Mitochondrial dysfunction as a cause of optic neuropathies

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Abstract

Mitochondria are increasingly recognized as central players in the life and death of cells and especially of neurons. The energy-dependence of retinal ganglion cells (RGC) and their axons, which form the optic nerve, is singularly skewed. In fact, while mitochondria are very abundant in the initial, unmyelinated part of the axons anterior to the lamina cribrosa, their number suddenly decreases as the myelin sheath begins more posteriorly. The vascular system also presents different blood–brain barrier properties anterior and posterior to the lamina, possibly reflecting the different metabolic needs of the optic nerve head (unmyelinated) and of the retrobulbar optic nerve (myelinated).

Mitochondrial biogenesis occurs within the cellular somata of RGC in the retina. It needs the coordinated interaction of nuclear and mitochondrial genomes. Mitochondria are then transported down the axons and distributed where they are needed. These locations are along the unmyelinated portion of the nerve, under the nodes of Ranvier in the retrobulbar nerve, and at the synaptic terminals. Efficient transportation of mitochondria depends on multiple factors, including their own energy production, the integrity of the cytoskeleton and its protein components (tubulin, etc.), and adequate myelination of the axons. Any dysfunction of these systems may be of pathological relevance for optic neuropathies with primary or secondary involvement of mitochondria.

Leber’s hereditary optic neuropathy (LHON) is the paradigm of mitochondrial optic neuropathies where a primary role for mitochondrial dysfunction is certified by maternal inheritance and association with specific mutations in the mitochondrial DNA (mtDNA). Clinical phenocopies of this pathology are represented by the wide array of optic neuropathies associated with vitamin depletion, toxic exposures, alcohol and tobacco abuse, and use of certain drugs. Moreover, the recent identification of mutations in the nuclear gene OPA1 as the causative factor in dominant optic atrophy (DOA, Kjer’s type) brought the unexpected finding that this gene encodes for a mitochondrial protein, suggesting that DOA and LHON may be linked by similar pathogenesis. Polymorphisms in this very same gene may be associated with normal tension glaucoma (NTG), which might be considered a genetically determined optic neuropathy that again shows similarities with both LHON and DOA.

Exciting new developments come from first examples of mitochondrial optic neuropathies in animal models that are genetically determined or are the result of ingenious engineering of mitochondrial gene expression, or from biochemical manipulations of the respiratory complexes. Even more exciting is the first successful attempt to correct the LHON-related complex I dysfunction by the allotopic nuclear expression of the recoded mitochondrial gene. There is hope that the genetic complexities, biochemical dysfunctions, and integrated anatomical–physiological cellular relationships will soon be precisely delineated and that promising therapeutic and prophylactic strategies will be proposed.

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Keywords: Leber’s; LHON; DOA; OPA1; Mitochondria; Optic neuropathy; Retinal ganglion cell; Complex I

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1. Introduction

In 1988, Wallace et al. (1988) reported the first point mutation in the mitochondrial DNA (mtDNA) in patients with Leber’s hereditary optic neuropathy (LHON), a disorder which was first described by Leber (1871). This discovery was a turning point. For the first time, the recognized maternal inheritance (Giles et al., 1980) of a human disorder was explained by the cytoplasmic transmission of a genetic error in the mitochondrial genome, as had been anticipated (Erickson, 1972). At the same time, this observation showed that the optic nerve is highly dependent on mitochondrial function and that a wide category of optic neuropathies of both genetic and environmental origin, sharing some common peculiar features, are determined by mitochondrial dysfunction (Carelli et al., 2002a; Sadun, 2002).

In 2000 came the remarkable but not surprising news that the genetic cause of dominant optic atrophy (DOA), also known as Kjer’s optic neuropathy, had been identified. This turned out to involve a protein encoded by the nuclear gene OPA1 that is imported into the mitochondria (Delettre et al., 2000; Alexander et al., 2000). Thus, from mtDNA back to the nuclear genome, mitochondria maintain a central role in determining optic nerve pathology and retinal ganglion cell (RGC) death.

2. Retinal ganglion cells and the optic nerve: what makes them so uniquely vulnerable?

All 1.2 million fibers of the optic nerve derive from the RGC of the inner retina (Sadun, 1986; Sadun et al.,
1986a, b; Fredericks et al., 1988). These cells occupy the space between the inner plexiform layer and the overlying retinal nerve fiber layer (NFL). In addition to the RGC, there are some displaced amacrine cells in this layer. The blood supply is primarily retinal since the choroidal circulation can only supply the outer half of the retina.

The distribution of RGC is very non-linear. In the perifoveal macula, the RGC are concentrated and stacked six or seven cells deep (Polyak, 1941; Fine and Yanoff, 1979). Further from the fovea, the numbers of RGC decreases, becoming relatively sparse in the periphery with each of the cells quite distant from the others. It can also be noted that not all RGC look alike (Wall and Sadun, 1989). Those near the fovea tend to be smaller with much smaller dendritic fields. Their axons are also of smaller caliber. As one moves peripherally some larger RGC are also encountered. The largest probably belong to a separate class of neurons, the magno (M)-cells (Livingstone and Hubel, 1987), which are more fully discussed below. However, both these M-cells, and the smaller parvo (P)-cells are smaller near the fovea and larger further out (Shapley and Perry, 1986).

Each RGC contributes an axon, which travels inwardly (toward the vitreous) into the NFL and then toward the optic nerve head. There, the fibers turn and exit the eye posteriorly as the thick optic nerve (Sadun, 1998b; Ogden, 1983) (Fig. 1). Recently, the organization of the intraretinal ganglion cell axons has been studied in greater detail in humans and non-human primates. Single axons were described as forming varicosities rich in mitochondria and desmosome- and hemidesmosome-like junctions with other axons or glial cells (Wang et al., 2003). These findings were interpreted as functional sites with local high-energy demand, possibly relevant for signal transmission. These NFL axons, with some retinopy (Ogden, 1983; Hoyt and Luis, 1962), coalesce and form the optic nerve head, much as spokes converging to the axle of a wheel. The 1.2 million axons then make an orthogonal turn posteriorly and pierce the many collagen plates that form the lamina cribrosa (Glaser and Sadun, 1990). Posterior to the lamina

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**Fig. 1.** RGC system: diagrammatic view of pattern for staining of COX. On the left side is a sagittal view of the human eye. The boxed part is enlarged and schematically illustrated, with emphasis on the RGC cellular system. Mitochondrial distribution is shown from the RGC somata through the unmyelinated (COX+) portion of the axon in the NFL and penetrating the lamina cribrosa at the optic nerve head. Mitochondrial accumulations are represented in the varicosities within the NFL, and in the prelaminar region. There is a drastic decrease in mitochondrial numbers within the retrolaminar portion (COX−). Mitochondria, in this part of the RGC axon, are mostly located under the unmyelinated nodes of Ranvier. On the right side is illustrated a histological sagittal section of a normal human eye showing a parallel transition from the retina to the optic nerve head, the lamina cribrosa and a portion of the myelinated retrolaminar optic nerve. The ocular tissue was stained by a method using immunoperoxidase for myelin basic protein.
staining suddenly decreases posteriorly in concert with the NFL and the prelaminar–laminar RGC axons. This ulation (OXPHOS). Intense COX staining characterizes myelination (Minckler et al., 1976; Andrews et al., 1999; relationship between the mitochondrial activities and

Recent studies of human retina and optic nerve specimens using electron microscopy and histoenzymatic stains for the mitochondrial retinal and optic nerve specimens using electron microscopy and histoenzymatic stains for the mitochondrial respiratory complexes elegantly showed an inverse relationship between the mitochondrial activities and myelination (Minckler et al., 1976; Andrews et al., 1999; Bristow et al., 2002). Cytochrome c oxidase (COX) accumulates at the sites of greatest oxidative phosphorylation (OXPHOS). Intense COX staining characterizes the NFL and the prelaminar–laminar RGC axons. This staining suddenly decreases posteriorly in concert with the appearance of myelin posterior to the lamina (Andrews et al., 1999; Bristow et al., 2002). These findings indicate an asymmetric distribution of mitochondria along the RGC axons that matches the energy and functional requirements of the axons related to their state of myelination (Fig. 1).

By virtue of their unmyelinated stretches, RGC axons are bioenergetically weak elements of the central nervous system. This is more remarkable if we consider that the brain itself has tremendous energy needs. Although it makes up only about 2% of the average person’s body weight, the human brain accounts for 20% of the body’s total energy consumption (Raichle and Gusnard, 2002; Shulman et al., 2003). Furthermore, the relative oxygen consumption of the retina is higher than that of the brain, being one of the highest oxygen-consuming tissues of the body (Yu and Cringle, 2001). Interestingly, a neuron-specific respiratory protein called neuroglobin has been found widely distributed in the murine retina in strict association with the subcellular localization of mitochondria and with the relative oxygen demands, including RGC (Schmidt et al., 2003).

About 50 mm posterior to the eye, the two optic nerves converge to form the optic chiasm, where a partial decussation (of about 53%) occurs (Kupfer et al., 1967). Only the axons from the nasal retina (temporal visual field) cross over joining the contralateral optic nerve’s temporal retinal fibers and form the contralateral optic tract. From the optic tract, the retinoferugal fibers terminate into one of several primary visual nuclei. At the chiasmal/tract junction, fibers diverge and enter one of three hypothalamic nuclei (the supraoptic nucleus, the superchiasmatic nucleus, and the paraventricular nucleus, Sadun et al., 1986b). The main body of the optic tract proceeds further posteriorly, with most of the retinoferugal fibers in man terminating in one of six layers of the lateral geniculate nucleus (LGN) (Sadun, 1986). Layers 1 and 2 (which are most proximal to the optic tract entrance) receive M-cell RGC afferents and layers 3–6 receive P-cell afferents (see below) (Livingstone and Hubel, 1987). Furthermore, afferents to layers 2, 3, and 5 are from the ipsilateral eye, and those to layers 2, 4, and 6 are from the contralateral eye. Additionally, a smaller percentage of optic tract fibers bypass the LGN and continue on toward the brachium of the superior colliculus. Most of these RGC fibers terminate into the superior colliculus and the pretectum just anterior to it (Sadun et al., 1986a). A few fibers proceed anteriorly and laterally to terminate in the pulvinar and the accessory optic system (Sadun et al., 1986a; Fredericks et al., 1988).

Over 90% of the RGC in the retina are P-cells (Sadun, 1992). These cells are non-linearly distributed, with a major peak in the macular area. The M-cells make up about 5–10% of the RGC in the retina, and they are only slightly more concentrated in the macula. Hence, the ratio of P-cells to M-cells is very high in the macula and closer to one-to-one in the far periphery (Glaser and Sadun, 1990). The P-cells and their projections to layers 3–6 of the LGN subserve the visual function of discrimination. For example, spatial resolution (visual acuity), color vision, texture, and high spatial frequency contrast sensitivity are mediated by the P-cell system (Livingstone and Hubel, 1987). In contrast, M-cells, and their projections to layers 1 and 2 of the LGN, subserve low spatial frequency contrast sensitivity, depth perception, and motion (Livingstone and Hubel, 1987). In some sense, the M-cell system gives more global information and is more concerned with addressing the issue of where in the visual system attention should be directed (Sadun and Bassi, 1990).

The papillomacular bundle (PMB) is composed of smaller axons. Most of these are P-cells. The few M-cell RGC found in the PMB are of considerably smaller caliber (Sadun et al., 2000). Hence, the small size of axons in the PMB may impart anatomical constraints for the axoplasmic transport of mitochondria. Mitochondria are typically about 0.4 µm across. Their biogenesis is carried out within the RGC soma and they are replicated through organelle splitting and budding. Hence, the RGC must transport the mitochondria along their very long axons to distribute them along strategic, energy-dependent locations in the optic nerve (see below for discussion; Grafstein, 1995). These are mainly the unmyelinated portions of the axons within the eye. Some particular areas that require concentrations of mitochondria are the gap junctions, described among the varicosities of the NFL; the prelaminar and laminar regions of the optic nerve head.
that allow for many cells to die at once. For example, it is intriguing that after decades of mitochondrial duress, as considered above, most of the 1.2 million axons abruptly die all at once, about three decades after birth (Carelli, 2002). For this to happen, RGC must either share a common reservoir of trophic factors or be able to quickly communicate to each other with a pro-apoptotic signal. One such type of connection that would allow for the propagation of a wave of apoptosis may be the gap junctions that lie between RGC axons, especially along the varicosities in the retinal NFL (Wang et al., 2003; Dermietzel and Spray, 1993). Indeed, some studies suggest that, by allowing for the passage of calcium and ROS, such gap junctions are responsible for the centripetal extension of spreading neuron death and that agents that close off these gap junctions can limit the propagation of apoptosis from a nidus of injury (Rawanduzy et al., 1997).

3. Mitochondria are traveling powerhouses

Mitochondria are double-membrane cytoplasmic organelles that carry multiple copies of their own 16569-bp circular mtDNA. mtDNA is located in the matrix, the internal compartment delimited by the inner mitochondrial membrane (Nass and Nass, 1963; Anderson et al., 1981). It encodes for 13 proteins, which are essential subunit components of the OXPHOS enzymatic complexes (complexes I, III, IV, and V). Each complex is a multi-subunit enzyme embedded in the inner mitochondrial membrane. The remaining protein subunits (~80), which are components of the OXPHOS complexes, are encoded by the nuclear genome and assembled under a strictly coordinated control, together with the 13 mtDNA-encoded subunits. There are also 24 genes in the mtDNA that contribute to the local protein synthesis of these 13 structural genes. Two mitochondrial specific ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs) are needed because of the slight coding differences between mtDNA and nuclear DNA (nDNA). Thus, the OXPHOS system, which provides most of the energy to eukaryotic cells, is under this dual genetic control. Diseases caused by its dysfunction may be due to nDNA defects and thus follow the Mendelian genetic rules; or they may be due to mutations that occur in the mtDNA, which express their pathogenic potential according to the peculiar rules of mitochondrial genetics (Wallace, 1999; Smeitink et al., 2001).

Mitochondria host numerous metabolic pathways, including for example amino acid metabolism and fatty acid oxidation, but the functions on which we will focus here are those of oxidative energy metabolism, the related production of reactive oxygen species (ROS), and the role played by mitochondria in promoting and regulating the apoptotic death of the cell. We will also
consider the factors that regulate the organization of the mitochondrial network within the cell and affect these processes.

3.1. Respiratory chain and oxidative phosphorylation

Mitochondrial respiration is driven by a chain of sequentially organized redox reactions fed by reducing equivalents derived from the oxidative degradation of carbon substrates. The respiratory complexes shuttle electrons one to the other with the help of two specific co-factors: coenzyme Q (ubiquinone, CoQ) and cytochrome c (cyt c).

Complex I (NADH:ubiquinone oxidoreductase), the first site of the respiratory chain, transfers electrons from nicotinamide adenine dinucleotide (NADH) to CoQ, thereby generating ubiquinol (CoQH$_2$), which then shuttles two electrons to complex III (ubiquinol:ferricytochrome c oxidoreductase, cytochrome bc$_1$ complex) (Walker, 1992). Complex I is produced by the assembly of about 35–37 nDNA-encoded subunits (Carroll et al., 2002) and 7 mtDNA-encoded subunits; it is the most represented respiratory enzyme in the mtDNA. To date no specific assembly proteins have been identified for complex I, but their existence is not in doubt given the large number of subunits that must be integrated in its build up. Complex III has only one mtDNA-encoded subunit, cytochrome b; the other 10 subunits are nDNA-encoded, and at least one nDNA-encoded protein has been reported to be essential for the enzyme assembly (Berry et al., 2000; Zeviani et al., 2003). Ubiquinol is also produced by complex II (succinicate:ubiquinone oxidoreductase), which, in a pathway parallel to that of complex I, transfers electrons from flavin adenine dinucleotide (FADH$_2$) to CoQ. Thus, complex III also receives electrons from this parallel path, which bypasses complex I. Complex II is the only respiratory enzyme completely encoded by mtDNA (ATPase6 and ATPase8), which include mitochondrial inner membrane-embedded lipophilic co-factors, such as CoQ and alfa-tocopherol (vitamin E), mitochondrial intermembrane space-located proteins such as the Cu/ZnSOD (Mattiazi et al., 2002) and the apoptosis-inducing factor (AIF) (Klein.

3.2. Reactive oxygen species

The mitochondrial respiratory chain is also a major cellular source of ROS. ROS are generated by loose electrons spilling mainly from complexes I and III, and reacting with molecular oxygen to form the superoxide anion (O$_2^-$). The O$_2^-$ is readily converted into hydrogen peroxide (H$_2$O$_2$) by manganese superoxide dismutase (MnSOD, SOD2); H$_2$O$_2$ is further metabolized by glutathione peroxidase (GPx) into H$_2$O. Alternatively, H$_2$O$_2$ may also generate the hydroxyl radical (OH$^*$) in the presence of transition metals through the Fenton reaction. Furthermore, O$_2^-$ may react directly with nitric oxide (NO$^*$) to produce peroxynitrite (ONOO$^-$). There is also evidence that NO$^*$ can be produced endogenously in mitochondria by a mitochondrial nitric oxide synthase (mtNOS) (Giulivi, 2003).

Excessive ROS production from the respiratory chain may cause local damage to the Fe–S center of respiratory enzymes, such as complexes I, II and III, as well as to tricarboxylic acid cycle (TCA) enzymes, such as aconitase (Rotig et al., 1997; Melov et al., 1999). Moreover, the highly reactive ONOO$^*$ can nitrate tyrosine residues of nearby proteins and both complex I and MnSOD have been reported to be damaged by this process (Riobo et al., 2001; Yamamoto et al., 2002; Yamakura et al., 1998). Complex I can also be permanently damaged by ONOO$^*$ through thiol-nitrosylation (Clementi et al., 1998). The mtDNA itself is highly exposed to oxidative stress, causing the accumulation of multiple deletions, and lipid peroxidation may damage the mitochondrial membranes. The above-mentioned anti-oxidant enzymatic machinery is located in the matrix. An increasing number of other anti-oxidant mechanisms have also been reported. These include mitochondrial inner membrane-embedded lipophilic co-factors, such as CoQ and alfa-tocopherol (vitamin E), mitochondrial intermembrane space-located proteins such as the Cu/ZnSOD (Mattiazi et al., 2002) and the apoptosis-inducing factor (AIF) (Klein.
et al., 2002), and outer mitochondrial membrane-bound proteins, such as the proto-oncogene Bel-2 (Degli Esposti et al., 1999).

### 3.3. Apoptosis

The most recent focus on mitochondria relates to their role in controlling apoptotic cell death (Kroemer and Reed, 2000; Ravagnan et al., 2002; Mayer and Oberbauer, 2003). The seminal observation was that cyt c, besides being the electron shuttle to complex IV, can be released under specific circumstances of mitochondrial permeabilization. It moves from the mitochondrial intermembrane space into the cytosol, to form the complex now known as “apoptosome”, which is a central event in the apoptotic cascade (Liu et al., 1996; Li et al., 1997). The anti-apoptotic protein Bcl-2 blocks cyt c release, through its interaction with the outer mitochondrial membrane (Kluck et al., 1997; Yang et al., 1997). Similarly, other members of the same protein family, namely Bax, Bak, Bim, and Bid, act as pro-apoptotic factors (Kroemer and Reed, 2000). Moreover, a growing number of mitochondrial factors are reported as being released by mitochondria and inducing or regulating apoptosis; these include pro-caspases (2, 3 and 9), AIF, Endo G, and Smac/Diablo (Ravagnan et al., 2002).

Thus, mitochondria are a crossroad for multiple signaling pathways that regulate the downstream execution of apoptosis. Respiratory function, oxidative stress, mitochondrial inner membrane potential, and calcium fluxes are all intimately involved in controlling the opening of the mitochondrial permeability transition pore (MPTP) through which the pro-apoptotic factors are released into the cytosol (Zoratti and Szabo, 1995; Green and Reed, 1998). Most factors involved in the apoptotic cascade have an otherwise “normal function”. The classic example is cyt c, which doubles as an electron carrier in the inner mitochondrial membrane and as apoptosis assembrer. Similarly, AIF has oxidoreductase and free radical scavenging properties in the inner mitochondrial membrane and large-scale DNA fragmentation properties when translocated into the nucleus; thus it functions as a caspase-independent pro-apoptotic factor released from mitochondria (Miranar et al., 2001; Klein et al., 2002). In addition, Bel-2 has recently been reported to influence respiratory function, regulating the adenine nucleotide exchange between mitochondria and cytosol, aside from its anti-apoptotic properties (Manfredi et al., 2003).

It is noteworthy that recent studies have shown that complex I and CoQ are involved in the regulation of MPTP opening and the activation of apoptosis (Fontaine et al., 1998; Fontaine and Bernardi, 1999; Chauvin et al., 2001). Remarkably, the mtDNA pathogenic mutations for LHON all affect complex I ND subunits and may influence its interaction with the quinone substrate (Carelli et al., 1997, 1999; see ahead for discussion). Even more remarkably, another protein embedded in the inner mitochondrial membrane that seems to be involved in multiple functions, including mitochondrial fission/fusion, cristae organization and apoptosis, is the dynamin-like GTPase encoded by the OPA1 gene. This has recently been found to be associated with DOA. The OPA1 gene illustrates the likely involvement of mitochondrial distribution in the life and death of neurons, particularly of RGC and their axons.

### 3.4. Mitochondrial fission/fusion and network

Mitochondria are traveling powerhouses that need to be located within the cytoplasm according to the local metabolic needs of the cell. Mitochondrial movements within neuronal axons occur as a bi-directional, anterograde, “saltatory” flux, traveling with the so-called “fast component” of axonal transport (Grafstein, 1995; Hollenbeck, 1996). The mitochondrial distribution within the cytoplasm is based on their interaction with the cytoskeleton, in particular with the motor proteins. Thus, microtubule (MT)-based motility is supported by kinesin for anterograde transport, and by dynein for retrograde transport. Mitochondria can also use actin microfilaments, most likely representing an auxiliary system involved in local transport (Morris and Hollenbeck, 1995). A newly discovered protein named Milton may act as an adaptor complex, which links the kinesin motor to mitochondria and has been found to be essential for the proper localization of mitochondria to the axon and synaptic terminal in Drosophila (Stowers et al., 2002). Two human cDNAs (huMilt1, huMilt2) showing high homology with Milton were also identified. Both kinesin and dynein are associated with an ATPase activity that is activated by MT binding. Thus, mitochondrial axonal transport is an ATP-dependent process (Ochs and Hollingsworth, 1971; Sabri and Ochs, 1972), and the mitochondria themselves are the major source of ATP. Other factors are likely to be involved with the regulation of axonal transport of mitochondria, such as the receptor mediated effects of Endothelin-1, which markedly decreases the anterograde axonal transport of mitochondrial subcomponent in rat optic nerve (Stokely et al., 2002).

Mitochondria may occur as discrete organelles or as an interconnected network (Rizzuto et al., 1998). Fission and fusion mitochondrial proteins regulate these different states of cytoplasmic organization (Bereiter-Hahn and Voth, 1994). Although the OPA1 gene encodes for one of these proteins, there is more to come, particularly from the study of the Fzo1 gene (fuzzy onions), a nuclear encoded mitochondrial transmembrane GTPase controlling mitochondrial fusion in yeast.
In fact, at least two human Fzo1 homologues mitofusin (Mfn) 1 and 2 have been identified so far (Santel and Fuller, 2001; Rojo et al., 2002). Very little is known at this point about gene expression and function of either OPA1 or Mfn1/Mfn2 fission/fusion proteins in the human retina and optic nerve. It seems an important issue that needs further investigation for the understanding of mitochondrial function in the peculiar RGC neuronal system, which is very dependent on mitochondria distribution, and ultimately for understanding the pathophysiology of mitochondrial optic neuropathies.

4. Leber’s hereditary optic neuropathy (LHON): a paradigm

LHON is an inherited form of acute or subacute loss of central vision affecting predominantly young males. It was initially noted by Von Graefe (1858) and was formally defined as a clinical entity by Leber (1871). LHON is now recognized as the most frequently occurring mitochondrial disease (Chinnery et al., 2000), having a minimum point prevalence of 3.22:100,000 in the northeast of England (Man et al., 2003).

4.1. Clinical features

The typical presentation of LHON is characterized by rapid loss of central vision in one eye. The condition is usually painless and is associated with fading of colors (dyschromatopsia) in the one eye, followed by similar involvement of the other eye, within days, months, or rarely years (Newman, 1998a; Carelli, 2002). Visual acuity reaches stable residual values at or below 20/200 within a few months. The accompanying visual field defect usually involves the central vision in the form of a large centro-cecal absolute scotoma.

Fundus examination during the acute/subacute stage in most cases reveals characteristic changes summarized in a triad of signs (Smith et al., 1973; Nikoskelainen et al., 1983, 1984):

1. circumpapillary telangiectatic microangiopathy,
2. swelling of the NFL around the disc (pseudoeoedema),
3. absence of leakage on fluorescein angiography (in contrast to true edema).

Thus, the optic disc appears hyperemic, occasionally with peripapillary hemorrhages. Characteristically, axonal loss in the PMB rapidly leads to temporal atrophy of the optic disc. With time, the optic disc eventually turns pale. These fundus changes may be absent or minimal in some cases. The microangiopathy may be present in a number of asymptomatic at-risk family members along the maternal line, in whom it may remain stable over the years (Nikoskelainen et al., 1982).

Optic atrophy with permanent severe loss of central vision but with relative preservation of pupillary light responses is the usual endpoint of the disease. However, spontaneous recovery of visual acuity has occasionally been reported even years after onset (Stone et al., 1992; Mackey and Howell, 1992; Pezzi et al., 1998). Visual function may improve progressively, sometimes suddenly, with contraction of the scotoma or reappearance of small islands of vision within it (fenestration). A young age of onset is a favorable prognostic factor, and the rate of visual recovery seems closely related to the type of pathogenic mutation, the 14484/ND6 mutation being most prone to it (Oostra et al., 1994; Riordan-Eva et al., 1995).

In long-lasting LHON, cupping of the optic disc has frequently been reported as a sign of the chronic stage of the pathological process (Ortiz et al., 1992; Weiner et al., 1993; Mashima et al., 2003).

4.2. Histopathology

Only a few LHON cases have been studied histopathologically, none during the acute stage of the disease (reviewed in Carelli, 2002). Five of these cases were molecularly defined, and only three were studied by both light and electron microscopy. They harbored the 11778/ND4, 14484/ND6 plus 4160/ND1, and 3460/ND1 mutations, respectively (Sadun et al., 1994, 2000, 2002; Kerrison et al., 1995). The age at death for these cases ranged from 68 to 81 years, several decades after the onset of visual loss. A drastic loss of RGC and NFL was invariably reported as the major retinal finding. In the 14484/4160 case some residual RGC with swollen mitochondria and double-membrane bodies with calcium inclusions were seen (Kerrison et al., 1995). This case stands apart because of the double mutation and the “LHON plus” phenotype recurrent in this Australian pedigree (known as QLD1), with a number of patients suffering LHON and a mitochondrial encephalomyopathy, lactic acidosis, stroke-like syndrome (MELAS)-like neurological disorder in infancy (Wallace, 1970; Howell et al., 1991a, b; Howell, 1999). The other two cases were investigated in our laboratories by serial sagittal- and cross-sectioning through the optic nerve head and the post-laminar optic nerve (Sadun et al., 1994, 2000, 2002; Carelli et al., 2002a). A complete loss of central fibers with various degrees of axonal sparing in the periphery was observed by light microscopy, with the absence of inflammatory signs (Fig. 2). Larger axon profiles were selectively spared and found in the reactive gliotic tissue filling in the fiber loss. The optic nerve head displayed some excavation of the optic disc, which was better seen on the sagittal sections.
Comparison of the two 11778/ND4 and 3460/ND1 mutations revealed comparable ultrastructural changes in the spared axons of the retrolaminar myelinated portion (Carelli et al., 2002a). Axoplasmic abnormalities, such as patchy accumulations of mitochondria, cytoplasmic debris and bodies, and cytoskeleton changes, were observed. Wide variability in myelin thickness was also evident, with some axons being almost denuded of myelin sheath. Oligodendrocytic cytoplasmic tongues were frequently seen to intercalate the myelin lamellae, sometimes displaying degenerative features similar to those observed in the cuprizone-induced demyelination (Ludwin, 1995). Interestingly, most of the demyelinated fibers showed mitochondrial accumulation, sometimes completely filling the axonal profile (Fig. 3). In some instances, evidence of remyelination was also seen, indicating that the physiopathological process may be much more dynamic than originally thought. Some activated astrocytes, with an abundance of mitochondria and filaments into the cytoplasm, were closely opposed to the spared axons. Numerous glial cells and occasional macrophages filled with lipofuscin inclusions were also present adjacent to the areas of axonal sparing. Overall, these findings in both axons and myelin are surprisingly suggestive of a still ongoing low-grade degenerative process long after the clinical onset of LHON (Carelli, 2002; Carelli et al., 2002a).

4.3. Genetics

The maternal pattern of inheritance of LHON, clearly recognized only a few decades ago (Erickson, 1972), led to the identification of a few pathogenic mtDNA point mutations, the three at positions 11778/ND4, 3460/ND1, and 14484/ND6 being the most common worldwide (an updated list is provided in Table 1). A number of putatively pathogenic mutations have also been
reported in single cases or families and need confirmation (Table 2). Other mutations, most of which are common polymorphic variants, have been associated with LHON as weak genetic determinants. These are defined as ‘secondary/intermediary mutations’ by some authors (Wallace et al., 1999) (Table 3). Their role, if any, is still controversial, apart from those population-specific polymorphisms defining the mtDNA background known as haplogroup J, for which a modifying role is suspected (see discussion ahead).

However, at least two features remain that are not easily explained solely by the mtDNA mutation: the male prevalence and the incomplete and variable penetrance (Carelli, 2002). Despite the fact that most LHON families carry the mtDNA pathogenic mutation in the homoplasmic condition (100% of the mtDNA molecules are mutant), not all maternally related individuals develop LHON. The existence of further genetic determinants, such as nuclear modifying genes, has been largely hypothesized (Bu and Rotter, 1991) and debated (Carelli et al., 2003a). Chromosome X has been the main target for the search of a nuclear modifying gene, because it would also explain the male prevalence. To date, however, although we have formal compatibility of the disease segregation within LHON pedigrees with a two-locus model (mitochondrial pathogenic plus nuclear modifying), no modifying gene has been clearly identified yet (Bu and Rotter, 1991, 1992; Carelli et al., 2003b).

The most compelling evidence for a modifying role actually comes from the genetic mtDNA background, i.e. the mtDNA haplogroup hosting the LHON mutational event. An mtDNA haplogroup is a particular group of mitochondrial genomes defined by a unique set of variants acquired from the same ancient common female ancestor (Wallace et al., 1999). Independent

### Table 1
Pure LHON pathogenic mutations

<table>
<thead>
<tr>
<th>Position</th>
<th>Gene</th>
<th>Amino acid</th>
<th>Heteroplasmy</th>
<th>Homoplasmy</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>G11778 A (frequent)</td>
<td>ND4</td>
<td>R340 H highly conserved</td>
<td>Yes</td>
<td>Yes</td>
<td>Wallace et al. (1988)</td>
</tr>
<tr>
<td>G3460 A (frequent)</td>
<td>ND1</td>
<td>A52 T moderately conserved</td>
<td>Yes</td>
<td>Yes</td>
<td>Huoponen et al. (1991) and Howelletal. (1991b)</td>
</tr>
<tr>
<td>T14484 C (frequent)</td>
<td>ND6</td>
<td>M64 V low conserved</td>
<td>Yes</td>
<td>Yes</td>
<td>Mackey and Howell (1992) and Johns et al. (1992)</td>
</tr>
<tr>
<td>A14495 G (rare-2 families)</td>
<td>ND6</td>
<td>L60 S highly conserved</td>
<td>Yes</td>
<td>—</td>
<td>Chinnery et al. (2001)</td>
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<tr>
<td>T14482 A/G (rare-3 families)</td>
<td>ND6</td>
<td>M64 I low conserved</td>
<td>Yes</td>
<td>Yes</td>
<td>Howell et al. (1998), Luberichs et al. (2002) and Valentino et al. (2002)</td>
</tr>
<tr>
<td>C4171 A (rare-2 families)</td>
<td>ND1</td>
<td>L289 M highly conserved</td>
<td>Yes</td>
<td>Yes</td>
<td>Kim et al. (2002)</td>
</tr>
<tr>
<td>C14568 T (rare-2 families)</td>
<td>ND6</td>
<td>G36 S moderately conserved</td>
<td>—</td>
<td>Yes</td>
<td>Besch et al. (1999) and Fauser et al. (2002a,b)</td>
</tr>
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</table>

Fig. 3. Human myelinated nerve fibers; transmission electron microscopy (TEM). (A) Normal myelinated axon and (B) myelinated axon from an LHON optic nerve. Note both the accumulation of mitochondria within the axoplasm and the thin myelin sheath of this obliquely sectioned fiber (both A and B; magnification ×10,200, bar 1 μm).
studies have shown that one continent-specific mtDNA haplogroup of Eurasian origin, classified as haplogroup J, is associated with the 11778/ND4, and particularly the 14484/ND6 LHON pathogenic mutations (Brown et al., 1997; Torroni et al., 1997; Hofmann et al., 1997; Lamminen et al., 1997). The most accepted explanation is that this particular mtDNA haplogroup, including some specific polymorphisms altering amino acidic positions in ND subunit genes of complex I (see Table 3), can increase the penetrance of LHON, justifying the most frequent recognition of the 11778/ND4 and 14484/ND6 mutations in association with haplogroup J. On the contrary, there is no explanation for why the 3460/ND1 mutation is distributed in all haplogroups at frequencies similar to the control population.

Cellular and functional studies suggest alternative explanations for a haplogroup modifying role and the topic is still hotly debated (Carelli et al., 2002b; Lodi

<table>
<thead>
<tr>
<th>Position</th>
<th>Gene</th>
<th>Heteroplasmy</th>
<th>Homoplasmy</th>
<th>LHON case</th>
<th>References</th>
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<tr>
<td>G5244 A</td>
<td>ND2</td>
<td>Yes</td>
<td>—</td>
<td>Single case</td>
<td>Brown et al. (1992)</td>
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<tr>
<td>G13730 A</td>
<td>ND5</td>
<td>“De novo” mutation</td>
<td>Yes</td>
<td>Single case</td>
<td>Howell et al. (1993)</td>
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<td>C4025 T</td>
<td>ND1</td>
<td>—</td>
<td>Yes</td>
<td>Pedigree with at least two maternally related cases</td>
<td>Huoponen et al. (1993)</td>
</tr>
<tr>
<td>T12811 C</td>
<td>ND5</td>
<td>—</td>
<td>Yes</td>
<td>Pedigree with at least two maternally related cases</td>
<td>Huoponen et al. (1993)</td>
</tr>
<tr>
<td>A13637 G</td>
<td>ND5</td>
<td>—</td>
<td>Yes</td>
<td>Pedigree with at least two maternally related cases</td>
<td>Huoponen et al. (1993)</td>
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<tr>
<td>G9804 A</td>
<td>COX III</td>
<td>—</td>
<td>Yes</td>
<td>Multiple unrelated single cases</td>
<td>Johns and Neufeld (1993), Wakakura et al. (1998), Dogulu et al. (2001) and Howell et al. (2003a)</td>
</tr>
<tr>
<td>T9101 C</td>
<td>ATPase 6</td>
<td>—</td>
<td>Yes</td>
<td>Single case</td>
<td>Lamminen et al. (1995)</td>
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<tr>
<td>C14498 T</td>
<td>ND6</td>
<td>Yes</td>
<td>—</td>
<td>Multigenerational pedigree</td>
<td>Wissinger et al. (1997)</td>
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<td>G3635 A (T8551 C)*</td>
<td>ND1</td>
<td>—</td>
<td>Yes</td>
<td>Multigenerational pedigree</td>
<td>Brown et al. (2001)</td>
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<tr>
<td>C4640 A</td>
<td>ND2</td>
<td>—</td>
<td>Yes</td>
<td>Multigenerational pedigree</td>
<td>Brown et al. (2001)</td>
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<tr>
<td>T11253 C (T11978 A)*</td>
<td>ND4 (ND4)*</td>
<td>—</td>
<td>Yes</td>
<td>Single case</td>
<td>Lee-Kottler et al. (2002)</td>
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<tr>
<td>G 14831 A</td>
<td>Cyt b</td>
<td>—</td>
<td>Yes</td>
<td>Single case</td>
<td>Fauser et al. (2002a, b)</td>
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<td>G3700 A</td>
<td>ND1</td>
<td>—</td>
<td>Yes</td>
<td>Single case</td>
<td>Fauser et al. (2002a, b)</td>
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<td>T10237 C</td>
<td>ND3</td>
<td>—</td>
<td>Yes</td>
<td>Single case</td>
<td>Horvath et al. (2002)</td>
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<td>T14325 C</td>
<td>ND6</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Single case</td>
<td>Howell et al. (2003a)</td>
</tr>
<tr>
<td>G13051 A</td>
<td>ND5</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Multigenerational pedigree</td>
<td>Howell et al. (2003a)</td>
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<th>Position</th>
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<th>Homoplasmy</th>
<th>Haplogroup</th>
<th>References</th>
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<td>T4216 C</td>
<td>ND1</td>
<td>—</td>
<td>Yes</td>
<td>J or T</td>
<td>Johns and Berman (1991) and Brown et al. (1992)</td>
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<tr>
<td>G13708 A</td>
<td>ND5</td>
<td>—</td>
<td>Yes</td>
<td>J or X</td>
<td>Johns and Berman (1991) and Brown et al. (1992)</td>
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<td>A4917 G</td>
<td>ND2</td>
<td>—</td>
<td>Yes</td>
<td>T</td>
<td>Johns and Berman (1991) and Brown et al. (1992)</td>
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<tr>
<td>T3394 C</td>
<td>ND1</td>
<td>—</td>
<td>Yes</td>
<td>J (French-Canadian-Dutch 14484 founder)*</td>
<td>Brown et al. (1992), Johns et al. (1992) and Howell et al. (2003a)*</td>
</tr>
<tr>
<td>G15257 A</td>
<td>Cyt b</td>
<td>—</td>
<td>Yes</td>
<td>J2</td>
<td>Johns and Neufeld (1991) and Brown et al. (1992)</td>
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<tr>
<td>G15812 A</td>
<td>Cyt b</td>
<td>—</td>
<td>Yes</td>
<td>J2</td>
<td>Johns and Neufeld (1991) and Brown et al. (1992)</td>
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<td>G7444 A</td>
<td>COX I</td>
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<td>Yes</td>
<td>—</td>
<td>Johns and Neufeld (1993) and Brown et al. (1994)</td>
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<td>G9438 A</td>
<td>COX III</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
<td>Johns and Neufeld (1993) and Brown et al. (1994)</td>
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<tr>
<td>A13637 G</td>
<td>ND5</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
<td>Huoponen et al. (1993)</td>
</tr>
<tr>
<td>G3316 A</td>
<td>ND1</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
<td>Matsumoto et al. (1999)</td>
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<td>G3496 T</td>
<td>ND1</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
<td>Matsumoto et al. (1999)</td>
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<tr>
<td>C3497 T</td>
<td>ND1</td>
<td>—</td>
<td>Yes</td>
<td>—</td>
<td>Matsumoto et al. (1999)</td>
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</table>
et al., 2000). In particular, haplogroup J has been implicated as having a protective effect in successful aging (De Benedictis et al., 1999) and Parkinson’s disease (van der Walt et al., 2003), and seems to be associated with lower cardiovascular risk in a study of a very large LHON family (Sadun et al., 2003). Moreover, recent studies provided strong evidence that at least one particular subbranch of haplogroup J is associated with a unique, very old founder event for the 14484/ND6 mutation, dated approximately 900–1800 years ago (Howell et al., 2003a). This founder is responsible for their tight association in some specific populations, such as the French-Canadians (Macmillan et al., 2000) and the Dutch (Howell et al., 2003a). The 14484/ND6-haplogroup J combination seems related to a very low penetrance in females, as reflected by the highest male to female ratio (8:1) compared to the 11778/ND4 (∼4:6:1) and 3460/ND1 (∼3:1) (Carelli, 2002). As a consequence the 14484/ND6 mutation, and to a certain extent also the 11778/ND4 mutation, tends to persist longer within the population when associated with haplogroup J, potentially explaining the biased recognition of their association. The observation that the male to female ratio is variable with the type of pathogenic mutation and that it might be also modulated by the specific association of some of these mutations with the haplogroup J is against the hypothesis of a chromosome X-linked modifying factor (Pegoraro et al., 2003). Various studies failed to find any relationship with genes encoded by chromosome X by linkage analysis (Chen et al., 1989; Carvalho et al., 1992; Chalmers et al., 1996), by investigating the X-inactivation pattern (Pegoraro et al., 1996; Oostra et al., 1996; Pegoraro et al., 2003), or by directly investigating candidate genes (Man et al., 2002). It is interesting to note that within the same extended LHON pedigree the observable variability in penetrance among different nuclear families proportionally follows the same gender ratio. Thus, when penetrance is very reduced, only males are affected (Sadun et al., 2003; Carelli et al., 2003b). In other words, the gender difference seems more explainable as a specific metabolic factor, which influences the pathogenicity of the mtDNA mutation. In fact, the protective role of estrogens on oxidative stress has recently been shown in animal models (Borras et al., 2003) and has been an explanation proposed as for gender bias in LHON (Pegoraro et al., 2003).

4.4. Biochemistry

As reviewed by Brown (1999), it appears that the LHON mutations have different biochemical signatures, which generally induce modest or subtle changes in measurable aspects of complex I function. A possible exception is represented by the 3460/ND1 mutation, which has consistently been shown to decrease the electron transport activity of complex I (Majander et al., 1991; Smith et al., 1994). Nevertheless, both 3460/ND1 and 11778/ND4 mutations may induce a complex I-dependent impairment of mitochondrial respiration (Majander et al., 1991, 1996). We have investigated the activity and inhibitor sensitivity of complex I in platelet-derived submitochondrial particles from a large number of individuals carrying the three major LHON mutations (Degli Esposti et al., 1994; Carelli et al., 1997, 1999; Ghelli et al., 1997). We did not observe a significant reduction of complex I activity with the 11778/ND4 and 14484/ND6 mutations, although this reduction was evident with the 3460/ND1 mutation (Carelli et al., 1997, 1999). These findings are similar to those reported by others (Brown, 1999). However, less predictably, we consistently found that mitochondria carrying the 11778/ND4 and 3460/ND1 mutations showed a decreased sensitivity to rotenone, a powerful complex I inhibitor that acts as an antagonist of Q intermediates (Degli Esposti et al., 1994; Carelli et al., 1997). Our unpublished results with the 14484/ND6 mutation indicated the same tendency. Variable degrees of decreased rotenone sensitivity in 11778/ND4 and 3460/ND1 mutant complex I have also been observed by others (Cock et al., 1999; Majander et al., 1996). Rotenone affects the binding of semiquinone intermediates and of the quinol product in complex I. Changes in rotenone sensitivity, as measured in LHON, are suggestive of an altered stability of the semiquinone intermediates formed during the catalytic cycle of complex I. The latter terminates with the release of a quinol product derived from controlled dismutation of the semiquinones.

We have also evaluated the sensitivity of complex I activity to product inhibitors such as quinols and myxothiazol and found that the 14484/ND6 LHON mutation induced an increased sensitivity of complex I to myxothiazol and nonyl-benzoquinol (Carelli et al., 1999). These results are consistent with those previously reported in cell lines carrying the rare LHON/dystonia/Leigh 14459/ND6 mutation, which lies close to the more common 14484/ND6 mutation in a conserved region of the ND6 protein (Jun et al., 1994, 1996). Interestingly, a quinol inhibition was also reported for the 3460/ND1 mutation (Majander et al., 1996), and we have evidence that the same applies to the 11778/ND4 mutation (Carelli, V., Ghelli, A., Degli Esposti, M., unpublished results). Thus, alterations in the affinity for quinol products, as reflected by changes in their inhibitory titers, consistently suggest that LHON mutations affect the interaction of complex I with the Q substrate (Degli Esposti et al., 1994; Ghelli et al., 1997). Biochemical evidence in LHON patients indicates that all LHON pathogenic mutations affect complex I function to induce an increased product inhibition during catalysis. What are the possible implications of
increasing the production of ROS (Degli Esposti et al., 1996; Barbiroli et al., 1995; Lodi et al., 1997, 2000). More recently, the evaluation of complex I-dependent ATP synthesis in transmitochondrial hybrid cell lines (cybrids, see below) carrying the 11778/ND4 mutation revealed an unexpectedly severe impairment (Guy et al., 2002; Komaki et al., 2003; Baracca, A., Solaini, G., Carelli, V., manuscript in preparation). However, the real impact of this dysfunction on the total cellular ATP, in particular at the level of the optic nerve, remains uncertain.

Alternatively, subtle but significant alterations in the stabilization of semiquinones by complex I may result in significant downstream effects. In particular, unstable semiquinone radicals can react with molecular oxygen, increasing the production of ROS (Degli Esposti et al., 1994; Carelli et al., 1997, 1999, 2002a; Brown, 1999). There is mounting evidence that increased ROS production and oxidative stress may be relevant for LHON pathophysiology. Initial studies on 11778/ND4 mutant cybrid cells obtained from a single LHON patient showed that they were significantly more sensitive to H2O2 exposure than control cybrids (Wong and Cortopassi, 1997). In another study, telomere shortening in two LHON patients was considered to be due to ROS injury (Oexle and Zwirner, 1997). More recently, an increase of ROS production was described as the main feature in a neuronal (NT2-derived) cybrid cell model of LHON carrying the 11778/ND4 and 3460/ND1 mutations only after differentiation was induced by retinoic acid (Wong et al., 2002). Other authors had evidence of altered vitamin E content in LHON patients (Klivenyi et al., 2001), and we have recently investigated the anti-oxidant mitochondrial machinery in osteosarcoma (143B.TK–)–derived LHON cybrids showing glutathione depletion and decreased MnSOD activity (Napoli, E., Carelli, V., Martinuzzi, A., manuscript in preparation).

The cybrid cell system has been widely used to model LHON pathophysiology at the cellular level. This cellular model is based on the repopulation with patient-derived mutant mitochondria of an immortalized, usually cancer-derived, cell line previously devoid of its original mtDNA (King and Attardi, 1989). The result is a transmitochondrial cell line in which only the features dependent on the patient mtDNA are co-transferred, dissected from the original nuclear genome and placed in a “neutral” nuclear background, i.e. that of the cancer-derived parental cell line. The initial cybrid studies of LHON have been aimed to co-transfer and reproduce into this cell system the biochemical features observed in patient-derived tissues (Vergani et al., 1995; Hofhaus et al., 1996; Cock et al., 1998). The most extensive study by Brown et al. (2000) closely reproduced into the cybrid cells all the respiratory and enzymatic features seen in LHON patients.

This model has recently been used to study cell death induced by complex I dysfunction in LHON. Using the approach of Fas-induced apoptosis, the LHON cybrids were more prone to undergo apoptotic cell death than control cybrids (Danielson et al., 2002). Similar results were also reached by another approach, i.e. growing LHON cybrids in glucose-free galactose-supplemented medium. This is a well-established method to reveal an OXPHOS dysfunction by forcing the cells to rely on the mitochondrial respiratory chain to synthesize ATP (Robinson et al., 1992). Thus, LHON cybrids in galactose medium display massive, apoptotic cell death (Ghelli et al., 2003). Control cybrids, in contrast, are still able to grow with a reduced rate. Interestingly, the most recent results using the galactose model suggest that the LHON cybrid apoptotic death is caspase-independent, possibly due to a rapid ATP depletion observed early in the time-course experiment, and the direct nuclear DNA cleavage by AIF and Endo G (Zanna et al., 2004).

An interesting cybrid model of partial complex I dysfunction set up by Barrientos and Moraes (1999), recapitulates the findings on LHON cybrids. Xenomitochondrial cybrids, constructed by transferring ape-derived mitochondria into the classic osteosarcoma-derived (143B.TK–) human recipient, display approximately a 40% decrease in complex I activity, possibly because of the mtDNA sequence divergence between humans and apes. In these cells 35–40% complex I inhibition was needed to impair respiration, whereas the increase of ROS production, oxidative damage, and apoptotic cell death were linearly related to complex I dysfunction as modeled through rotenone titration (Barrientos and Moraes, 1999).

4.5. Mutation-related features

The comparison of combined clinical, biochemical, and genetic features of the three most frequent LHON pathogenic mutations suggests some interesting observations. The only clinical feature that seems to be mutation-dependent is the spontaneous recovery of visual acuity, which is clearly more frequent with the 14484/ND6 mutation (Mackey, 1994; Oostra et al., 1994; Riordan-Eva et al., 1995; Nikoskelainen et al., 1996; Yamada et al., 1999). The visual recovery also seems to be related to a younger age. The higher rate of visual recovery with the 14484/ND6 mutation does not seem related to the tight association of this mutation with the European haplogroup J (Torróni et al., 1996; Yamada et al., 1997; Nishioka et al., 2003).
Nevertheless, there are reports of visual recovery or better final visual outcome in a few cases of 11778/ND4 mutation associated with haplogroup J2, as characterized by the presence of the 15257/cytb and 15812/cytb polymorphisms (Salmaggi et al., 1994; Oostra et al., 1994; Nikoskelainen et al., 1996). However, visual recovery is infrequent with this LHON mutation (Newman et al., 1991; Mackey, 1994; Oostra et al., 1994; Riordan-Eva et al., 1995; Nikoskelainen et al., 1996; Yamada et al., 1999).

The severity of the biochemical defect in LHON, as evaluated by the decrease in complex I activity, shows a clear gradient going from the 3460/ND1 (most severe) to the 14484/ND6 (milder), passing through the intermediate features of the 11778/ND4 mutation (Carelli et al., 1997, 1999; Brown, 1999). The same gradient seems to be reflected in other parameters. The frequency with which both LHON probands and families are heteroplasmic in our experience decreases from the 3460/ND1, the most frequently heteroplasmic (Jacobi et al., 2001), to the 11778/ND4, and to the 14484/ND6, which is only exceptionally found to be heteroplasmic (Carelli, V., Valentino, M.L., Barboni, P., unpublished results). The same gradient is reflected in two other features. The 3460/ND1 mutation is randomly distributed among the mtDNA haplogroups, while the 11778/ND4 has a weak association with haplogroup J (Brown et al., 1997; Torroni et al., 1997; Hofmann et al., 1997; Lamminen et al., 1997). The most striking association is observed between the 14484/ND6 mutation and haplogroup J (Brown et al., 1997; Torroni et al., 1997; Hofmann et al., 1997). Following the same trend, the male to female ratio is lowest with the 3460/ND1 mutation, intermediate with the 11778/ND4 mutation, and most skewed with the 14484/ND6 mutation (Carelli, 2002), indicating a progressively lower penetrance in females.

Taken together, these observations indicate that LHON pathogenic mutations do not express a similar pathogenic potential. The 3460/ND1 mutation seems the most severe: it consistently impairs the electron transport through complex I, it is the most penetrant when homoplasmic and presents with less gender difference, it is not modified by the mtDNA haplogroup, and it has a low rate of spontaneous visual recovery. In our experience, most LHON cases with 3460/ND1 mutation are recent mutational events, as reflected by the very frequent occurrence of heteroplasmly and the small size pedigrees or single cases. The 11778/ND4 seems to be intermediate, being partially associated with haplogroup J, only slightly impairing complex I activity, having a more skewed male to female ratio, and being more rarely heteroplasmic. The 14484/ND6 clearly stands apart, with its tight association with haplogroup J, which in a set of families seems clearly related to a common founder (Howell et al., 2003a), its frequent occurrence of spontaneous visual recovery, its very skewed male to female ratio, and consequently its low penetrance in females. This last feature may account for the founder effect, the very rare finding of heteroplasmy and the tendency to present as large, well-established homoplasmic pedigrees. Finally, the 14484/ND6 mutation has been found on two occasions in the absence of LHON within normal population mtDNA screenings, while the 11778/ND4 has been seen only once, and the 3460/ND1 has never been observed apart from LHON pedigrees (Howell et al., 2003b; Torroni pers. comm.).

4.6. Environmental factors may influence penetrance

A controversial issue is the influence that certain environmental factors may exert on LHON penetrance, triggering the pathological features in previously unaffected mutation carriers. Tobacco smoking and alcohol consumption are the most likely risk factors (Newman, 1998a; Carelli, 2002), but exposure to a variety of less common toxics have been described (Sadun et al., 2003), as has head trauma (Riordan-Eva et al., 1995; Redmill et al., 2001), non-controlled diabetes (DuBois and Feldon, 1992), and pharmaceutical agents that interfere with mitochondrial metabolism such as ethambutol (Dotti et al., 1998; De Marinis, 2001), and anti-retroviral therapy in HIV patients (Warner and Ries, 2001; Mackey et al., 2003).

Recently, two studies carried out in large LHON pedigrees with 11778/ND4 mutation and haplogroup J showed convincing evidence of a strong association between tobacco use and affected individuals (Tsao et al., 1999; Sadun et al., 2003). In both families, penetrance was remarkably high in the older generations, as previously noted for LHON families carrying the association of the 11778/ND4 mutation and haplogroup J (Carelli, V., Barboni, P., Valentino, M.L., unpublished observation). This apparently contradicts the conclusions of a case/control study, which found no correlation between tobacco and LHON (Kerrison et al., 2000). A possible explanation is that LHON patients with visual loss precipitated by exposure to tobacco or alcohol may be a subset particularly predisposed to these environmental factors. This predisposition is likely to be genetically determined with a possible role played by polymorphic variation of mitochondrial genetic background. A striking example of this scenario is represented by a specific mutation in complex I which increases ethanol sensitivity in Caenorhabditis elegans (Kayser et al., 2003). LHON pathogenic mutations themselves may enhance the influence of some environmental factors on complex I function, and this effect may differ depending on the mutation type.

It would not be surprising if environmental factors such as tobacco and alcohol increase penetrance in
LHON pedigrees. In fact, the clinical presentation of toxic and nutritional optic neuropathies is remarkably similar to that of LHON (Carelli et al., 2002a). However, during the recent outbreak of a toxic-nutritional optic neuropathy in Cuba (Sadun, 1998a) that affected tens of thousands of individuals, the penetrance in some defined LHON Cuban families did not apparently change (Kerrison et al., 2000).

4.7. New concepts about LHON

The traditional view considers LHON a disease with visual impairment leaving off 1 year after onset (Newman, 1998a; Carelli, 2002). As a consequence, both LHON patients and their clinicians usually lose interest and lose contact with each other. However, we have evidence that these patients continue in a “chronic” state characterized by a low-grade degenerative process that may consistently worsen the residual vision over the years. Furthermore, the preclinical period in mutation carriers, when the patient is apparently healthy, may in fact be subclinically abnormal with objectively observable fluctuations in fundus appearance, mild visual field changes, and electrophysiologically abnormal patterns in visual evoked potentials. Our histological findings, with evidence of the variable state of myelination of spared axons, rare evidence of remyelination, and signs of active axonal degeneration in patients who suffered the acute phase of the disease decades before, are all indicative of a continued active process. It is particularly tempting to speculate that reparative processes such as remyelination of axons may provide a substrate for the spontaneous visual recovery that sometimes occurs, years after the acute phase of the disease.

5. Microvascularization of the optic nerve head: a link connecting LHON with MELAS and Leigh syndromes

Vascular changes in LHON have been noted since the initial descriptions, and their role in the pathophysiology of the disease has been debated (Nikoskelainen, 1994). The microangiopathy in LHON may persist as a subclinical sign over a number of years in asymptomatic family members without the development of an optic neuropathy (Nikoskelainen et al., 1982, 1987). However, in those individuals undergoing the active phase of the disease, these vascular abnormalities become more pronounced, accompanying the pathological evolution at the optic nerve head. Typically, there is an observable increase in arteriovenous shunting in the telangiectatic vascular bed, with dilatation of retinal artery branches and tortuosity of peripapillary arterioles (Nikoskelainen et al., 1983, 1984). The optic disc appears hyperaemic with occasional peripapillary hemorrhages (Carelli et al., 1998b). As the vision loss stabilizes, loss of the PMB becomes apparent, leading to temporal atrophy that will eventually evolve to complete pallor of the optic disc and disappearance of the microangiopathic signs (Nikoskelainen et al., 1983, 1984; Newman, 1998a).

In our histological investigations, we looked with particular interest at the blood vessels in the optic nerve head. There, we found dramatic collections of abnormal mitochondria in both endothelial and smooth muscle cells of the blood vessel wall (Ross-Cisneros et al., 2001; Ross-Cisneros, F.N., Carelli, V., Sadun, A.A., unpublished results). Similar findings were classically reported in the blood vessels of patients with MELAS syndrome (Sakuta and Nonaka, 1989; Hasegawa et al., 1991). In MELAS, a pathogenic role for such vascular changes has been hypothesized, with particular reference to the stroke-like episodes. Another mitochondrial disease, Leigh syndrome, is also characterized by vascular changes and by small vessel proliferation in the anatomical areas surrounding the necrotizing lesions that are the histological hallmark of this very severe central nervous system disease (Cavanagh and Harding, 1994).

The prelaminar region of the optic nerve head, the metabolic choke-point characterized by unmyelinated axons rich in mitochondria, has been reported to have unusual blood–brain properties in the microvasculature (Hofman et al., 2001). In particular, the small vessels seem to be characterized by a non-specific permeability, possibly mediated by vesicular transport, instead of the classical blood–brain barrier. These observations are intriguing when some other elements are taken into consideration. Unpredictably, a few mutations affecting ND subunit genes (ND5 and ND6) have recently been described to underlie a syndromic form of LHON, presenting with the combined phenotype of LHON/MELAS/Leigh syndrome (Pulkes et al., 1999; Corona et al., 2001; Chol et al., 2003; Liolitsa et al., 2003; Kirby et al., 2003) or LHON/dystonia/Leigh syndrome (Jun et al., 1994; Shoffner et al., 1995; Kirby et al., 2000) (see also Table 4 for an updated list). A few cases of slowly evolving or adult-onset Leigh syndrome have also been reported in LHON patients who carry one of the major mutations, 11778/ND4, 3460/ND1, or 14484/ND6 (Funalot et al., 2002; these cases were reported as “LHON Plus” phenotypes by the old literature (Nikoskelainen et al., 1995; Newman, 1998a; Carelli, 2002). Overall, these observations point to a possible common pathogenic motif, with different degrees of central nervous system involvement, where the LHON-like optic neuropathy represents only the tip of the iceberg. It is noteworthy that the few histopathological studies reporting the ocular findings in Leigh cases present with features indistinguishable from LHON (Cavanagh and Harding, 1994; Hayashi et al., 2000; Carelli and Sadun, 2001). The link between LHON, MELAS and Leigh...
syndromes, all of which share the common feature of a mitochondrial angiopathy, strongly suggests the need to reevaluate the role of vascular pathology in the complex sequence of events that trigger the acute/subacute occurrence of LHON. LHON could be considered a small scale MELAS or Leigh syndrome limited to the optic nerve head, possibly having the characteristics of a sudden metabolic imbalance with a complicated interplay of partial demyelination, impaired axonal transport, and dysfunctional vascular supply.

6. Nuclear genome defect (OPA1) leads to dominant (Kjer’s) optic atrophy (DOA): another mitochondrial problem

DOA is clinically and genetically distinct from LHON. DOA is a form of slowly progressive optic neuropathy, usually with its onset in the first decade of life, which has a clear autosomal dominant pattern of inheritance. DOA, also known as Kjer’s optic neuropathy (Kjer, 1959), has been reported to have a prevalence of 1:50,000 to 1:10,000 in Denmark (Newman, 1998b), and is considered the most common form of autosomally inherited (non-glaucomatous) optic neuropathy (Votruba et al., 1998).

6.1. Clinical features

The typical presentation in DOA is characterized by a slowly progressive, roughly bilaterally symmetrical visual loss in childhood, accompanied by temporal pallor of the optic discs. Examination also demonstrates centrocaecal scotomas and impairments of color vision (tritanopia). The patient is often unaware of a vision defect, and the disease is recognized by chance during routine vision testing (school eye screenings). The disease progression may be quite variable within the same family, ranging from mild cases with visual acuity that stabilizes in adolescence, to slowly but constantly progressing cases, to cases with sudden, step-like decreases of visual acuity. Peripheral vision is usually spared, and a central, paracentral, or cecocentral scotoma is typical. Variability of clinical expression is reflected by the extent of optic atrophy reached by different patients.

Despite the remarkable difference of the clinical evolution, the endpoint of the pathological process in Kjer’s is clinically indistinguishable from LHON in form if not in degree, as both a visual defect and a fundus appearance (Jacobson and Stone, 1991). It is noteworthy, for further discussion, that a frequent feature of DOA’s end-stage fundus examination is optic disc excavation (Votruba et al., 2003). Such excavation is also frequently reported in LHON (Ortiz et al., 1992; Weiner et al., 1993; Mashima et al., 2003) and
specifically characterizes the optic neuropathy in normal tension glaucoma (NTG) (Buono et al., 2002). A recent detailed study of optic disc morphology in DOA patients with OPA1 mutations showed optic disc excavation with enlarged cup to disc ratio, frequent peripapillary atrophy, and temporal gray crescent, most of which are features also seen in glaucomatous optic neuropathy (Votruba et al., 2003). Overall, despite a remarkably different natural history, the two genetic optic neuropathies, LHON and DOA, share a similar endpoint with a predominant involvement of the PMB. In fact, optic disc pallor, prevalent on the temporal side, is typically seen in DOA patients. Moreover, both diseases show a highly variable penetrance and degree of clinical involvement in the affected subjects. This happens despite the fact that mtDNA mutations in LHON are homoplasmic in most cases, and that the mutations in DOA are heterozygous in the affected individuals, as would be the case for a Mendelian, autosomal dominant disorder.

6.2. Histopathology

DOA histopathology is limited to a few cases. These showed a selective loss of RGC, in particular from the macular area, with a substantially normal appearance of the rest of the retina (Johnston et al., 1979; Kjer et al., 1983). The optic nerve showed axonal loss and swelling, demyelination, and increased content of collagen tissue prevalently in the temporal aspect. This suggests that there is, in DOA, a particular vulnerability of the smallest fibers of the PMB, similar to that seen in LHON (Sadun et al., 2000; Carelli et al., 2002a). No sign of inflammatory reaction was present in DOA in the optic nerve or the retina, similar to the lack of inflammation seen in LHON. There was some evidence of transsynaptic degeneration in the lateral geniculate body, but this did not extend further to the calcarine cortex.

6.3. Genetics

DOA has been linked to two different loci, the large majority of cases being mapped to chromosome 3q28-qter (OPA1) (Eiberg et al., 1994). Only one family of German descent has been mapped to chromosome 18q12.2–12.3 (OPA4) (Kerrison et al., 1999). Further genetic heterogeneity probably exists; for example, the variant of DOA associated with sensorineural deafness does not link to the above loci (Ozden et al., 2002).

In 2000, two different groups simultaneously communicated the identification of the OPA1 gene (Delettre et al., 2000; Alexander et al., 2000), reporting that the protein encoded is a dynamin-related GTPase targeted to mitochondria. Large series of DOA patients have since been investigated and over 60 mutations have been reported, including missense, nonsense, deletion/insertion, and splicing mutations (Pesch et al., 2001; Toomes et al., 2001; Delettre et al., 2001; Thiselton et al., 2002). Mutations are found throughout the gene, but three clusters often occur: the leader sequence for mitochondrial import, the GTPase domain, and the –COOH terminus (Thiselton et al., 2002). Most mutations induce a truncated protein, and haploinsufficiency is the suggested mechanism underlying DOA. Missense mutations are clustered in the GTPase domain. They probably lead to a loss of function of the protein and to haplotype insufficiency. However, a cluster of truncation mutations affects the C-terminus, a putative dimerization domain, and a dominant negative effect has been hypothesized in these cases (Delettre et al., 2002). Alleles behaving in a semidominant way have also been reported, with compound heterozygote patients having a much more severe disease than the heterozygotic parents or siblings (Pesch et al., 2001). A number of asymptomatic carriers of OPA1 mutations have been identified within families, leading to the re-calculation of a consistently lower penetrance (Toomes et al., 2001). One mutation, the 2708del (TTAG) frameshift mutation, seems to be the most frequent in white patients and may be a hot spot (Delettre et al., 2001; Thiselton et al., 2002).

The genotype–phenotype correlation is weak, with great variability in both penetrance and clinical severity, even within families with the same mutation. Thus, as in LHON, other still unknown genetic or epigenetic/environmental factors may play a role in the phenotypic expression of DOA. For instance, a consistent number of polymorphic variants described in the OPA1 gene may contribute to this variation in phenotypic expression.

6.4. Biochemistry

The OPA1 gene encodes for a protein that is targeted to mitochondria by a leader sequence. This protein is closely related to a family of proteins involved in the mitochondrial network organization. These GTPases of the dynamin family include the yeast protein Mgm1/ Msp1, responsible for mitochondrial maintenance and inheritance (Pelloquin et al., 1998). Another dynamin-related GTPase protein, the human DRP1, homologue to the yeast Dnm1, has been shown to induce changes in mitochondrial distribution and morphology when mutated (Smirnova et al., 1998).

OPA1 is the human ortholog of Mgm1/Msp1 and has been shown to be anchored to the mitochondrial inner membrane, facing the intermembrane space (Olichon et al., 2002). OPA1 has eight mRNA isoforms resulting from alternative splicing. These are expressed in a variety of tissues, with the highest levels in retina, brain, testis, heart, and muscle (Delettre et al., 2001). Studies
on the mouse ortholog of OPA1 showed results similar to human OPA1 in terms of tissue distribution (Misaka et al., 2002). According to another study, two isoforms, which are prevalently expressed in HeLa cells, are differently located within mitochondria: the 88-kDa protein is associated with the mitochondrial outer membrane, while the 93-kDa protein is associated with the inner membrane (Satoh et al., 2003). Moreover, mitochondrial membrane potential, and drastic disorganization of the cristae was proposed (Satoh et al., 2003). Double labeling of COS7 cells with antibodies against mouse OPA1 and cytc showed a substantial co-localization of OPA1 with cytc (Misaka et al., 2002). Furthermore, the transfection of COS7 and HeLa cells with a construct containing a mutant OPA1 changed the mitochondrial network from tubular to fragmented vesicular, no longer distributed throughout the cytoplasm but accumulated adjacent to the nucleus (Misaka et al., 2002).

The conclusions that could be drawn from these experiments are that OPA1 may be a major organizer of the mitochondrial inner membrane, contributing to cristae maintenance and mitochondrial morphology, and possibly involved in cytc sequestration. Extrapolating, RGC death in DOA may be apoptotic, resembling by analogy the cybrid model of LHON mutants dying of apoptosis in galactose medium (Ghelli et al., 2003). There is no information so far about respiratory function in OPA1 mutant mitochondria, which may link even more tightly the biochemical features of LHON and DOA.

7. Are Leber's and Kjer's variations on the same theme?

There are multiple parallels that link LHON and DOA, the two major neurodegenerative disorders of RGC leading to optic atrophy mediated by a mitochondrial dysfunction. We can summarize these analogies in the following points.

7.1. Clinical similarities

There is a remarkable difference in timing with the degenerative processes in that the events are acute/subacute, only rarely slowly progressive, in LHON (Nikoskelainen et al., 1996), and always slowly progressive with frequent stable conditions in DOA (Votruba et al., 1998). However, the clinical endpoint of both optic neuropathies is identical. For each, there is a loss of central vision with relative sparing of the peripheral field that reflects the selective and early involvement of the small, thinly myelinated fibers of the PMB. For both diseases, the extent of optic disc temporal pallor depends on the loss of the PMB; if the atrophy is severe it extends above and below the optic disc, with the nasal quadrant last to be involved. Dyschromatopsia is prevalent in both diseases. The end-stage cupping of the optic disc is another frequently overlapping feature in both disorders, even though some differences may exist (Votruba et al., 2003; Mashima et al., 2003).

7.2. Histopathological similarities

The anatomical substrate in LHON and DOA is identical and involves the selective degeneration of RGC in the retina in association with the loss of axons and optic nerve atrophy. Again, the timing is different, being a synchronized wave of cellular death in LHON but a slow parcellar loss of RGC in DOA. The few histopathological studies in both diseases showed the same features of massive RGC losses in an otherwise intact retina. Demyelination and loss of axons in the central and temporal aspects of the optic nerve cross-section were seen in the post-laminar, myelinated portions of the optic nerve.

Unfortunately, only a few cases of DOA have been studied by histopathology and electron microscopy, and none of these had molecular definition. Furthermore, all of the cases studied were in the late atrophic stage, many years after the age of onset; there remains no information available on the early stages of the active degenerative process in DOA. LHON ultrastructural studies strongly suggested an abnormal distribution of mitochondrial numbers along the spared axon profile (Fig. 3). This can be interpreted as a compensatory redistribution of mitochondria as a result of the changing energy requirements of axons needed to maintain the saltatory transmission of action potentials. Alternatively, the accumulation may reflect profoundly impaired axonal transport of mitochondria. In both cases, a partial demyelination of the axons contributes to the organization of mitochondrial network and to the pathological aggregation of mitochondria.

7.3. Biochemical similarities

A final common path of mitochondrial dysfunction in LHON and DOA seems to be a predisposition of neuronal cells for apoptotic death. In LHON this feature is mediated by a deficient complex I that leads to an impaired efficiency of the respiratory chain and a
chronic increase in ROS production. Under stressful conditions, as produced by the use of galactose medium, the unstable metabolic balance rapidly fails, causing a profound ATP depletion, followed by release of cytc, and the caspase-independent apoptotic death of cells, possibly mediated by AIF and Endo G (Ghelli et al., 2003; Zanna et al., 2004). The direct participation of complex I and the quinone substrate in MPTP opening has been suggested, but it remains a poorly explored hypothesis.

It is not known if something similar occurs in DOA, i.e. a respiratory function defect with decreased bioenergetic efficiency and/or ROS overproduction; but it seems a reasonable hypothesis that this occurs also in DOA, given the inner membrane localization of the OPA1 protein. However, defective OPA1 function, as predicted by haploinsufficiency induced by most DOA mutations, leads to cytc release and a caspase-dependent apoptotic death of cells (Olichon et al., 2003). These events are also accompanied by profound changes in mitochondrial morphology and the organization of mitochondrial network. Histopathological findings in LHON document a drastic redistribution of mitochondria within spared axons, indirectly suggesting that mitochondrial accumulations within the neuronal cell may be a major feature common to both disease profiles (Fig. 3).

7.4. Possible scenarios for LHON pathogenesis

The construction of the LHON puzzle remains incomplete. However, one can delineate a hypothetical scenario for the sequence of pathological events leading to optic neuropathy.

There are two main consequences of complex I dysfunction in LHON (Brown, 1999), i.e. reduction of ATP synthesis, and increase of ROS production. Both of these may have multiple simultaneous consequences at the cellular level. The reduced availability of ATP may slow the axoplasmic transport, especially of mitochondria, particularly if stressful conditions such as exposure to toxic environmental factors occur. Oxidative stress may influence vulnerable cells, such as oligodendrocytes, and the turnover of axonal myelination may suffer (Smith et al., 1999). Respiratory impairment may produce some compensatory proliferation of mitochondria in specific tissues. This seems to be documented to some extent in skeletal and specialized muscle (Larsson et al., 1991; Sadun et al., 1994; Carelli, 2002) and in the endothelial and smooth muscle cells of blood vessels (Ross-Cisneros et al., 2001).

All these features may have minor consequences in the setting of metabolic homeostasis, even in LHON. Young adult males may undergo substantial changes in metabolism, and this may represent a risky period for the efficient compensation of a mitochondrial defect (Sadun, 1998a). Similarly, metabolic imbalance may be induced by other instances, such as non-compensated diabetes (DuBois and Feldon, 1992).

Over time, the system may become progressively less well compensated. The myelin sheath may become thinner than normal. This may induce some compensatory changes in axons, such as rearrangements of cytoskeleton (Sanchez et al., 1996; Griffiths et al., 1998; Brady et al., 1999; Witt and Brady, 2000), and a need for increased mitochondrial numbers (Mutsaers and Carroll, 1998). These changes would put the system under pressure causing increases in axonal diameter that manifest as axonal swelling visible at the fundus examination. Vascular changes may also play a role in the supply of oxygen and metabolites, particularly at the vulnerable optic nerve head level. Subclinical signs often appear before the patient complains of any visual loss. These consist mainly of pseudodema caused by swelling of the NFL, particularly evident on the superior and inferior arcades at the entrance of the optic disc and vascular changes, including telangetasias, tortuosity, some arteriovenous shunting and dilatation of the small vessels. The patient then notes visual loss, and the PMB rapidly disappears. The PMB loss is not accompanied by comparable swelling of its own thin fibers or by inflammatory changes; fluorescein angiography shows no leakage. The PMB enters the optic nerve and becomes organized as the most central of the fibers, while the superior and inferior nerve fiber arcades, which are visibly swollen, run along the periphery of the optic nerve. It is intriguing that the larger axons from the arcades swell dramatically but are largely spared by the wave of cellular death. The small fibers of the PMB are also believed to have highly disadvantageous metabolic and functional conditions that would make them more prone to a synchronized and rapid loss (Sadun, 1998a; Sadun et al., 2000). The swelling of the superior and inferior nerve fiber arcades diminishes just as the PMB is lost. The nasal periphery is the last set of fibers to be involved, as reflected by the consistent sparing of axonal profiles at the nasal margin on cross-section of optic nerve specimens in LHON histopathological studies (Sadun et al., 1994, 2000; Sadun, 1998a; Carelli et al., 2002a).

There is also evidence that astrocytes may play an active role, through changes of their functional state, in neurodegeneration of the optic nerve. A good example comes from studies of glaucomatous optic neuropathy, where the activated astrocytes that synthesize and release NO play a crucial role (Liu and Neufeld, 2000). There are multilevel consequences of NO as a possible trigger for rapid axonal loss (Carelli et al., 2002a). LHON leads to chronically increased ROS production that predicts a consequent increase of ONOO. Indirect evidence for this is seen in investigations of tyrosine nitration in optic nerve specimens from
LHON patients (Carelli et al., 2000). Two important targets for ONOO\(^-\)-related protein damage are complex I (Clementi et al., 1998; Stewart et al., 2000) and MnSOD (Yamakura et al., 1998). This target damage may consistently accelerate and worsen the mitochondrial dysfunction. Furthermore, NO\(^+\) can reversibly block nerve conduction in the presence of demyelinated axons (Redford et al., 1997), thus precipitating the functional conduction blockage of axons that precedes their eventual atrophy. The influence of NO\(^+\) on the functionality of small vessels is another issue to be investigated, especially in light of the vasculopathic changes we observed in LHON optic nerve specimens (Ross-Cisneros et al., 2001).

It remains difficult to account for all of these pathological events that may take part in the active pathophysiology of optic neuropathy in LHON. However, we can say that the mitochondrial redistribution in demyelinated axons and impairment of axoplasmic transport of mitochondria, the vascular changes at the optic nerve head, the activation of astrocytes and increase of NO\(^+\) synthesis and release, the possible mechanical dynamics between the swollen peripheral axons, and the vulnerable small fibers of the PMB all combine to lead to a synchronized wave of cellular death that propagates through RGC. This cellular death is most probably apoptotic, as it may be for glaucoma (Kerrigan et al., 1997), ischemic optic neuropathy (Levin and Louhab, 1996), and optic nerve axotomy (Berkelaar et al., 1994). The LHON cybrid studies strongly suggest that apoptosis is a consequence of complex I dysfunction (Danielson et al., 2002; Ghelli et al., 2003; Zanna et al., 2004), and the possible direct involvement of complex I and quinone in this process is suspected (Fontaine and Bernardi, 1999). There are peculiarities in the apoptotic path followed by LHON cybrids in galactose since apoptosis is caspase-independent despite the release of cytochrome c (Ghelli et al., 2003; Zanna et al., 2004). The recently reported regulation of MPTP opening in RGC is also peculiar (Vrabec et al., 2003). It is important to understand the exact timing of the events leading to RGC death and of the specific apoptotic paths in order to identify the windows of opportunity for therapeutic intervention during the acute phase of the disease and the setup of efficient anti-apoptotic drugs.

Two further, previously underemphasized, elements in LHON need to be considered, i.e. the chronic stage and visual recovery. Visual loss in LHON is usually a single episode disease, followed by a post-visual loss chronic stage. This phase, when apparently not much is happening, may actually be characterized by a continuous worsening of the residual visual function caused by low-grade activity of the degenerative process, as suggested by histological evidence (Sadun et al., 1994; Carelli et al., 2002a). Only in rare cases have recurrent episodes of visual loss throughout life, leading to further worsening of vision, been described (Chuman et al., 1999; Newman-Toker et al., 2003). At the same time, on rare occasions patients report a gain of visual acuity, which may also occur years after the onset of the disease. We have observed demyelinated axons that are otherwise normal. We have also described oligodendrocytic cytoplasmic tongues wrapping denuded axons, strongly suggestive of ongoing axonal remyelination. Both of these findings point to a dynamic process occurring with the surviving axons in LHON. A number of axons may remain functionally silent but still viable; in rare cases these axons may undergo remyelination and gain the functional properties of conducting action potentials. The understanding of the anatomo-microphysiological substrate for the phenomena of late visual recovery may be very important for developing a therapy for the chronic phase of LHON to limit further RGC death and restore axonal function.

7.5. Possible scenarios for DOA pathogenesis

Our understanding of DOA is much more limited and incomplete than our understanding of LHON. Hypothesizing the pathological events for DOA is an even more slippery exercise. However, the progressive course of the optic neuropathy and the early age of onset compared to LHON suggest a different pathological process that progresses with a dissimilar tempo. In DOA, RGC loss is not synchronized and is not associated with an apoplectic loss of vision. The cellular death, most probably apoptotic, proceeds slowly and almost silently, but still follows a pattern of selective involvement of the small fibers of the PMB, similar to that of LHON. The functional and anatomical characteristics of these smaller and less myelinated axons make them more vulnerable to mitochondrial dysfunction. However, none of the preclinical signs of LHON is reported in DOA, in particular the axonal swelling and the vascular changes. Moreover, the propagation of a synchronized wave of cell death seen in LHON does not seem to apply in DOA.

8. Is normal tension glaucoma another mitochondrial optic neuropathy?

NTG is a subtype of glaucomatous optic neuropathy, sharing most of the same clinical characteristics at fundus examination and visual field defects. However, NTG is not associated with elevated intraocular pressures. Hence, the NTG clinical features may overlap with both DOA and LHON, and misdiagnosis is a frequent occurrence. Because of these similarities, mitochondrial respiratory chain functionality has been investigated in NTG and LHON mutations have been
screened in NTG patients without success (Brierley et al., 1996; Opia et al., 2001).

Recently, a group of NTG patients have also been screened for mutations in the OPA1 gene, and in two cohorts of patients single nucleotide polymorphisms (SNPs) have been found associated (Aung et al., 2002a). The same polymorphisms have also been investigated in a cohort of patients with high-tension primary open angle glaucoma without success (Aung et al., 2002b). Furthermore, when the same authors compared NTG patients carrying the OPA1 SNPs with similar patients not bearing these SNPs, they reported that no significant clinical difference distinguished the two groups (Aung et al., 2003). These studies open some interesting possibilities, adding NTG to the list of optic neuropathies that may be linked to mitochondrial dysfunction (Buono et al., 2002). This seems an attractive hypothesis based on the clinical similarities with DOA and LHON. However, the association of DOA with SNPs in the OPA1 gene needs to be reproduced in other populations and on a larger scale. Moreover, the functional relevance of these SNPs needs to be elucidated since both IVS8 + 4 and IVS + 32 are intronic, thus not involving the coding sequence, but potentially interfering with RNA splicing.

9. What animal models teach us

Modeling the conditions to reproduce optic nerve pathology in animals has been widely used to advance our knowledge of the pathophysiology in glaucoma and ischemic optic neuropathies (Goldblum and Mittag, 2002). The rapidly growing field of human mitochondrial disorders has produced some attempts to develop a “mito-Mouse” to investigate the complexities underlying the pathogenesis of this category of human pathology (Hirano, 2001). Technically, the issue is not simple, particularly when the purpose is to reproduce a truly genetic model of mtDNA mutations in mice, homologous to those pathogenic for humans (Wallace, 2001). It was much easier to deal with the nuclear genes that also encode for mitochondrial proteins, and a few such mice models have been generated (Wallace, 2001). Biochemical manipulations have also been adopted to induce mitochondrial dysfunction by such means as the systemic administration of drugs or toxins that specifically interfere with the respiratory chain, as recently reported for MPTP- or rotenone-induced Parkinson’s disease in animal models (Betarbet et al., 2002).

The eye features in some of these nDNA and mtDNA mito-mice models are now being reported (Sligh et al., 2000; Sandbach et al., 2001; Bioussé et al., 2002). Some other animal models were created with the specific purpose of reproducing a mitochondrial optic neuropathy by biochemical manipulations (Sadun, 1998a; Zhang et al., 2002), or more recently, by a newly designed ingenious engineering of gene expression (Qi et al., 2003a, b).

9.1. nDNA mito-mice

This first group of mito-mice models recently established are the MnSOD-, GPx1-, ANT1-, and Tjα-deficient mice (Wallace, 2001). All of them were obtained by inactivating nuclear genes encoding for mitochondrial proteins. Both MnSOD- and GPx1-deficient mice models have been generated to explore the consequences of increased mitochondrial ROS production (respectively O$_2^-$ and H$_2$O$_2$) when each of these anti-oxidant enzymes is inactivated. While the GPx1-deficient mouse had only a relatively minor phenotypic expression (Esposito et al., 2000), the null MnSOD +/- animals were characterized by neonatal death (~8 days) caused by dilated cardiomyopathy when using the Sod2$^{tm1Cje}$ mouse strain on a CD1 background (Li et al., 1995) or by perinatal death (~18 days) caused by a neurological/myocardial syndrome when using the Sod2$^{tm1Leb}$ mouse strain on a B6 background (Lebovitz et al., 1996). Interestingly, the Sod2$^{tm1Cje}$ animals, when rescued with the SOD mimetic manganese 5,10,15,20-tetrakis (4-benzoic acid) porphyrin (MnTBAP) which does not cross the blood–brain barrier, no longer suffered the dilated cardiomyopathy, but extended their life-span and developed a neurological syndrome that resembles the human disorder Leigh syndrome (Melov et al., 1998). The biochemical features characterizing these MnSOD-deficient animals included the inactivation of mitochondrial enzymes containing Fe–S centers like complex I, complex II, and aconitase, the consequent respiratory impairment, and an increased tendency toward MPTP opening. Some of these features, like the preferential damage of the Fe–S center-containing enzymes, reproduce some of the biochemical findings in Friedreich ataxia (FA), a human mitochondrial neurodegenerative disease for which an increased oxidative stress was reported (Rotig et al., 1997; Wong et al., 1999). Interestingly, a subset of FA patients develop an optic neuropathy with some similarities with DOA (Carelli et al., 2002a). These studies open some interesting possibilities, adding NTG to the list of optic neuropathies that may be linked to mitochondrial dysfunction (Buono et al., 2002). This seems an attractive hypothesis based on the clinical similarities with DOA and LHON. However, the association of DOA with SNPs in the OPA1 gene needs to be reproduced in other populations and on a larger scale. Moreover, the functional relevance of these SNPs needs to be elucidated since both IVS8 + 4 and IVS + 32 are intronic, thus not involving the coding sequence, but potentially interfering with RNA splicing.

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The ocular phenotype of the MnSOD-deficient mouse model has recently been reported (Sandbach et al., 2001). This study compared old (20–21 days) and younger (9–10 days) MnTBAP-treated animals, with treated and untreated control animals. A thinning of the
retina involving all but the outer nuclear layer characterized the mutant treated animals at 20–21 days of age. All the other layers, including the ganglion cell layer and the photoreceptor layer, were very inviolated compared with controls. At the ultrastructural level, clumps of abnormally swollen mitochondria, with pale matrix and disorganized cristae, were present in the retinal pigmented epithelium (RPE) of the older mutant animals treated with MnTBAP. The cross-sectional area of the optic nerve was smaller in the mutant animals compared with controls, as expected by the thinning of the inner retinal layers. However, no ultrastructural abnormalities were detected in the axons and myelins. Abnormally pale and swollen mitochondria with dis abnormalities were detected in the axons and myelins. Abnormally pale and swollen mitochondria with disorganized cristae were also noted in the extraocular muscle of 16-day-old untreated mutant mice, while treated animals displayed no obvious abnormality (Sandbach et al., 2001).

Taken together these findings do not faithfully reproduce any of the known ocular phenotypes caused by mitochondrial dysfunction. The mitochondrial abnormalities noted in the RPE may somehow resemble the retinitis pigmentosa-like retinal dystrophy more typically seen in Kearn-Sayre syndrome (KSS), neuroopathy, ataxia, retinitis pigmentosa (NARP) or MELAS (McKechnie et al., 1985; Chang et al., 1993). However, the thinning of the inner retinal layers seems to indicate a developmental impairment of the mouse retina more than an active pathological process involving the RGC. This observation is corroborated by the apparent lack in ultrastructural abnormalities in axons and myelins of the optic nerve. As expected, the MnTBAP treatment rescued the extraocular muscle mitochondrial abnormalities, which were seen only in untreated animals. Although all retinal layers are post-blood–brain barrier and, thus, are exposed to the MnSOD deficiency, only the RPE clearly displayed mitochondrial abnormalities.

The \textit{ANTI-deficient mouse} is considered a model of ATP deficiency since ANT is the transporter protein deputed to exchange the ATP synthesized at the matrix side with ADP across the inner mitochondrial membrane (Graham et al., 1997). Both the \textit{ANT1} and \textit{ANT2} isoforms are expressed in the eyes and optic nerves (Napoli et al., 2001); thus, a pathological phenotype may affect these ocular tissues. The main clinical features reported for this animal model involve the skeletal muscle, evidenced by a typical mitochondrial myopathy exhibiting ragged-red-fibers (RRF), and the heart, manifested by a hypertrophic cardiomyopathy (Graham et al., 1997). The lack of exchange of ADP/ATP is expected to produce a hyperpolarization of the inner mitochondrial membrane, to inhibit the respiratory chain, and ultimately, to increase the spilling of electrons leading to ROS formation. A consistent upregulation of the anti-oxidant enzymes (MnSOD and GPx) was in fact reported for the skeletal muscle, though not for the heart; consequently a relevant accumulation of mtDNA multiple deletions was observed as compared with the skeletal muscle (Esposito et al., 1999). Mutations in the human \textit{ANT1} gene are associated with autosomal dominant progressive external ophthalmoplegia (adPEO) and mtDNA multiple deletions (Kaukonen et al., 2000). However, the generation of mtDNA multiple deletions in this human pathology has been proposed to be differently mediated by a perturbation of the nucleotide precursors’ pool, thus interfering with the mtDNA replication process by analogy with another human disease characterized by accumulation of mtDNA multiple deletions and depletions, namely the mitochondrial neuro-gastro-intestinal encephalomyopathy (MNGIE) syndrome. This latter syndrome is caused by mutations in the thymidine phosphorilase gene (Nishino et al., 2001). Ocular dysfunction, other than ophthalmoplegia, has only rarely been reported in these human disorders (Nishino et al., 2000). Yet, when the eyes of the \textit{ANTI-deficient mice} were recently evaluated, electroretinograms showed the unexpected feature of supranormal responses (a trend toward larger b-wave amplitudes for higher light stimulus), and there were no gross abnormalities at pathological examination of the retina (Pardue et al., 2002). At the ultrastructural level, more numerous and smaller mitochondria were described in the photoreceptors of mutant mice. The optic nerve and extraocular muscle examinations have yet to be performed.

The \textit{Tfam-deficient mouse} has been produced by genetic inactivation of the nuclear encoded mitochondrial transcription factor (Tfam) and is considered a model of mtDNA depletion, given that Tfam-dependent synthesis of the RNA primer by transcription from the light-stranded promoter also regulates initiation of replication of the leading strand of mtDNA (Larsson et al., 1998). This mouse model has been produced in multiple flavors by a conditional mutation strategy to manipulate the expression of Tfam in specific tissues, with systemic (Larsson et al., 1998), cardiac-specific (Wang et al., 1999; Li et al., 2000), pancreatic \beta-cell-species (Silva et al., 2000), and skeletal muscle-specific (Wredenberg et al., 2002) mice models. However, the ocular features of the systemic model have not yet been investigated, and no ocular-specific version of this mouse model has yet been designed.

9.2. \textit{mtDNA mito-mice}

This second group of mito-mice models includes the only true animal models carrying an mtDNA pathogenic defect: a single deletion in one case (\textit{ΔmtDNA4696}) (Inoue et al., 2000; Nakada et al., 2001) and a single point mutation (2433T>C in the 16S rRNA) responsible for chloramphenicol-resistance.
(CAPR) in the other (Sligh et al., 2000). The ΔmtDNA4696-mice model is intended to reproduce the pathological features of the human diseases linked with single mtDNA deletion, in particular the KSS. The clinical phenotype of the ΔmtDNA4696-mice does not faithfully reproduce the KSS pathological features (Inoue et al., 2000); however, these animals displayed some of the key features of the human disorder and proved to be an exciting model to study mitochondrial diseases (Nakada et al., 2001). So far, no investigations of the ocular system in the ΔmtDNA4696-mice have been reported. It is predicted that retina and optic nerve may show some of the typical pathology seen in human mitochondrial disorders.

The CAPR-mice suffered frequent perinatal or in utero lethality; surviving mice displayed growth retardation, myopathy, and dilated cardiomyopathy (Sligh et al., 2000). These animals resulted from the germ-line transmission of the 2433T>C mtDNA point mutation in the 16S rRNA, in both homoplasmic and heteroplasmic fashion, the latter being the survivors. The chimeric animals, initially obtained by injection of the embryonic stem cell mutant cybrids into the blastocysts, were characterized by congenital cataracts and reduction of the ERG b-wave amplitudes, with both rods and cones similarly affected (Sligh et al., 2000). The histopathological investigation of the eyes from the CAPR-mice was characterized by pigmented epithelium vacuolization and full preservation of the photoreceptors. Moreover, hamartomatous-like changes were also described at the optic nerve head, though some concern was raised in the interpretation of this finding since similar features have been described at the emergence of the central retinal artery at the optic nerve head of normal mice (May and Lutjen-Drecoll, 2002). Thus, the retinal defects observed in the CAPR-mice are reminiscent of the retinitis pigmentosa-like retinal dystrophy in KSS or NARP syndromes in humans.

We recently examined the ocular histopathology from B6SJL mice fed with increasing concentrations of chloramphenicol, which, to some extent reproduced the same features as the genetic model of CAPR-mice, although more severely (Ross-Cisneros et al., 2002). High dose chloramphenicol administration (1000 mg/kg/day for 15 days) induced devastating changes in the murine eye, affecting mostly the outer retina. The changes included severe loss of the photoreceptor and outer nuclear layers, and focal patches of dense pigment in the RPE. Degenerative features were also evident in the inner retina; swollen RGC and a preferential loss of small axons in the optic nerve were noted (Ross-Cisneros et al., 2002). Thus, a marked retinal dystrophy mimicking the KSS and NARP syndromes of humans was observed. Additionally, the involvement of small axons in the optic nerve was reminiscent of LHON. Chloramphenicol has a well-known inhibitory effect on mitochondrial protein synthesis, which, in turn, leads to a respiratory chain deficiency and hence models mitochondrial optic neuromyopathies. Indeed, an iatrogenic phenocopy of LHON is represented by reported cases of chloramphenicol-induced optic neuropathy in humans (Harley et al., 1970; Carelli et al., 2002a).

9.3. Biochemical mito-mice

The third group of animal models of optic neuropathy is based on biochemical manipulations designed to reproduce certain conditions of impaired respiratory function that may reflect nutritional and toxic optic neuropathies. One such strategic approach was aimed to reproduce the nutritional and toxic conditions characterizing the Cuban epidemic of optic neuropathy (CEO). The rationale was to combine folate deficiency with chronic methanol toxicity so as to increase formate production to match the serum levels measured in patients blinded by CEO. Formate inhibits COX leading to a mitochondrial respiratory defect (Nicholls, 1975, 1976), which probably recapitulates the pathology produced by vitamin deficiency and methanol intake seen in CEO (Sadun, 1998a; Eells et al., 2000). The histological features of the animals from the folic acid-deficient diet/methanol-injected mice group revealed extensive vacuolation in the fibers of the optic nerve head, anterior to the lamina cribrosa as compared to controls (Sadun, 1998a). Ultrastructural examination showed accumulation of intraxonal debris, neurofibrillary fragmentation, focal swelling, and a relative abundance of mitochondria, many of which were swollen and with disrupted cristae (Sadun, 1998a). Overall, most of these features are also highly evocative of the histopathological descriptions in LHON (Sadun et al., 1994; Carelli et al., 2002a).

A further model of LHON-like optic neuropathy has recently been obtained in mice by intravitreal administration of the complex I inhibitor rotenone (Zhang et al., 2002). The thickness of the RGC layer in the rotenone-mice group was significantly reduced compared to controls, mimicking once again the histological findings in LHON. This is an easy and rapidly available animal model, but it does not faithfully reproduce the tissue specificity of LHON. Any molecule toxic to the respiratory chain that is injected into the vitreous would prevalently affect the RGC inducing their degeneration, since they are the first cellular layer encountered. It would be interesting to examine the eyes from the rotenone-mice model generated by systemic administration of rotenone, as has already been done to reproduce Parkinson's disease (Betarbet et al., 2002). Experimental conditions in terms of rotenone concentration and adequate time-course protocol could be found not only to induce Parkinson's disease but possibly to reach a selective RGC degeneration in an otherwise intact
retina, thus reproducing a complex I-dependent optic neuropathy that mimicks LHON.

9.4. Ribozyme-engineered mito-mice

The most recent and ingenious experimental approach used to reproduce an LHON-like optic neuropathy in mice involves intravitreal administration of an adeno-associated virus (AAV), which has been engineered to express a hammerhead ribozyme targeted to suppress either the gene expression of the MnSOD gene (Qi et al., 2003a), or the nuclear-encoded NDUFA1 subunit gene of complex I (Qi et al., 2003b). In both ribozyme-MnSOD mice and ribozyme-NDUFA1 mice models the animals developed an optic neuropathy closely resembling the histopathological features of LHON, indirectly suggesting that extreme conditions of respiratory chain blunting the histopathological features of LHON, indirectly suggesting that extreme conditions of respiratory chain deficiency, as induced by profoundly deficient complex I lacking NDUFA1 (Au et al., 1999), and overproduction of ROS, as induced by abolished MnSOD activity, may lead to a similar pathological result. Retinal thinning due to RGC loss and optic nerve head swelling characterized both models. Intracellular edema and mitochondrial accumulation in the unmyelinated axons at the optic nerve head contributed to the swelling. Mitochondria were pathologically distended and with dissolved cristae. Moreover, in both models, demyelination of axons preceded their loss and the degeneration of RGC bodies in the retina. Axons denuded of myelin and others with signs of remyelination were also reported.

Taken together, the histopathological details and the sequence of events reported in these last two mice models were very similar to our histopathological findings in human optic nerve specimens from LHON patients. Thus, the scenario of pathological events we outlined previously gets some substantial confirmation about the interconnected involvement of demyelination, axonal transport impairment, and local vulnerability of the prelaminar unmyelinated axons at the optic nerve head.

9.5. Other mice models of interest

One more animal model of great interest is the recently reported “Harlequin” (Hq) mutant mouse, in which a proviral insertion in the AIF gene induces about 80% reduction in AIF expression (Klein et al., 2002). Interestingly, the Hq-mutant-mice suffer progressive degeneration of terminally differentiated cerebellar and retinal neurons. In particular, loss of RGC was the earliest phenomenon in the retina, followed by a progression of retinal degeneration, involving first the inner, then the outer, nuclear layers, and finally all cell layers. Dying neurons in the aged Hq-mutant-mice showed evidence of oxidative stress (increased peroxide sensitivity), and they re-entered the cell cycle before undergoing apoptosis (Klein et al., 2002). These features suggest that AIF plays a role as a free radical scavenger, aside from being a pro-apoptotic factor. It is particularly intriguing that AIF seems also to be one of the key mediators of apoptosis in the LHON cybrid model with galactose, in which a caspase-independent activation of apoptosis was documented (Ghelli et al., 2003; Zanna et al., 2004). Furthermore, this animal model highlights again the importance of oxidative stress in RGC degeneration and apoptosis as the preferred death pathway in neuronal degeneration. Plenty of animal models of cell death have been produced so far, knocking out most of the known genes involved in the multi-step apoptotic cascade (Ranger et al., 2001), but the eye is rarely investigated. The study of ocular pathological features in these animal models may turn out to be extremely informative with regard to the retinal physiopathological processes.

Finally, the Bst (belly spot and tail) mutant mice, which show atrophy of the optic nerves, as well as a more complicated syndrome, and which was proposed as a possible model for DOA, have been recently shown not to be linked to the murine ortholog of the human OPA1 gene responsible for DOA (Delettre et al., 2003).

10. Gene therapy, neuroprotection and other treatments: possible therapeutic modalities may offer

Therapy for mitochondrial disorders and neurodegenerative diseases remains elusive. There is no effective therapy for LHON, nor for DOA currently available. The obvious therapeutic strategy should be the correction of the genetic defect in LHON and DOA.

Some important steps have recently been undertaken in this direction for LHON. The complementation of complex I genetic defects has been accomplished by two different strategies. In one model, the trasfection of the complex I-deficient Chinese hamster CCL16-B2 cells with the Saccharomyces cerevisiae NDII gene effectively restored the respiratory deficiency (Seo et al., 1998). The next step was to reproduce this approach using a human cell line carrying a lethal, frameshift mutation in the mitochondrially encoded ND4 subunit of complex I (Bai et al., 2001). Transfection with the Saccharomyces cerevisiae NDII gene completely restored the NADH dehydrogenase activity, though the original defect was very severe, since there is no assembly of the mDNA-encoded complex I subunits with such mutation (Hofhaus and Attardi, 1993). Thus, the Saccharomyces cerevisiae NDII gene was successfully integrated into the mammalian nuclear genome, expressed, transported to mitochondria, and assembled into the inner membrane. There it was able to transfer electrons from NADH to CoQ and feed the downstream respiratory chain,
restoring malate/glutamate-dependent respiration. These studies highlight the potential of the yeast ND4 gene for the therapy of mitochondrial diseases involving complex I deficiency.

The second, recently developed, model is based on the so-called “allotopic expression” in the nucleus of a recorded and adequately engineered mtDNA gene (Guy, 2000; Larsson, 2002). The general idea is to express the mtDNA-encoded protein in the nucleus, having corrected the mutant nucleotide. Such a corrected protein, expressed in the cytoplasm, is then targeted to mitochondria and assembled within the specific enzymatic complex in the inner mitochondrial membrane, competing with the mutant version of the same gene expressed locally by the mtDNA. The slight differences in the genetic code of mitochondria, with respect to the nuclear DNA, require to recode the mtDNA gene according to the nuclear genetic code, correcting also the LHON mutant nucleotide position. Further, this recoded gene required the addition of a specific mitochondrial targeting sequence at the amino terminus, to import the expressed protein within the mitochondria. Additionally, to monitor the localization of this recoded and corrected protein, different sequence tags had to be added at the carboxy terminus, such as green fluorescent protein or smaller FLAG tags. This transgene construct was then inserted within a suitable vector to be transfected to cells. The choice for these experiments was an AAV, which has successfully been used to introduce genes into the visual system of experimental animals (Guy et al., 1999; Owen et al., 2000). Transgene expression was finally driven by the chicken β-actin promoter and cytomegalovirus enhancer.

Using this technique, the complementation of defective mitochondria was accomplished in two different cellular hybrid cell lines carrying, respectively, the mtDNA NARP 8993T > G mutation (Manfredi et al., 2002), and the LHON 11778G > A mutation (Guy et al., 2002). In the case of LHON, two criteria have been used to confirm that the allotopic expression successfully corrected the biochemical deficiency. The LHON mutant cybrids restored the ability to grow in galactose medium and greatly increased their ATP synthesis rate, reaching the normal range. The use of the AAV to introduce genes into cells of the visual system has previously been accomplished in experimental animals. Thus, this approach to express the recoded ND4 gene by the nucleus of the RGC of LHON patients seems a promising avenue for a succesful gene therapy. The same considerations apply in the case of NARP-related retinal dystrophy.

Despite the excitement for the scenario opened by such genetic approaches to prevent optic atrophy through drug-based therapies. Three different lines of therapeutic intervention can be identified:

- therapy to increase the respiratory activity and the ATP synthesis,
- therapy to titrate the excess oxidative stress,
- therapy to limit or inhibit the apoptotic cascade.

These different approaches for LHON address different stages of the pathological process. The first two are thought to work as a chronic, preventive therapy before the patient reports any visual loss. Their effectiveness, if at all, would be only before any visual loss is reported. The symptomatic phase of LHON would be too late for correcting the biochemical defect, given the rapidity with which the PMB is lost and the rapid spreading of the wave of apoptotic death to RGC. At the onset of visual loss, the therapeutic approach would be an anti-apoptotic strategy (Waldmeier, 2003). The details with which the pathological events occur in the transition from the subclinical changes observable at the ophthalmoscopic examination to the frank onset of visual loss are essential to define the window of opportunity for a therapeutic intervention. The clinical presentation in LHON is quite stereotypical, and the interval between the involvement of the first and the second eye may be considered as a window for starting such a therapy. The rescue of the second eye, or its better outcome, may represent a convincing demonstration of the efficacy of neuroprotection therapy. A controlled study, would be very difficult in LHON.

Therapeutic attempts in LHON have recently been based on the use of CoQ10 or its short chain derivative idebenone (Cortelli et al., 1997; Carelli et al., 1998a, b; Mashima et al., 2000). There is a double rationale for the use of quinones in LHON: the restoration of electron flow and the increase of anti-oxidant defenses (Geromel et al., 2002). Moreover, a potential enhancement of shuttling electrons through the alternative pathway of complex II is considered as a possible way to maintain the electron flow to the downstream respiratory chain (Degli Esposti et al., 1996). However, the effectiveness of idebenone therapy in LHON is controversial, and the timing with which the therapy is started may partially determine the different outcome in patients. Other promising ROS scavengers are being studied experimentally but are not yet being used in human drug trials (Melov, 2002). We continue to advise LHON patients to not smoke or drink alcohol and avoid exposure to a variety of toxins; there is good evidence indicating that exposure to these environmental factors may be of great importance (Sadun et al., 2003).

The use of pharmaceuticals, such as idebenone, in an attempt to correct the biochemical dysfunction, would probably be more effective as a preventive measure rather than after visual loss. There are many new
strategies which fall under the wide rubric of “neuro-protection” that may be more appropriately used to contrast cell death. Many of these strategies are intent on altering the delicate balance that exists within a cell between the mechanisms that promote the apoptotic cascade and those that exert an anti-apoptotic effect, favoring the latter. One such agent that has recently drawn some attention is the alpha-2 receptor agonist brimonidine (Yoles et al., 1999; Tatton et al., 2001; Wheeler et al., 2003). The neuroprotective properties of brimonidine are exerted by maintenance of mitochondrial membrane potential and Bcl-2 upregulation. This agent currently used in glaucoma may be a suitable candidate for a therapy after visual loss in LHON, particularly in the attempt to rescue the second eye as previously outlined.

Studies in glaucoma have also demonstrated the potential benefit of inhibitors of inducible nitric oxide synthase (NOS-2) (Neufeld et al., 1999). Aminoguanidine, a relatively specific inhibitor of NOS-2, has been shown to provide neuroprotection of RGC in a rat model of chronic glaucoma. NOS-2 expression is increased in reactive astrocytes clustered in the areas of nerve damage in the prelaminar and lamina cribrosa of glaucomatous optic nerve heads. The excess NO produced by reactive astrocytes led to degeneration of RGC (Liu and Neufeld, 2000). Our histological evidence of low-grade axonal degeneration, activated astrocytes and nitrotyrosine labeling in LHON optic nerve heads may suggest a similar pathophysiology (Carelli et al., 2000, 2002a). Thus, LHON and glaucoma, as well as DOA and NTG, may all share similar mechanisms of RGC degeneration, and therapeutic strategies for one disease may prove useful for the others.

Therapeutic photobiomodulation represents a completely different approach to neuroprotection (Eells et al., 2003). LED photobiomodulation by red to near-IR radiation has been shown to stimulate COX activity, thus promoting cell survival in vitro using primary neuronal cells (Wong-Riley et al., 2001). The use of this approach in vivo was successfully applied to protect the retina in a rodent model of methanol intoxication, which is mediated by the accumulation of toxic levels of formic acid and consequent inhibition of COX (Eells et al., 2003). Hence, photobiomodulation shows promise in the therapeutic approach of retinal diseases including LHON, in which mitochondrial dysfunction is postulated to play a role. However, a concern may arise from the enhanced generation of ROS that is reported as accompanying red to near-IR light stimulation of mitochondrial electron transport (Karu, 1999).

Finally, neuronal regeneration may represent the Holy Grail for optic neuropathies (Horner and Gage, 2002). The capability of neurons to regrow is very limited due to a number of inhibitory factors. However, growth-promoting substrates are also being identified and studies on the possible reactivation of embryonic axonal growth, essential to induce regeneration in adult neurons, is actively being pursued in the RGC system (Goldberg et al., 2002a, b).

11. Future directions

It has been over 15 years since the molecular basis of LHON was discovered to be point mutations in mtDNA. Yet we are still faced with several extraordinary enigmas with this disease. Male prevalence, incomplete penetrance, tissue specificity and age-related delayed onset all remain unexplained. The pathogenic pathway, from the biochemical dysfunction of complex I to RGC death, is not yet clear. In this review, we have undertaken the exercise of assembling different available pieces of the puzzle to suggest a few possible scenarios. The recent demonstration that other optic neuropathies are also related to a primary mitochondrial dysfunction, such as DOA and possibly others, may accelerate the degree of our understanding of RGC physiopathology.

Cellular models, such as cybrids, are very useful for delving into the complexities of biochemical dysfunction, and to test possible therapeutic agents. The construction of more reliable cell lines, as recently accomplished with neuronal-like differentiated cybrids, is likely to reveal even more. Further investigations will look more closely at, and compare the role played by the nuclear genome in affected individuals in contrast to unaffected mutation carriers in order to isolate the elusive nuclear modifying gene postulated in LHON. The combination of in vitro and in silico investigations may efficiently complement more traditional genetic strategies, such as linkage analysis of large pedigrees, in the search for the subtle nuclear-mitochondrial-environmental interactions. Furthermore, parallel investigations of the apoptotic cascade in both LHON and DOA need to be undertaken in the near future, as well as the study of the respiratory chain in DOA and of mitochondrial cellular distribution in LHON.

However, most likely the true breakthrough will come from animal models that can clarify the early pathological stages leading to RGC death and optic nerve atrophy. In particular, the inter-relationships of myelin turnover, axonal transport and physiology, vascularization, and neuronal–glial metabolic exchanges, will need such investigation. The difficulties in generating a true mtDNA mutant animal model carrying a defect equivalent to LHON may now be overcome by the availability of the nuclear-encoded OPA1 gene responsible for DOA. Knocking out OPA1 will be the obvious next step. Overall, we expect the generation of a reliable animal model that faithfully mimics human optic nerve degeneration, as an extremely valuable tool with which to test new and promising therapeutic strategies.
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