed a buildup of axoplasmic material proximal to the strictures. Similarities were drawn between this and normal optic nerve despite obvious differences between these 2 tissues. Many researchers followed, observing increased accumulation of mitochondria with increased intraocular pressure. Minckler et al found increased numbers of mitochondria at the level of the lamina in the rhesus monkey, but failed to show any reduction in axonal diameter during passage through the lamina. In addition,
bearing in mind that mitochondria are fluid structures able to assume different shapes to conform to their environment, the study demonstrated no change in the size of mitochondria to support a constriction theory. A more recent study of active transport components in the guinea pig optic nerve by Ou et al reported a reduction in active transport components responsible for mitochondrial carriage at the level of the lamina and a corresponding increase in mitochondrial numbers. This and other studies have developed our understanding of mitochondrial movement, indicating a much more active ordered mechanism than previously thought.

Our study of normal human optic nerve tissue demonstrates a distinct distribution of mitochondrial enzyme activity. Unmyelinated prelaminar and laminar regions display high mitochondrial enzyme activity when compared with postlaminar myelinated regions.

We found that histochemical and immunocytochemical labeling is useful and can be used to make comparisons between differing regions of individual optic nerves.

We sought to look closely at the relationship of mitochondrial activity to the laminar structure and mye- lination. This is a difficult task in the human optic nerve because the posterior border of the lamina is usually closely related to the onset of myelination.

Furthermore, it is difficult to define the posterior limit of the laminar structure on longitudinal section. Despite these limitations, there are regional differences in individuals and the mitochondrial enzyme activity more closely correlates with myelination than with laminar structure. While most mammals have a similar optic nerve arrangement, the rabbit provides an excellent opportunity to test our hypothesis.

Demonstration of a transition in mitochondrial enzyme activity from high to low in the nerve fiber layer at the point of myelination on the retina supports our hypothesis.

A study of pituitary stalk unmyelinated nerve fibers showing similar mitochondrial enzyme activity to the unmyelinated region of the human optic nerve provides further evidence to suggest that levels seen in the optic nerve are not artificially high due to primary axoplasminc stasis but rather reflect a dynamic functional requirement of this part of the nerve.

The main function of mitochondria is the production of adenosine triphosphate energy, which is essential for any cell’s survival. Individual cells have differing metabolic requirements, and this is also true at an intracellular level. Previous studies have shown that, in the retina, differing cell layers have varying cytochrome-c oxidase activities. Furthermore, within the photoreceptor cells, the outer segments show a high degree of cytochrome-c oxidase activity when compared with inner segments because they require an enormous amount of energy for maintenance of their membrane potential. This region of the cell is packed with mitochondria. We suggest that the retinal ganglion cell and its axon also display differing requirements. Myelinated nerve fibers conduct by saltatory conduction, while unmyelinated fibers require more energy to repolarize the plasma membrane. Therefore, the unmyelinated prelaminar and laminar regions require more mitochondrial enzyme activity. While this study is based on histochemical demonstration of mitochondrial activity, other structural investigations support this idea. Electron microscopic studies conducted by Hollander et al demonstrated more mitochondria on both sides of the human lamina cribrosa in unmyelinated fibers in the human optic nerve and no accumulations in myelinated fibers. In rabbits, in which most fibers were myelinated, accumulations of mitochondria were not seen. While these findings were interpreted as evidence of antegrade and orthograde flow restriction in unmyelinated fibers by the lamina cribrosa, this study would also support our hypothesis. Our observations differ in that we did not find a significant gradient of cytochrome-c oxidase activity relating to laminar position along the length of the unmyelinated fibers. Other work has shown that, in myelinated fibers, mitochondria collect in the nodes of Ranvier, where the membrane potential undergoes flux.

Hepplemann et al studied the peripheral afferent nerve fiber in the cat, commenting that the unmyelinated region of the fiber contained more mitochondria than the myelinated segment. A study of mutant rats with...
unmyelinated optic nerves reported an increase in the numbers of mitochondria compared with controls. Finally, a study\(^1^0\) of cats in which optic nerve demyelination was induced while preserving axoplasmic transport showed an initial increase in mitochondrial numbers in the demyelinated segment, which then decreased to normal levels during remyelination.

Our work and that of others cited suggest that mitochondrial enzyme activity is tailored to the needs of specific regions and that this relates not only to different tissues but also to individual cells. It is possible that, in disease processes, mitochondrial movement is altered in an attempt to compensate and maintain function.

Given that the unmyelinated optic nerve has a high relative demand for mitochondrial enzyme activity, this region may also be extremely sensitive to mitochondrial deficits. This might explain why optic neuropathies occur in those with mitochondrial inherited diseases. Leber hereditary optic neuropathy specifically targets the optic nerve, but other mitochondrial inherited diseases, including myoclonic epilepsy and ragged red fibers, chronic progressive external ophthalmoplegia, Leigh syndrome, and mitochondrial encephalopathy, lactic acidosis, and strokelike episodes syndrome are also associated with optic nerve dysfunction.\(^2^0\) While these mitochondrial inherited diseases are relatively rare, our hypothesis also challenges our approach to other more common optic neuropathies, such as glaucoma. More work is needed to advance our understanding of the dynamics of mitochondrial distribution, movement, and function in disease processes.

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