CHAPTER THREE

Mitochondria in Multiple Sclerosis: Molecular Mechanisms of Pathogenesis

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Abstract

Mitochondria, the organelles that function as the powerhouse of the cell, have been increasingly linked to the pathogenesis of many neurological disorders, including multiple sclerosis (MS). MS is a chronic inflammatory demyelinating disease of the central nervous system (CNS) and a leading cause of neurological disability in young adults in the western world. Its etiology remains unknown, and while the inflammatory component of MS has been heavily investigated and targeted for therapeutic intervention, the failure of remyelination and the process of axonal degeneration are still poorly understood. Recent studies suggest a role of mitochondrial dysfunction in the neurodegenerative aspects of MS. This review is focused on mitochondrial functions under physiological conditions and the consequences of mitochondrial alterations in various CNS disorders. Moreover, we summarize recent findings linking mitochondrial dysfunction to MS and discuss novel therapeutic strategies targeting mitochondria-related pathways as well as emerging experimental approaches for modeling mitochondrial disease.

1. INTRODUCTION

Mitochondria are organelles with a complex structure and function. They are derived from an α-proteobacterium-like ancestor, due to an ancient "invasion" that occurred more than a billion years ago (Dyall et al., 2004). The acquisition of mitochondria equipped the eukaryotic cell with bioenergetic and biosynthetic factories, which were essential for its evolutionary success.

The mitochondrion is composed of a double-membrane system with a medial component traditionally referred to as the intermembrane space (IMS). The mostly permeable outer membrane (OMM) is quite similar to the phospholipid bilayer of the eukaryotic cell membrane, which allows the passage of ions and metabolites up to 10 kDa in size. In contrast, the highly selective inner mitochondrial membrane (IMM) is similar in lipid composition to the bacterial cell membrane and is characterized by invaginations termed cristae, which enclose the core of the organelle, known as the mitochondrial matrix. The matrix contains several proteins involved in the tricarboxylic acid (TCA) cycle and fatty acid oxidation as well as one circular molecule of mitochondrial DNA (mtDNA), which is double-stranded and contains 37 genes encoding subunits of the mitochondrial respiratory chain required for ATP production, rRNAs, or tRNAs (Scarpulla, 2008) (Fig. 1).

Within the inner membrane, it is possible to distinguish two distinct regions: the inner boundary membrane (IBM) and the cristae membrane.

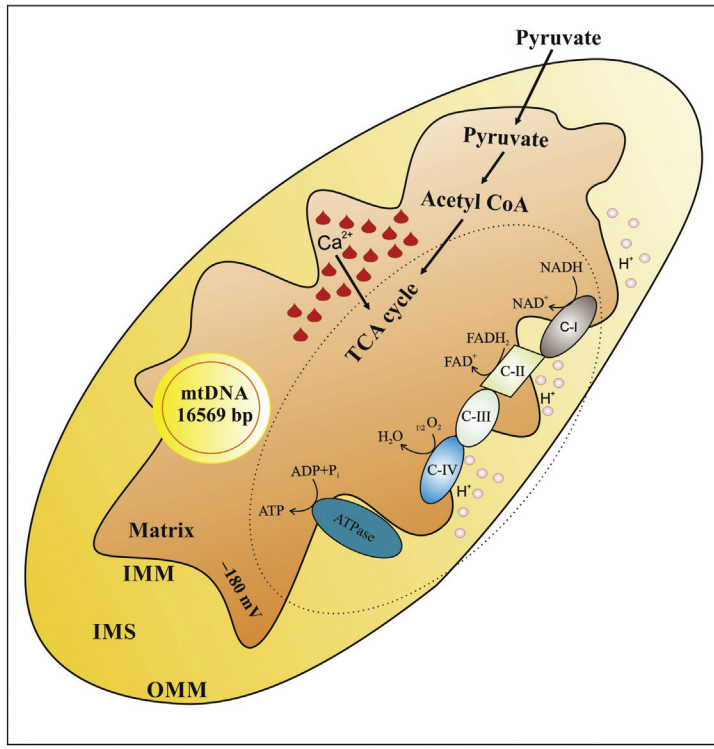

Figure 1 The mitochondrial compartment. Mitochondria are essential components of eukaryotic cells with a structure that is distinct from that of other organelles. They contain two membranes: the outer mitochondrial membrane *(OMM)* and the inner mitochondrial membrane *(IMM)*, which separates the intermembrane space *(IMS)* from the matrix. Within the mitochondrial matrix resides the mitochondrial DNA *(mtDNA)*. mtDNA is composed of 16,569 base pairs and encodes only 37 genes. Mitochondria are involved in a diverse set of cellular processes, including cellular signaling, apoptosis, and cell growth. They are also known as the powerhouse of the cell because they regulate ATP production. After glycolysis, one molecule of glucose is converted into two molecules of pyruvate, which then enters the mitochondria, where it is converted into acetyl CoA, the fuel of the citric acid cycle (TCA). As a result, the total number of molecules generated through the oxidation of pyruvate includes 2 ATP, 8 NADH, and 2 $FADH_2$ molecules. NADH and $FADH_2$ donate electrons to the respiratory chain complexes (complexes I–III–IV and II–III–IV, respectively), which transfer electrons across the membrane to O_2, reducing the O_2 molecule to water. The flow of electrons from NADH to O_2 across these complexes allows protons to be pumped from the mitochondrial matrix to the IMS, generating a potential difference of up to 180 mV. This gradient produces a proton motive force, which supports the proton flux through ATP synthase.

Cristae are attached to the IBM by narrow, tubular openings termed "crista junctions." This specific architecture creates a barrier that prevents the diffusion of metabolites, such as protons and ADP (Zick et al., 2009). The IBM is enriched in structural proteins and components of the import machinery of mitochondria (Vogel et al., 2006). Mitochondrial morphology differs depending on the tissue and undergoes dramatic changes depending on the physiological state and developmental stage (Rizzuto et al., 1998). This heterogeneity results from the balance between fusion and fission via a process termed mitochondrial dynamics. The extreme complexity of the mitochondrial structure mirrors the various functions of the organelle and its involvement in a wide variety of cellular processes.

2. BIOLOGICAL IMPORTANCE OF MITOCHONDRIA

Within cells, the energy required to sustain processes, such as macromolecule synthesis, muscle contraction, active ion transport, and thermogenesis is provided by oxidation of carbon-based metabolic fuels, primarily carbohydrates, lipids, and proteins. The free energy produced by oxidation is not dispersed as heat—as it is during combustion—but is instead stored in the high-energy bonds of different types of molecules, the most important of which (in terms of abundance and the number of known reactions catalyzed) is adenosine-5'-triphosphate (ATP).

The primary carbon source for cellular metabolism is glucose, which is catabolized through three consecutive processes, namely glycolysis, the TCA cycle, and oxidative phosphorylation (OxPhos), to produce 38 molecules of ATP per molecule of glucose. Glycolysis occurs in the cytoplasm and converts one molecule of glucose into two molecules of pyruvate, which are then transported into the mitochondria and converted to acetyl coenzyme A (acetyl-CoA). Through the TCA cycle, acetyl-CoA undergoes a series of oxidative reactions that generate energy-rich electrons. The flow of electrons across the respiratory chain complexes (complex I–IV) is utilized during OxPhos to pump protons from the mitochondrial matrix to the IMS. The energy accumulated via this proton gradient is ultimately used by the F1/FO ATP synthase (also known as complex V) to phosphorylate ADP and generate 36 molecules of ATP per molecule of glucose (Bonora et al., 2012; Madeira, 2012) **(**Fig. 1**)**.

2.1 Mitochondrial Regulation of Energy Metabolism in the CNS

The CNS, and particularly the brain, utilizes a large fraction of the body's energy production, estimated to correspond to approximately 20% of the total O_2 consumed (Mink et al., 1981). This energy is mostly represented by cytosolic ATP, which is utilized as a cofactor of Na^+/K^+ ATPase activity to equilibrate the intracellular ion concentration following an action potential, to facilitate neurotransmitter uptake and, to a lesser extent, other cellular processes, including biomolecule synthesis and intracellular trafficking. Nevertheless, different cells of the CNS (specifically neurons, astrocytes, and oligodendrocytes) show highly specific metabolic profiles, a concept known as "metabolic compartmentalization."

Within neurons, energy consumption is predominantly attributed to the synapses, and mitochondria are preferentially located at pre- and postsynaptic terminals, where most energy is predicted to be required (Harris et al., 2012). Notably, the synaptic terminals degenerate earlier in neurodegenerative diseases associated with impaired energy metabolism, such as Parkinson's disease (PD) (Winklhofer and Haass, 2010) and Alzheimer's disease (Bonda et al., 2010). Recently, Pathak and coworkers observed that even when the mitochondria are not in close proximity to synaptic boutons, the mitochondrial ATP produced at synaptic terminals diffuses rapidly to support energetic activities in all proximal boutons. Their study also showed that the energy demand is mostly related to the endocytic vesicle fusion phase required for synaptic vesicle recycling (Pathak et al., 2015). It has been suggested that synaptic mitochondria (sMITO) become specialized by activating specific metabolic pathways. In contrast to nonsynaptic mitochondria (ns-MITO), sMITO downregulate components of the respiratory complexes and specific mitochondrial calcium channels, possibly to preserve the synaptic terminal from detrimental activation of cell death pathways caused by ROS or high Ca^{2+} levels (Bonora et al., 2015). This explanation is corroborated by the observation that the number of mitochondria at the synaptic terminal is greater than would be required by the predicted energy consumption at that site (Wong-Riley, 1989; Harris et al., 2012). In a recent study, Völgyi and coworkers (Volgyi et al., 2015) observed reduced levels of superoxide dismutase 2 (SOD2), an ROS scavenger, suggesting that sMITO could be more sensitive to ROS. Furthermore, their findings clearly indicate that the protein composition of sMITO is modified in response to specific synaptic functions.

Neurons predominantly rely on OxPhos for energy production (Lin et al., 2010). However, most of the reduced carbon is not in the form of glucose, but lactate, provided by neighboring astrocytes, at least under resting conditions (Bouzier-Sore and Pellerin, 2013; Dienel and Cruz, 2004).

The transfer of lactate between astrocytes and neurons is referred to as the astrocyte-neuron lactate shuttle (ANSL). Astrocytes consume most of the available glucose in the brain to sustain their energy demand, but the resulting pyruvate is converted to lactate and extruded, rather than being utilized for OxPhos. The extruded lactate is then taken up by neurons, where it is reconverted to pyruvate to support respiration (Belanger et al., 2011).

In line with this model, analyses of metabolic enzymes have shown low levels of pyruvate dehydrogenase (PDH) in astrocytes, reinforcing the idea that pyruvate does not primarily enter the TCA cycle **(**Fig. 2**)**.

Although the role of ANSL in brain energy metabolism remains controversial (Hall et al., 2012; Patel et al., 2014), increasing evidence supports

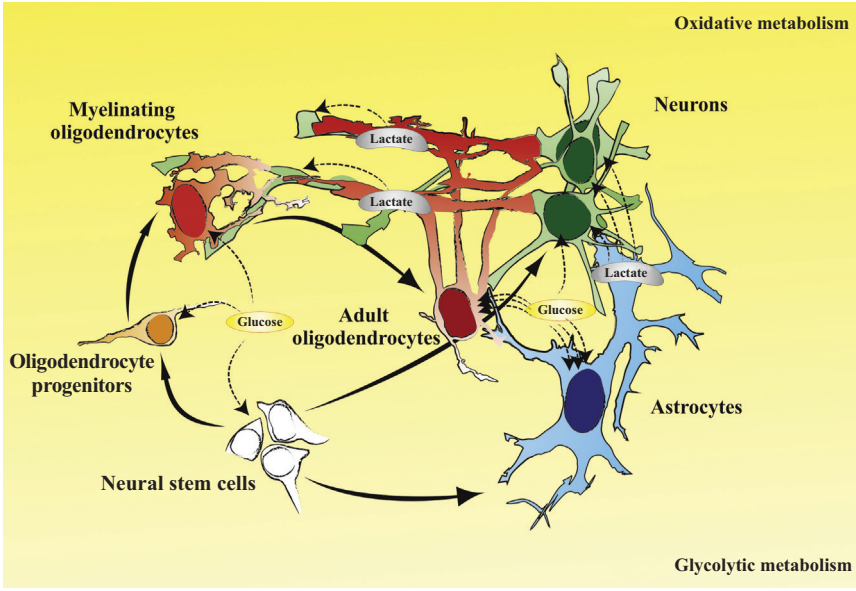

Figure 2 Major routes of metabolism. Proliferating neural stem cells (*white* cells) and progenitors (*yellow* cells) rely on glycolytic supply of metabolic demands as well as adult astrocytes (*blue* cells) or adult myelin forming oligodendrocytes (*red* cells). The latter two are responsible of largest glucose (*orange* balloon) consumption and production of important lactate (*gray* balloon) amount. Lactates in turn support glucose in feeding oxidative metabolism in neurons (*green* cells) and myelinating oligodendrocytes (*orange* cells).

the occurrence of metabolite exchange between astrocytes and neurons. Indeed, it has been demonstrated that astrocytes can rapidly take up the glutamate released during synaptic transmission so that it can be recycled. Through the glutamate–glutamine cycle, astrocyte-specific glutamine synthetase converts glutamate into glutamine, which is then transported to neurons and converted back to glutamate.

Metabolic compartmentalization is also observed in oligodendrocytes, the myelinating cells of the CNS. Through conditional KO of COX10 (a member of respiratory complex IV), it was shown that myelinating oligodendrocytes use glycolysis to meet their energy demands, rather than OxPhos (Funfschilling et al., 2012). Interestingly, Lee and coworkers observed that myelinating oligodendrocytes express the highest levels of monocarboxylate transporter 1 (MCT1), which they use to extrude lactate. Pharmacological inhibition or genetic deletion of MCT1 leads to axonal damage, indicating that adult oligodendrocytes participate in the ANSL (Lee et al., 2012). Amaral et al. (2016) observed that oligodendrocytes use great amounts of glucose in several metabolic pathways and largely interact with both astrocytes and neurons through the release/uptake of metabolites, such as lactate. It has also been proposed that the differentiation of oligodendrocyte progenitors depends on mitochondrial metabolism (Schoenfeld et al., 2010), to sustain the massive structural rearrangements required to form myelin sheets. This hypothesis is further supported by the observation that cultures enriched in myelinating oligodendrocytes import extracellular lactate to maintain their mitochondrial activity (Rinholm et al., 2011) **(**Fig. 2**)**.

Specialized cells within the adult brain are all derived from neural stem cells (Ming and Song, 2005). Gene expression analyses have revealed that the transition of stem cells to mature cells is associated with numerous transcriptional changes in genes linked to metabolism and energy sensing (Geschwind and Miller, 2001; Gurok et al., 2004; Ramalho-Santos et al., 2002; Homem et al., 2014). The metabolism of embryonic stem cells is predominantly based on glycolysis, in contrast to respiration in their adult counterparts, and mitochondria participate in cell physiology via anaplerotic replenishment of metabolites to sustain self-renewal (Carey et al., 2015). This concept has been partially illustrated in *Drosophila* neural stem cells, in which inhibition of mitochondrial respiration was proposed to induce accumulation of metabolites, promoting stemness (Homem et al., 2014).

Overall, mitochondrial metabolism is fundamental for proper brain development and activity and provides energy through respiration, as predominantly occurs in adult neurons and differentiating oligodendrocytes,

or through anaplerotic supplementation of metabolites, as observed in astrocytes, adult oligodendrocytes, and neural stem cells.

2.2 Mitochondrial Regulation of Cell Survival and Death

In addition to their primary role in the metabolic processes of OxPhos, mitochondria regulate cellular danger and damage responses. Indeed, different cellular stresses lead to direct mitochondrial damage, which is typically initiated by permeabilization of the OMM (Marchi and Pinton, 2014; Bonora et al., 2015), and result in the release of several proapoptotic factors from the IMS into the cytosol. Importantly, the calcium (Ca^{2+}) levels inside mitochondria play a central role in driving mechanisms that increase energy supply and that control cell death. Thus, altering mitochondrial activities by disrupting the morphology of the mitochondrial network, reducing ATP production and oxygen consumption, or modulating Ca^{2+} signaling could have a massive impact on all aspects of intracellular homeostasis. Indeed, whether a cell lives or dies depends on (at least) three distinct cell death pathways that are profoundly driven by mitochondria: (1) apoptosis (including intrinsic and extrinsic apoptosis), (2) autophagy, and (3) regulated necrosis **(**Fig. 3**)**.

2.2.1 Apoptosis

Extrinsic apoptosis can be triggered by the ligation of death receptors, such as FAS/CD95 or tumor necrosis factor receptor 1, leading to the assembly of a multiprotein complex that culminates in the activation of proapoptotic caspase 8 and caspase 3 to induce cell death (Muzio et al., 1996). In some cell types, this event occurs in the absence of mitochondrial involvement (Barnhart et al., 2003; Scaffidi et al., 1998). However, in other cell types, including brain cells (Franz et al., 2002), active caspase 8 cleaves BID, a BH3 domain-containing proapoptotic B-cell lymphoma-2 (Bcl-2) family member, and the COOH-terminal region of Bid translocates to mitochondria, where it triggers cytochrome *c* release (Li et al., 1998; Luo et al., 1998) **(**Fig. 3**)**. This evidence indicates that the mitochondrial amplification loop is essential for the apoptosis of selected cells and contributes to extrinsic apoptosis (Yin et al., 1999). While playing a marginal role in extrinsic apoptosis, several members of the Bcl-2 family, such as Mcl-1 (myeloid cell leukemia-1), Bcl-XL, Bcl-w, and Bfl-1/A1, are primarily antiapoptotic agents involved in the regulation of intrinsic apoptosis (Morciano et al., 2016; Pinton et al., 2001). In contrast, other Bcl-2 family members, such as Bcl-2, Bax, and Bak, are proapoptotic factors (Chipuk et al., 2010).

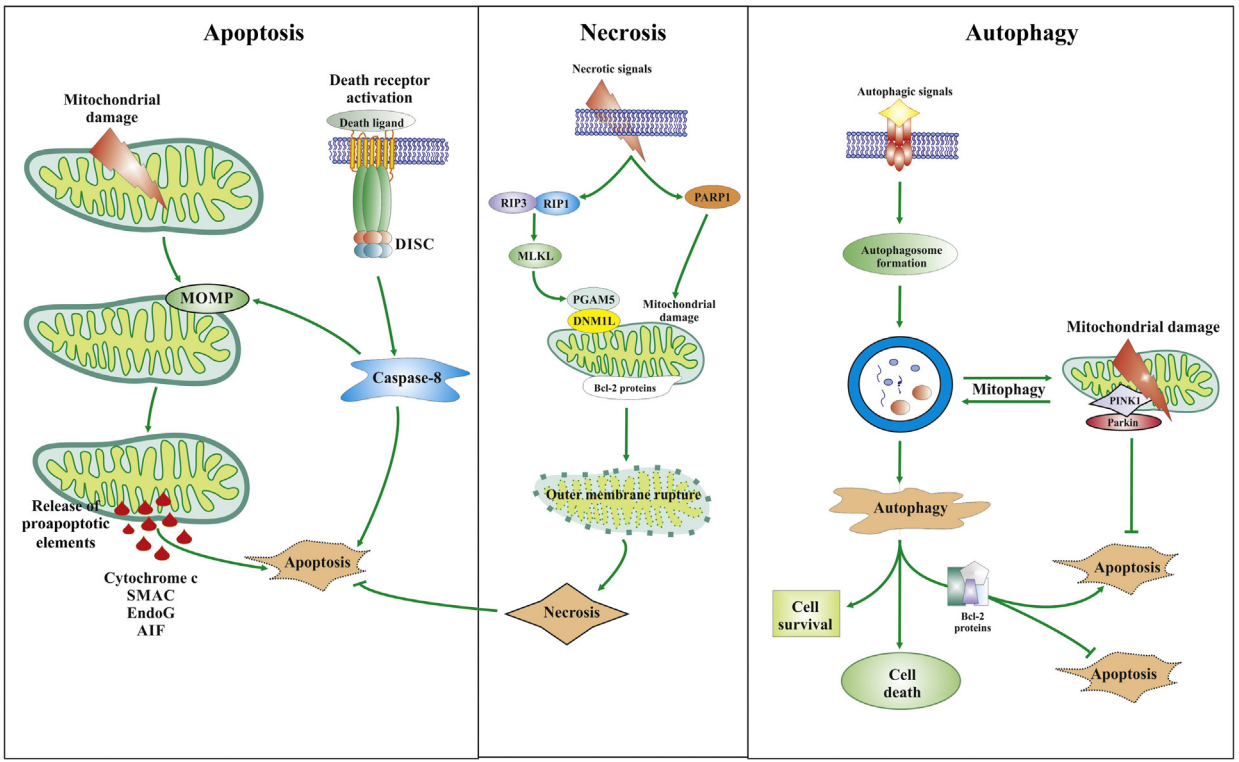

Figure 3 A mechanistic view of apoptosis and other cell death modalities. Upon a severe damage, mitochondria undergo mitochondrial outer membrane permeabilization *(MOMP)*, which results in the release of cytochrome *c* and other proapoptotic factors. Alternatively, apoptosis may be triggered in response to ligation of cell death ligands. This results in the recruitment of several proteins, which assemble DISC complex and

Intrinsic apoptosis can be initiated by several intracellular perturbations, such as DNA damage, cytosolic Ca^{2+} overload, oxidative stress, and endoplasmic reticulum stress, and this process involves a central step of regulation that is mediated by mitochondria (Kroemer et al., 2007).

Mitochondrial outer membrane permeabilization (MOMP) is the pivotal event that leads to the release of mitochondrial proapoptotic factors, such as cytochrome *c*, apoptosis-inducing factor (AIF), Smac/DIABLO, and endonuclease G **(**Fig. 3**)**. These components interact with cytosolic proteins to form a complex (apoptosome) that recruits and activates caspases, causing apoptotic cell death (Adams and Cory, 2002). The local regulation and execution of MOMP involves, in addition to Bcl-2 family proteins, mitochondrial lipids, proteins that regulate bioenergetic metabolite flux, and putative components of the permeability transition pore (mPTP).

Alternatively, mitochondria can lose their structural integrity after the so-called mitochondrial permeability transition (MPT), which refers to an abrupt increase in the permeability of the IMM to small solutes (Kroemer et al., 2007). The mPTP is a large-conductance channel located in mitochondrial membranes that can drive the MPT in response to several stressors, including but not limited to mitochondrial calcium (Ca^{2+}) ($[Ca^{2+}]_m$)

◀ activate caspase-8. Next, caspase-8 truncates the BH3 (Bcl-2 homology 3)-only protein Bid (BH3-interacting domain death agonist) into t-Bid, which coactivates the intrinsic pathway of apoptosis by translocating to mitochondria. Several stimuli (hypoxia, death ligands, ROS) drive necrosis via mitochondrial dysfunction by RIP1 and RIP3 assembly. Once activated, these cofactors are reported to activate MLKL. Next, MLKL boosts the activity of PGAM5 and DNM1L, which, in turn, leads to mitochondrial fragmentation and, finally, cell death. Necrotic signals may also modulate the activity of several Bcl-2 proteins and induce cell death in a caspase-independent manner. Another cell death modality that does not involve apoptotic or necrotic effector molecules is autophagy. Different stimuli activate this pathway. Among them, the most common are nutrient deprivation, hypoxia, ER stress, and ROS. Once activated, the autophagic program assembles a double-membrane vesicle (called autophagosome). Next, autophagosome fuses with lysosomes and the contents inside the autophagosome are degraded by lysosomal hydrolases. Even if autophagy has been described as a cytoprotective mechanism for the cell, a considerable body of literature reports this process as an alternative cell death mechanism to apoptosis. Of relevance, several Bcl-2 proteins are well-characterized regulators of both apoptosis and autophagy. Finally, autophagy can be involved in the elimination of damaged mitochondria in the process called mitophagy. The main regulator of this mechanism is the PINK1-Parkin axis. Recent evidences suggest that mitophagy may act as a rescue mechanism to escape from cell death.

overload and oxidative stress. Important components of the mPTP include cyclophilin D (CyPD) in the mitochondrial matrix, voltage-dependent anion channel-1 (VDAC1) in the OMM, adenine nucleotide translocase-1 (ANT), and the c subunit of mitochondrial ATP synthase in the IMM (Kroemer et al., 2007; Brenner and Moulin, 2012; Halestrap, 2009, 2014; Bonora et al., 2013, 2015, 2016; Morciano et al., 2015).

$[Ca^{2+}]_m$ overload is undoubtedly among the principal apoptotic stimuli that cause the release of specific mitochondrial proapoptotic factors into the cytosol (Giorgi et al., 2012). Moreover, several oncogenes and tumor suppressors are known to regulate proteins involved in Ca^{2+} homeostasis to exert their anti/proapoptotic activities. For example, Bcl-2 was first described to reduce the steady-state Ca^{2+} levels within the ER, and this event results in reduced Ca^{2+} transfer to mitochondria during apoptotic stimulation and reduced induction of mitochondrial fragmentation and apoptosis initiation (Pinton et al., 2000, 2001). Similar to Bcl-2, the serine/threonine kinase Akt another antiapoptotic factor, regulates the $[Ca^{2+}]_m$ levels by diminishing Ca^{2+} flux from the ER, thereby protecting cells from apoptosis (Marchi et al., 2008). Alternatively, the proapoptotic proteins FHIT and PML act at the mitochondrial and ER levels, respectively, to promote $[Ca^{2+}]_m$ accumulation (Rimessi et al., 2009; Giorgi et al., 2010). The discovery of a pore-forming subunit that regulates Ca^{2+} entry into the mitochondria, the mitochondrial calcium uniporter (MCU) complex, and microRNA miR-25 (Baughman et al., 2011; De Stefani et al., 2011; Marchi and Pinton, 2014), further emphasizes the importance of Ca^{2+} signaling and provides new tools for investigating apoptosis.

2.2.2 Autophagy

Mitochondria act as an intracellular checkpoint in autophagy, a catabolic process in which damaged and redundant cellular organelles or exogenous pathogens are sequestered within double-membrane vesicles termed autophagosomes, which are degraded upon fusion with lysosomes (Mizushima and Komatsu, 2011; Feng et al., 2014; Patergnani et al., 2013). Similar to apoptosis, autophagy is now recognized as an active mechanism of programmed cell death, referred to as autophagic cell death (ACD) (Galluzzi et al., 2012) **(**Fig. 3**)**.

Cell death through autophagy is reported to occur in various pathological conditions, including cancer, neurodegeneration, and aging (Shintani and Klionsky, 2004). Autophagy was first linked to cancer due to the identification and characterization of Beclin 1, a mammalian ortholog of

yeast Atg6/Vps30 that is part of a class III PI3K complex, which participates in autophagosome formation (Kihara et al., 2001). Additionally, Beclin 1 is a haploinsufficient tumor-suppressor gene that is frequently monoallelically deleted in human sporadic breast, ovarian, and prostate cancers (Levine and Klionsky, 2004). Beclin 1 is bound and inhibited by the antiapoptotic protein Bcl-2 (Pattingre et al., 2005); however, proapoptotic members of the same family are capable of disrupting this interaction to promote autophagy (Maiuri et al., 2007). Mammalian target of rapamycin (MTOR) is a major negative regulator of autophagy in human cells that partly associates with the OMM in a complex with the mitochondrial proteins Bcl-XL and VDAC1 (Paglin et al., 2005; Schieke et al., 2006; Ramanathan and Schreiber, 2009). Nevertheless, the functional role of autophagy as a "*pure*" cell death mechanism remains a matter of debate, and the term ACD is now under revision. In fact, in most known cases, ACD constitutes a cell death mechanism in which cells are killed *with* autophagy and not *by* autophagy (Shen et al., 2011). Several publications suggest this possibility and illustrate autophagy as a component of other lethal mechanisms responsible for cell killing, such as apoptosis or necrosis (Shen et al., 2012; Galluzzi et al., 2012). An example of this role may be observed when the autophagic process is suppressed by chemicals or through genetic means. Notably, RNA interference targeting the main autophagy-related genes (e.g., ATG5, Beclin1, or Ambra1) was shown to accelerate cell death in several tumor samples when treated with chemotherapeutic agents (Grander et al., 2009; Hwang et al., 2015; Jiang and Mizushima, 2014).

2.2.3 Necrosis

In contrast to the "programmed" nature of cell death during apoptosis and autophagy, necrosis has typically been considered a random, uncontrolled process that leads to the "accidental" death of the cell. However, a new concept of programmed necrosis has been developed, and it is now accepted that specific genes can induce necrosis in a regulated manner. RIPK1-like protein receptor-interacting protein kinase 3 (RIPK3) is considered to be a crucial regulator of death receptor-induced necrosis (He et al., 2009; Zhang et al., 2009). During necrosis, RIPK3 is activated and forms a supramolecular complex with RIPK1, known as the necrosome. This RIPK1–RIPK3-dependent necrosis may be induced by the engagement of death receptors, including TNFR1, either directly or under conditions of caspase inhibition. RIP kinases, possibly via translocation to the mitochondria, can induce reactive oxygen species (ROS) production

by complex I of the mitochondrial electron transport chain (mETC) (Zhang et al., 2009), and RIP1 or RIP3 can localize to mitochondria in response to a necrotic stimulus (Temkin et al., 2006; Davis et al., 2010). Once activated, RIP3 is able to phosphorylate two residues (Thr357 and Ser358) of a crucial protein in the necrosis pathway, mixed lineage kinase domain-like (MLKL) (Sun et al., 2012; Chen et al., 2013). The precise molecular mechanisms whereby MLKL exerts its essential nonenzymatic function in necroptosis remain elusive (Wu et al., 2013). It has been suggested that MLKL boosts the catalytic activity of phosphoglycerate mutase family member 5, mitochondrial (PGAM5), by promoting dynamin 1-like (DNM1L)-mediated mitochondria fragmentation (Linkermann et al., 2012; Galluzzi and Kroemer, 2011). However, this notion contrasts with several studies implying that necroptosis is a mitochondrion-independent form of cell death (Galluzzi et al., 2016) due to the fact that cells lacking mitochondria following a mitophagic response and cells from *Pgam5*$^{-/-}$ mice exhibit normal necroptotic signaling (Moriwaki et al., 2016).

One mitochondrion-dependent variant of necrosis is MPT-driven regulated necrosis, which is triggered by a rapid decrease in intracellular ATP availability (Kroemer et al., 2009).

Necrotic death stimuli can also activate PARP1, which may affect mitochondrial respiratory complex activities and ROS production in a calpain activation-dependent manner (Moubarak et al., 2007). Interestingly, Bcl-2 proteins involved in apoptotic mechanisms (Bax, Bak) and other factors, such as Bmf, BNIP3, and Nix can also induce necrosis by triggering the release of AIF from mitochondria via a caspase-independent mechanism (Ben-Ari et al., 2007; Bajt et al., 2008; Moubarak et al., 2007) **(**Fig. 3**)**. Taken together, these observations support the notion that mitochondria play an important, albeit nonexclusive, role in the control of regulated necrosis.

3. NEURODEGENERATION ASSOCIATED WITH MITOCHONDRIA

3.1 General Aspects

Neurodegenerative disorders are certainly emerging in the industrialized world as a leading cause of morbidity and their incidence will inevitably increase due to the extension of life expectancy. A better understanding of the pathogenic mechanisms underlying these incurable diseases is urgently needed to develop efficient therapies that halt disease progression.

Neurodegenerative diseases are characterized by the gradual, progressive, and/or selective loss of anatomically or physiologically related neuronal systems.

Clinical signs of these diseases involve alteration of cognition, movement, strength, coordination, sensation, vision, or autonomic control (Lin and Beal, 2006).

Complex multifactorial disorders typically involve interplay between epigenetic, genetic, and environmental factors (Landgrave-Gomez et al., 2015). Abnormal protein dynamics associated with defective protein degradation and aggregation, oxidative stress, free radical formation, impaired bioenergetics, mitochondrial dysfunction, or exposure to toxic metals and pesticides have been shown to be involved in their pathogenesis.

Not surprisingly, the majority of these diseases are characterized by impairment of mitochondrial energy metabolism and function (Schon and Manfredi, 2003). For example, it is well known that three primary point mutations in three complex I subunits, ND1 (nucleotide 3460), ND4 (nucleotide 11778), and ND6 (nucleotide 14484) cause Leber's hereditary optic neuropathy (LHON). This disorder is characterized by subacute degeneration of the optic nerve, predominantly affects young males, and causes bilateral visual failure leading to nearly complete blindness (Carelli et al., 2013, 2015). Mutations in the mtDNA ATPase 6 gene have been associated with a syndrome clinically characterized by neuropathy, ataxia, and retinitis pigmentosa (NARP) (Lenaz et al., 2004). Mutations in nuclear genes affecting OxPhos have also been linked to neurological disorders (Koopman et al., 2013). For example, Leigh syndrome (LS) is a severe condition characterized by subacute symmetrical necrotic lesions in the subcortical regions of the CNS. This pathology, which is caused by alterations of normal mitochondrial energy metabolism, can originate from a wide variety of molecular defects, among which cytochrome *c* oxidase (COX) deficiency is one of the most common (Lombes et al., 1991). Friedreich's ataxia (FRDA) is a triplet repeat disorder of chromosome 9q13, resulting in alteration of the frataxin protein. In the mitochondrial compartment, the frataxin precursor is cleaved by mitochondrial peptidases into its mature form. Under normal conditions, frataxin is necessary for appropriate iron homeostasis and iron–sulfur cluster synthesis (Muthuswamy and Agarwal, 2015).

Furthermore, mitochondrial dysfunction may be secondary, but it is still relevant to the development of neurological diseases, particularly Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS). HD

patients present with a general lack of coordination. As the disease advances, muscle coordination is lost, and mental abilities generally decline along with progressive emotional and cognitive disturbances. This disease is characterized by progressive degeneration of striatal neurons and by expansion of a CAG repeat in the IT15 gene on chromosome 4, which encodes a protein of unknown function termed huntingtin (Browne et al., 1997). Even if the exact mechanism by which this protein causes mitochondrial dysfunction is not yet well understood, evidence emphasizes the pivotal role of mitochondria in HD. In support of this conclusion, defects in energy metabolism and activity of the mETC have been found in HD patients, postmortem HD brains, and transgenic mouse models of HD (Tabrizi et al., 1999).

In contrast to HD, ALS affects the anterior horn cells of the spinal cord and the cortical motor neurons. As a consequence, neural activity to the muscles declines, and subsequently, the activity of the muscle ceases. Unable to function, muscles gradually weaken, atrophy, and perform very fine twitches. The basis for this disease is unknown in 90% of cases. However, approximately 10% are familial cases with mutations in the superoxide dismutase 1 (SOD1) gene (Rosen, 1993). Since SOD1 is responsible for the detoxification of harmful superoxide radicals and considering the high sensitivity of mitochondria to ROS, it is reasonable to speculate that mitochondria play a key role in ALS. In fact, mutant SOD1 mice exhibit abnormal mitochondrial respiratory chain function associated with accumulation of abnormal mitochondria (Mattiazzi et al., 2002). In addition, substantial alteration of $[Ca^{2+}]_m$ signaling and Ca^{2+}-dependent apoptosis has been found in mutant SOD1-transfected cells (Tan et al., 2014).

PD is one of the most common neurodegenerative disorders, and there are familial, sporadic, and toxin-induced forms of PD (Ryan et al., 2015). The main symptoms of PD-affected patients are bradykinesia, rigidity, and tremor, and these symptoms are due to the loss of dopaminergic neurons in the substantia nigra. Mitochondria appear to play a crucial role in the pathogenesis of PD (Subramaniam and Chesselet, 2013). First, the increased lipid peroxidation and DNA damage found in PD brains could be explained by dysfunction of complex I in the substantia nigra. Next, several mtDNA mutations, complex I deficiency, and oxidative damage have been found in the substantia nigra of PD patients, and cybrids carrying PD–mtDNA mutations display strong inhibition of complex I activity (Arduino et al., 2015).

Furthermore, suppression of mitophagy, a selective form of autophagy, leads to accumulation of aberrant mitochondria, ROS overload, and consequent mitochondrial dysfunction.

Studies have shown, for example, that disease-associated mutations in PARK2 (Parkin), PARK6 (encoding the Pink1 protein), and Fbxo7 resulted in defective mitophagy (Burchell et al., 2013; Zhou et al., 2015; Narendra et al., 2010).

Therefore, considering the impact of mitochondria on the onset and progression of numerous neurodegenerative disorders, it is logical to investigate the role of mitochondria in the development and progression of multiple sclerosis (MS).

4. MS

4.1 Background on MS

MS is a complex disease of unknown etiology that affects the CNS. The name "la sclerose en plaques," given by the French neurologist Jeanne-Martin Charcot in the 19th century, captures the cardinal feature of the disease: the presence of demyelinated lesions scattered throughout the brain and the spinal cord (Lucchinetti and Bruck, 2004). MS lesions are generally characterized by inflammation and gliosis; however, they can be highly heterogeneous with respect to the pattern of demyelination, the recruitment of inflammatory cells, the degrees of axonal degeneration, and the level of remyelination. MS typically manifests in young adults between the third and fourth decades of life, with an increased prevalence among women. MS lesions can lead to a broad range of clinical manifestations, including gait and sensory disturbances, visual loss, vertigo, and bladder problems. Neurological disabilities in MS are transient, followed by periods of complete or partial remission. This phase of the disease is defined as relapsing-remitting MS (RR-MS); the number of relapses is highly variable among patients, with an average of one every 2 years. Over time, the majority of MS patients transition to a so-called secondary progressive phase (SP-MS), which is distinguished by the absence of relapses and the steady accumulation of neurological disabilities (Compston and Coles, 2002). Approximately 10% of patients experience gradual worsening beginning from disease onset without experiencing acute relapses, and this form of MS is defined as primary progressive MS (PP-MS). Finally, fewer than 5% of MS patients

present with acute exacerbations in the context of a progressive course (progressive-relapsing MS, PR-MS). PP-MS typically presents at a later age compared to RR-MS and equally affects females and males. Due to its distinct clinical course, PP-MS has been at the center of a long-standing debate concerning whether it should be considered as a separate disease entity; however, genetic, imaging, and pathological studies indicate that PP-MS is on the extreme end of the MS disease spectrum (Antel et al., 2012) **(**Fig. 4**)**.

4.2 MS Pathology: Inflammation and Neurodegeneration

MS is considered an autoimmune disease that is primarily driven by CD4+ T helper type 1 lymphocytes in individuals carrying specific susceptibility allelic variants (Nylander and Hafler, 2012). Disruption of the blood–brain barrier (BBB) allows T cells to infiltrate the CNS and cause local inflammation, resulting in damage to myelin and axonal fibers. Lesions can be clinically silent or produce a broad range of symptoms, depending on their location. Many other components of the innate and adaptive immune systems, including Th17 cells, B cells, and humoral factors, have been implicated in this inflammatory assault on the CNS **(**Fig. 5**)**. All treatments currently available for RR-MS have been developed on the basis of the autoimmune hypothesis and these treatments act as immunomodulators to reduce the frequency of relapses. Both natalizumab, a monoclonal antibody against integrin-α4β1 that prevents lymphocytes from crossing the BBB, and fingolimod, a sphingosine-1-phosphate receptor agonist that traps lymphocytes in the lymph nodes, have shown greater capacity to decrease disease activity than older drugs, such as β interferons and glatiramer acetate (Ransohoff et al., 2015). The relevance of B cells to MS pathogenesis has recently been revised following the exciting results from clinical trials of monoclonal antibodies directed against CD20, a molecule expressed in mature B lymphocytes (Kappos et al., 2014). In contrast, inhibiting B cell functions using atacicept, a recombinant fusion protein that binds to the cytokines BLyS and APRIL, led to increased disease activity in a clinical trial. Furthermore, inhibiting TNF-α or blocking IL-12/IL-23, while promising in preclinical studies, did not show effective results in humans (van Oosten et al., 1996; Segal et al., 2008). This evidence highlights the complexity of the immunopathology of MS and the need for a deeper understanding of the distinct molecular pathways that promote or suppress inflammation. Nonetheless, the development of immunomodulatory drugs has dramatically changed the outlook for

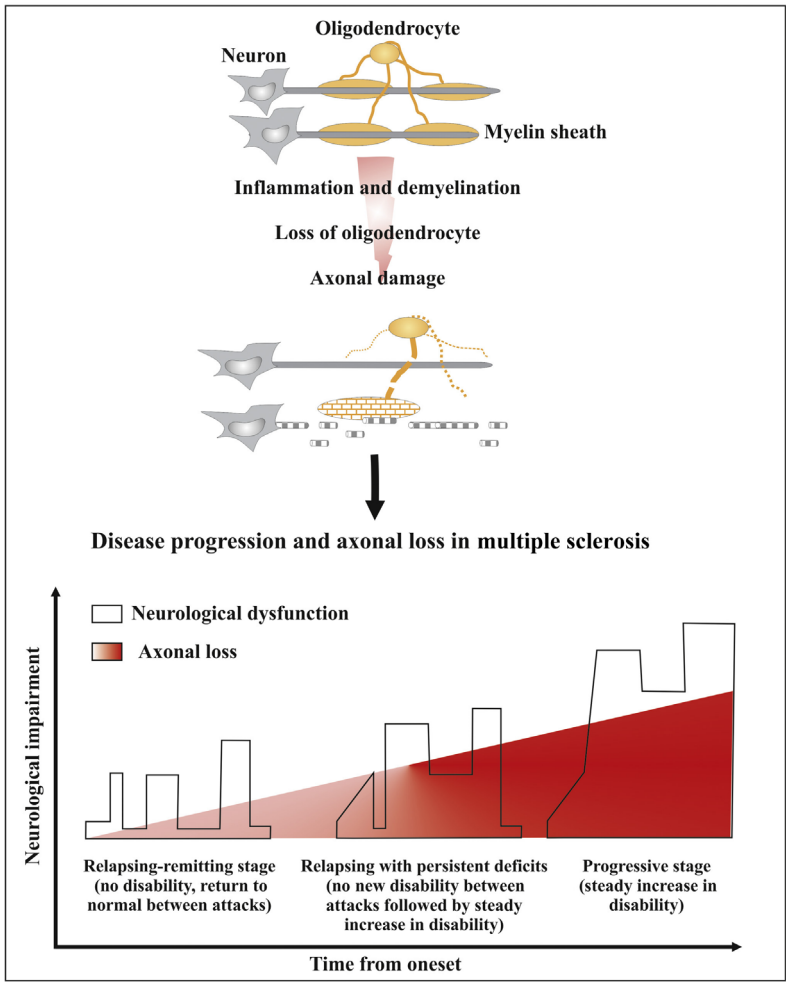

Figure 4 Disease progression in multiple sclerosis. The main characteristics of MS are inflammation and demyelination, which result in loss of oligodendrocytes and progressive axonal destruction. Neurological disabilities in MS are transient, followed by periods of complete or partial remission. This phase of the disease is defined as relapsing-remitting MS *(RR-MS)*; the number of relapses is highly variable among patients. Over time, the majority of MS patients transition to a secondary progressive phase *(SP-MS)*, distinguished by the absence of relapses and the steady accumulation of neurological disabilities.

RR-MS patients, as these drugs provide clear short-term benefits by significantly reducing disease activity. These drugs may have long-term effects, but longitudinal studies extending for at least a couple of decades

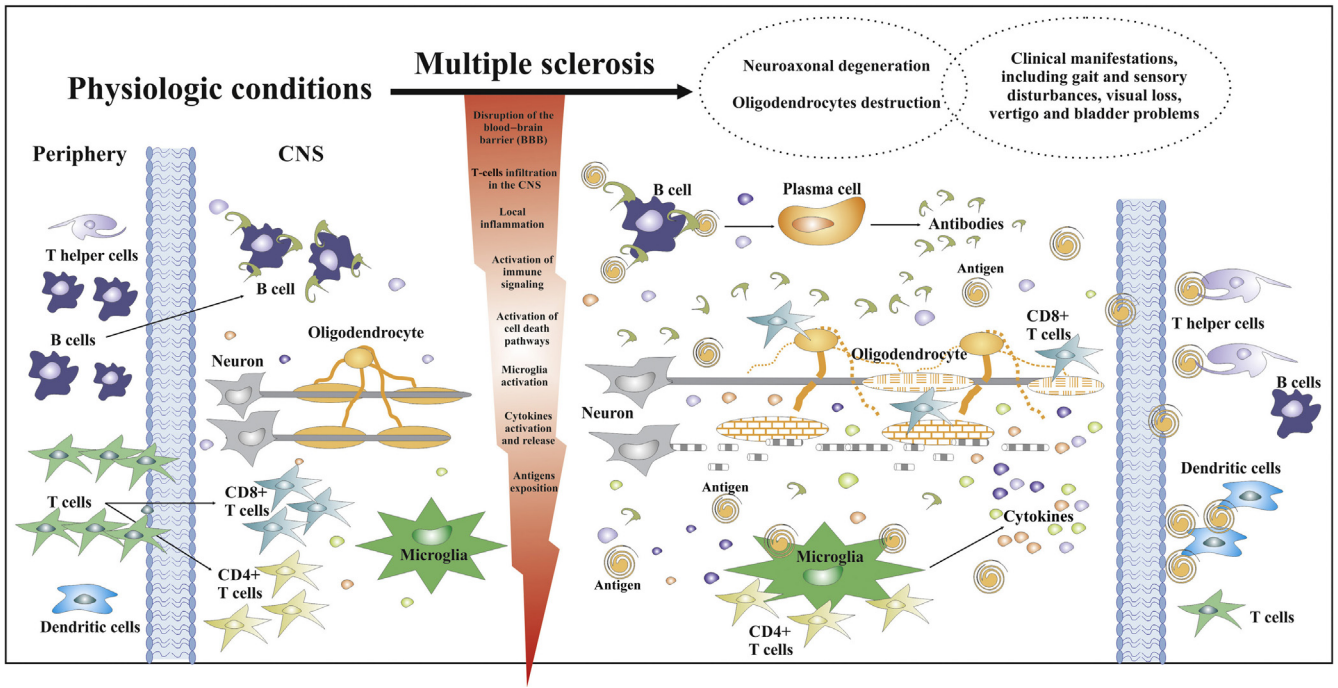

Figure 5 Pathogenesis of multiple sclerosis. The primary symptoms of MS are fatigue, muscle weakness, mobility problems, and pain. MS has a multifactorial etiology, including both genetic and environmental factors. The main characteristics of MS are progressive degeneration of neurons, demyelination, and oligodendrocyte destruction. T cells and B cells enter the CNS, where they secrete cytokines and activate microglia. Once the blood–brain barrier is breached, other inflammatory cells accumulate in the CNS. As a result, oligodendroglial cells are damaged, and B cells (plasma cells) concomitantly produce myelin-specific antibodies, which in turn produce complexes that further damage oligodendroglial cells. Dendritic cells residing in the periphery also participate in the presentation of antigens to T cells.

are required to demonstrate their long-term efficacy. In the meantime, the challenge in the MS field has shifted toward tackling progressive MS, as neither SP-MS nor PP-MS patients respond to any of the available treatments. Whereas inflammation is the cardinal feature of the RR-MS phase, irreversible neurodegeneration distinguishes the progressive forms of MS. Indeed, several radiological imaging and histological studies suggest that axonal damage is responsible for the accumulation of physical and cognitive disabilities, and these results could not be explained solely by the presence of focal lesions in the white matter (Anthony et al., 2000; Bruck, 2005; Filippi et al., 2003; Lucchinetti et al., 2005; Stadelmann et al., 2008; Trapp et al., 1998). In addition, it is now established that MS pathology influences both white and gray matter and that progressive cortical atrophy develops beginning from the very early stages of relapsing-remitting disease, independent of the frequencies of white-matter lesions (Charil et al., 2007). Importantly, gray-matter atrophy is emerging as a better predictor of disability than focal white-matter lesion load or white-matter atrophy (Manfredonia et al., 2007; Rovaris et al., 2006). Whether the degenerative process is a consequence of autoimmune demyelination or the immune response is secondary to early degeneration in the CNS remains uncertain (Geurts and Barkhof, 2008; Stys et al., 2012). Certainly, increasing our understanding of neuronal damage is urgently needed to identify new therapeutic targets and to develop neuroprotective strategies for progressive MS.

5. MITOCHONDRIA AND MS

As reported earlier, structural pathology and the immunopathogenetic mechanisms of injury are distinct between the early and late stages of MS. Despite this difference, several studies have underlined the important contribution of mitochondria during the establishment of MS. It is now possible to identify specific hallmarks of mitochondrial abnormalities observed during the development and progression of MS: (1) alterations in mtDNA and anomalous mitochondrial protein functions; (2) increased free radical production and oxidative damage; (3) cellular ionic imbalance; (4) apoptosis; and (5) cellular clearance mechanisms (Mao and Reddy, 2010; Kalman et al., 2007; Campbell et al., 2014) **(**Fig. 6**)**. Notably, the same hallmarks are found in other myelin disorders characterized by either damage to myelin [demyelinating disorders, such as acute disseminated

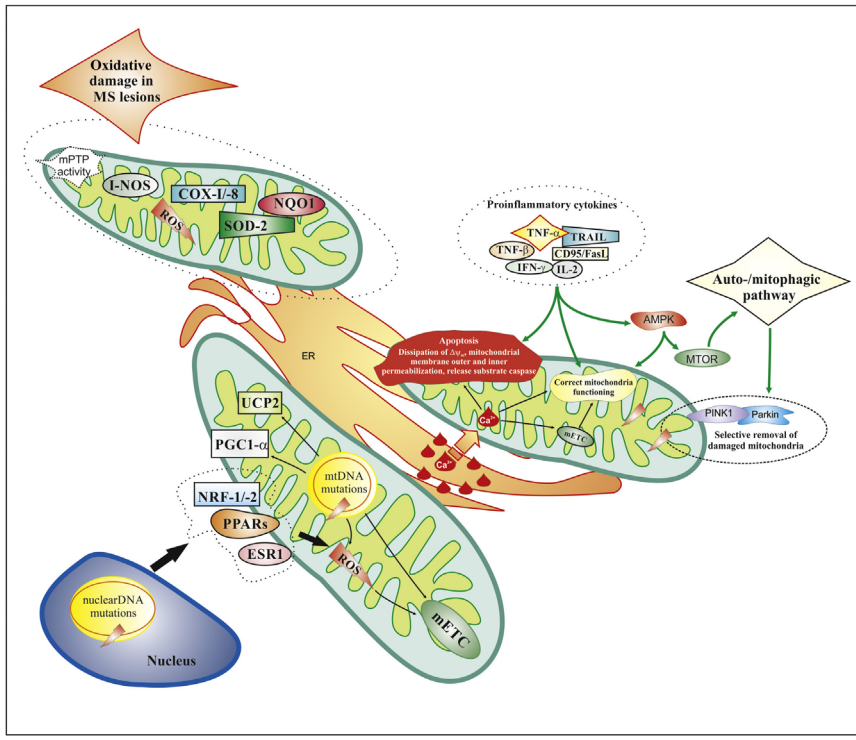

Figure 6 Mitochondrial defect in multiple sclerosis. mtDNA and nuclear DNA mutations may increase the risk of developing MS. These variations result in changes in the expression of several proteins involved in the regulation of oxidative stress and the activity of mETC subunits. In addition, MS lesions are characterized by oxidative damage, likely due to dysregulation of proteins involved in ROS production and detoxification, such as SOD2 and NQO1. Furthermore, ionic imbalance can trigger MS: while correct Ca^{2+} handling is important for mitochondrial functioning and cell death regulation, excessive exposure to proinflammatory cytokines in MS may alter mitochondrial Ca^{2+} homeostasis and block oligodendrocyte differentiation. Energy production and mitochondrial physiology are recognized as the main regulators of autophagy. Several studies highlight the importance of AMPK and mTOR in both mitochondrial functioning and oligodendrocyte maturation. Finally, selective autophagy of damaged mitochondria (mitophagy) is primarily regulated by the activity of the PINK1-Parkin axis. Interestingly, mutations in these genes have been found in MS patients.

encephalomyelitis (ADEM) or acute hemorrhagic leukoencephalitis (AHL)] or genetic mutations linked to abnormal myelin formation (dysmyelinating disorders, such as X-linked adrenoleukodystrophy) (Kuhlmann et al., 2008; Mahad et al., 2015). All these mechanisms will be described in further detail in the following paragraphs.

5.1 Alterations of mtDNA and Mitochondrial Proteins in MS

mtDNA mutations may increase the risk of developing MS (Blokhin et al., 2008) **(**Fig. 6**)**. The −866G/A mtDNA variation in a promoter region has been associated with susceptibility to MS in a German population. This variation results in a change in the expression of uncoupling protein 2 (UCP2), a mitochondrial member of the proton transport family that uncouples proton entry into mitochondria from ATP synthesis (Vogler et al., 2005). It is possible that this polymorphism leads to MS susceptibility by regulating the protein level of UCP2 in the CNS. The T4216C and G13708A mutations of UCP2 have also been linked to MS predisposition in European studies (Andalib et al., 2013, 2015). mtDNA variations in the ND2 and ATP6 genes, (A4917G, G9055A, and T14798C) which encode subunits of the mETC, further highlight the importance of mtDNA in MS (Andalib et al., 2015). Additional evidence was provided by Schoenfeld et al. (2010), who demonstrated through microarray analysis that both mtDNA content and mitochondrial gene expression were significantly increased during oligodendrocyte differentiation in rats and humans. Moreover, they showed that oligodendrocyte differentiation is particularly sensitive to mitochondrial toxins, and this observation corroborated the importance of mitochondria for proper oligodendrocyte maturation and consequent myelination. In addition to mtDNA mutations, the expression of key nuclear- and mitochondrial-encoded subunits of the mETC has been linked to MS susceptibility. For example, peroxisome proliferator-activated receptor (PPAR) gamma coactivator-1α (PGC-1α) is a transcription factor that regulates the expression of OxPhos subunits and mitochondrial defense. The latter function is achieved by controlling the expression of mitochondrial antioxidant proteins. Interestingly, the mRNA levels of PGC-1α were found to be significantly reduced in cortical samples from MS patients, and this reduction was associated with a significant loss of pyramidal neurons in deep cortical layers and with an increase in ROS production (Witte et al., 2013). Thus, the reduction of the neuronal PGC-1α levels may contribute to neuronal loss via impairment of OxPhos and mitochondrial redox imbalance.

Furthermore, recent findings indicate that altered expression of nuclear respiratory factor 1/2 (NRF-1/2), estrogen-related receptor α (ESR1), and PPARs affect the expression of OxPhos genes and cause oxidative as well as nitrosative damage. In particular, changes in NRF-2 associated with down-regulation of mETC genes and increased production of ROS have been

found in postmortem MS brains (Pandit et al., 2009). As the binding of NRF-2 to DNA is redox regulated (Kelly and Scarpulla, 2004), the overall increment in oxidative damage could affect NRF-2 outcomes. As a consequence, NRF-2 fails to bind to the promoter region of mETC genes, and the transcription and production of protein subunits of the mETC complexes are consequently affected.

5.2 Oxidative Stress in MS

As previously noted, mitochondria are primarily involved in ATP synthesis, Ca^{2+} regulation, and ROS production. Consequently, mitochondrial damage caused by inflammatory events in MS may lead to inadequate energy production and intracellular dysregulation.

Primary evidence of this link may be found in active lesions from acute MS patients based on immunohistochemical analysis of many mETC proteins (Mahad et al., 2008b). In their study, Mahad and coworkers identified a global reduction in total mitochondrial density, accompanied by selective loss of mitochondrial proteins, particularly COX-I and COX-IV. These mitochondrial defects were more abundant in oligodendrocytes, probably due to the higher susceptibility of these cells to oxidative stress. This observation is consistent with the results of other studies demonstrating how oxidative stress inhibits oligodendrocyte differentiation (French et al., 2009).

Another unique characteristic of MS lesions is the sustained activation of inducible nitric oxide synthase and myeloperoxidase (Bolanos et al., 1997), which causes continuous production of ROS and nitric oxide, ultimately affecting mitochondrial function. Additionally, MS lesions showed persistent microglial and macrophage infiltration accompanied by hypoxia-like damage. Overall, these findings implicate oxidative stress in microglial activation in MS lesions and a hypoxia-like tissue injury.

The production of superoxide via NADPH oxidase has been recognized as a microglia-mediated mechanism of neuronal damage (Block, 2008). Other markers of oxidative damage found in active demyelinating MS lesions include 4-hydroxy-2-nonenal (4-HNE) and 8-hydroxy-2-deoxyguanosine (van Horssen et al., 2008; Lu et al., 2000). The former is a highly reactive aldehyde derived from oxidative damage to cellular membrane lipids that is toxic to CNS cells, whereas the latter, reflects oxidative damage to nucleic acids and may be a marker of increased mtDNA damage or RNA oxidation.

The CNS is widely known to be susceptible to oxidative injury due to its high oxygen consumption and the presence of polyunsaturated fatty acids. Therefore, the brain is uniquely equipped with a wide array of antioxidant enzymes that regulate cellular redox status. Increased levels of SOD1/2, catalase, and heme oxygenase-1 have been found in active demyelinating MS lesions (van Horssen et al., 2008; Ruuls et al., 1995).

In addition to SOD2, other antioxidant systems that remove superoxide metabolites are present in mitochondria. Several of these systems have been found to be upregulated in MS lesions, including the thioredoxin (TrxR)-peroxiredoxin (Prx) system, which detoxifies mitochondria (and, thus, the entire cell) by clearing hydrogen peroxide and peroxynitrite. Global increases in the expression of certain Prx family members, particularly the antioxidant enzyme Prx-5, were found in MS samples (Holley et al., 2007; Plaisant et al., 2003).

Another antioxidant that protects the CNS from ROS damage is NAD(P)H:quinone oxidoreductase 1 (NQO1), a cytosolic flavoprotein essential for maintaining both α-tocopherol and coenzyme Q10 in their reduced antioxidative state. Interestingly, NQO1 is markedly upregulated in inflammatory MS lesions (van Horssen et al., 2006) **(**Fig. 6**)**. Additionally, the gene encoding glutathione peroxidase, which is important for the detoxification of hydrogen peroxide, was found to be significantly upregulated in MS lesions. Notably, in an animal model of inflammatory demyelination, treatment with this antioxidant enzyme exerted a protective effect during inflammation (Guy et al., 1989).

5.3 Cellular Ionic Imbalance in MS

A mitochondrial defect may also affect Ca^{2+} homeostasis. Notably, when $[Ca^{2+}]_m$ homeostasis is compromised, different pathological conditions can occur, depending on the cell type involved (Patergnani et al., 2011). Indeed, $[Ca^{2+}]_m$ appears to play a pivotal role in MS. For example, we showed that TNF-α, one primary proinflammatory cytokine involved in MS, blocks the maturation of oligodendrocyte precursor cells and axonal myelination by promoting appropriate mitochondrial function, including $[Ca^{2+}]_m$ homeostasis. Importantly, TNF-α, but not other proinflammatory cytokines, significantly reduces the ability of mitochondria to take up Ca^{2+} and, consequently, to drive OxPhos. $[Ca^{2+}]_m$ signaling is based on highly controlled mechanisms that induce bursts of Ca^{2+} uptake and release, which are mainly driven by the activity of MCU (responsible for Ca^{2+} influx) and the

mitochondrial Na^+/Ca^{2+} antiporter (mNCLX), which regulates Ca^{2+} efflux (Giorgi et al., 2012; De Stefani et al., 2015). In addition, the mPTP has been shown to be actively involved in Ca^{2+} release, mostly through CyPD, a prolyl isomerase located in the mitochondrial matrix. Interestingly, in an experimental animal model of MS, CyPD KO mice initially developed neuronal damage on par with WT mice but then partially recovered from their injury. Moreover, in mice lacking CyPD, the axonal damage was less extensive, and neurons were resistant to oxidative stress and displayed reduced axonal damage (Forte et al., 2007). Notably, it has recently been discovered that the c subunit of ATP synthase is an important component of the mPTP (Bonora et al., 2013). Since mtDNA deletions affecting various subunits of complex V have been found in individual neurons from MS brains, it will be of interest to determine whether mutations in the gene encoding the C subunit increase the risk for MS (Campbell et al., 2011).

Other findings underline the possible role of Ca^{2+} signaling in MS. Atypical expression of voltage-gated Ca^{2+} channels and glutamate receptors has been found in MS lesions (Kornek et al., 2000; Werner et al., 2001). The expression of TRPM4 cation channels in axons during inflammation may promote axonal and neuronal degeneration in both mouse models of MS and in human MS samples (Schattling et al., 2012). In addition, ultrastructural analysis of demyelinated spinal cord lesions demonstrated Ca^{2+}-mediated destruction of axons (Mahad et al., 2008a). This event may be explained by altered $[Ca^{2+}]_m$ transmission, which increases the intraaxonal levels of Na^+ by promoting an imbalance in Na^+/K^+ ATPase activity. As a consequence, Na^+ efflux in axons, which is required to maintain resting membrane potential or to conduct nerve impulses, is inhibited. Last but not the least, it should be emphasized that the activity of these pumps is primarily dependent on the hydrolysis of ATP. During MS progression, cytokines, oxidants, inflammatory mediators, and preexisting mtDNA mutations markedly affect mitochondrial function. Consequently, oxidative metabolism and ATP synthesis are compromised, and the activity of the Na^+/K^+ ATPase may be impaired (Trapp and Stys, 2009). Therefore, it appears that mitochondrial dysfunction and ionic imbalance may be major contributors to progressive neurological decline in MS.

5.4 Apoptosis in MS

The development of MS lesions results from both activated residential glial cells and circulating immune cells that enter the CNS through the BBB. Several pathways, including apoptotic processes, are triggered by this

infiltration. Although several studies have reported apoptotic cell death as a key pathogenic mechanism of MS, its detection remains controversial. This controversy is mainly due to limited access to brain specimens with early active lesions, the existence of alternative pathways of cell death, and the short duration of the apoptotic pathway. As a consequence, it is unclear whether apoptotic cell death is the trigger or a consequence of inflammation, whether apoptosis occurs during acute or chronic stages of the disease, or which cell type undergoes apoptosis. However, there is a general consensus that during the acute inflammatory stages of MS, activated immune cells express and release inflammatory products, such as TNF-α, IFN-β, TRAIL, and Fas-L (Kalman et al., 2007; Macchi et al., 2015; Friese et al., 2014) **(**Fig. 6**)**. Therefore, the ligand-receptor initiated apoptotic pathway is primarily induced by T-lymphocytes and affects neurons and oligodendrocytes. There are several initiators of this event. The main trigger is the prominent production of ROS and NO by activated leukocytes, macrophages, astrocytes, and microglia (Druzhyna et al., 2003, 2005; Munoz-Fernandez and Fresno, 1998). Oxidative damage affects DNA, particularly mtDNA because of its less-efficient repair and protective systems (Gruber et al., 2015; Gonsette, 2008). As reported earlier, mtDNA mutations increase the risk of developing MS, and the majority of these mutations target subunits of the mETC. A decrease in the activity and expression of the OxPhos complexes, concomitant with impairment of mitochondrial energy metabolism, and efficient removal of ROS/NO, significantly contributes to the initiation of the apoptotic process. Additionally, intramitochondrial iron accumulation may initiate oxidative damage. Notably, oligodendrocytes require iron due to intensive oxidative metabolism and as a cofactor for myelin synthesis (Hametner et al., 2013). The exact mechanism underlying pathological iron deposition remains unknown, but it has been demonstrated that excess iron accumulation drives increased production of oxygen and hydroxyl radicals, which induce further oxidative damage, mtDNA mutations, and, finally, apoptosis (Zhang et al., 2005).

The demyelination process is strongly activated by inflammatory cytokines that are released by activated T cells, such as TNF-β, TNF-α, TRAIL, CD95/FasL, interferon (IFN)-γ, and interleukin (IL)-2 (Fas et al., 2006; Hovelmeyer et al., 2005; Okuda et al., 2006). These factors were found to promote oligodendrocyte death through their corresponding death receptors. For example, TRAIL mediates oligodendrocyte apoptosis by engaging with the TRAIL receptor. Interestingly, it has been demonstrated that

apoptosis triggered by this pathway appears to be caspase-independent but dependent on AIF and DNA fragmentation (Barnett and Prineas, 2004). Alternatively, FasL binds to the death receptor CD95/Fas, which is expressed by oligodendrocytes and neurons, to induce cell death (Aktas et al., 2006).

Several studies have described oligodendrocyte death by apoptosis after exposure to TNF-α.

For example, it has been reported that administration of 100 ng/mL of human recombinant TNF-α to mature mouse oligodendrocytes resulted in 70% apoptotic cell death (Hisahara et al., 1997). Nevertheless, it has also been reported that TNF-α induced oligodendrocyte necrosis, and other studies have shown that TNF-α did not have cytotoxic effects on oligodendrocytes (Soliven et al., 1994; Scurlock and Dawson, 1999) but blocked their maturation (Bonora et al., 2014). Several possible explanations may shed light on these discrepancies. First, the results are highly dependent on the treatment duration, the culture conditions, and the experimental methods utilized in different laboratories. For instance, the expression levels of TNF receptors (TNFR1, involved in cell death, and TNFR2, involved in cell proliferation) are highly dependent on the culture conditions. Next, microglia and astrocytes express high levels of TNFR1 and produce TNF-α and are involved in microglial cell-mediated killing of oligodendrocytes (Merrill et al., 1993; Merrill and Scolding, 1999). Thus, the observed pro/antiapoptotic effects may depend on cellular contaminants.

The effects of IFN-γ on oligodendrocytes are also controversial. Studies have shown that this cytokine mediates both apoptosis and necrosis (Hisahara et al., 2000; Vartanian et al., 1995). However, recent work suggests that IFN-γ enhances oligodendrocyte damage rather than directly mediating their death (Pouly et al., 2000).

Taken together, these data indicate that mediators of apoptotic processes are important determinants of the loss of myelin and the survival of oligodendrocytes. Thus, these factors and the mechanisms controlling programmed cell death should be further investigated as novel targets for therapeutic intervention.

5.5 Cellular Clearance Mechanisms

Two elimination pathways are currently known to be involved in catabolic cellular processes. Both of these pathways control the quality of cellular components and maintain cellular homeostasis. Furthermore, they are highly involved in cell death mechanisms. These are the autophagic pathway (Eskelinen, 2008) and the ubiquitin-proteasome system (UPS)

(Dantuma and Bott, 2014), the latter of which is associated with several pathogenic mechanisms of neurodegenerative disorders and the so-called "proteinopathies," in which abnormally assembled proteins appear to play a central role. Well-known examples include AD, PD, HD, and ALS (McKinnon and Tabrizi, 2014). Unfortunately, less is known about the involvement of the UPS in MS (Bellavista et al., 2014). Published data indicate that in experimental allergic encephalomyelitis (EAE), pharmacological administration of a selective inhibitor of the UPS pathway decreased the progression of the disease (Vanderlugt et al., 2000), but further investigation is required to verify whether the UPS pathway may lead to a novel therapeutic option. Alternatively, autophagy is emerging as a highly interesting process to target for the treatment of MS, although its precise role remains under debate. First, autophagy is necessary for the survival of neurons. Considering their extreme polarization and size, neurons are particularly sensitive to unnecessary or aggregated cytosolic compounds, and autophagy is responsible for removing these superfluous "materials" (Klionsky et al., 2016). Next, studies using transgenic mice harboring specific autophagic gene mutations/deletions have shown that autophagy is crucial for the appropriate development of the CNS. Most importantly, a growing body of evidence indicates that autophagy may participate in MS. The role of mTOR in MS has been extensively investigated. Several studies have indicated that the mTOR signaling pathway restores the regrowth of axons in the CNS (Kim et al., 2011; Park et al., 2008). Notably, this process is important for remyelination, and mTOR is the main negative regulator of autophagy. mTOR is also a fundamental regulator of oligodendrocyte differentiation (Tyler et al., 2009; Zou et al., 2011) **(**Fig. 6**)**. As described earlier, the final step of autophagy is the fusion of an autophagosomal vesicle with a lysosome. This event suggests an important role of the lysosomal compartment in autophagy. Interestingly, in MS lesions, LAMP2 (a component of the lysosome membrane) expression is altered (Lindberg et al., 2004). Furthermore, in EAE animal models, autophagy appears to be repressed and protein aggresomes are not efficiently removed. These findings suggest that autophagy may improve the clinical outcome of MS. This result is in contrast to other data suggesting that autophagy is deleterious in MS. Quantitative real-time PCR analysis of blood samples obtained from EAE mice showed that the expression of the autophagic marker ATG5 correlates with the severity of the disease in these animals (Alirezaei et al., 2009). Similar results were obtained from RNA extracted from T cells derived from MS patients and from

immunohistochemical analysis performed in MS brain tissue samples (Lu et al., 2007; Li et al., 2006). Our laboratory demonstrated that exposure of oligodendrocyte cultures to proinflammatory cytokines leads to progressive mitochondrial impairment followed by AMPK activation (Bonora et al., 2014). Notably, when AMPK is activated, the autophagic machinery is stimulated. Furthermore, AMPK activity is known to be regulated by several stimuli, particularly energy deficiency and ROS. Additionally, mtDNA mutations/deletions appear to regulate this kinase **(**Fig. 6**)**. Based on these studies, AMPK may be a key cofactor related to the progression of MS.

Importantly, autophagy also represents a selective process that catabolizes specific intracellular and extracellular components. The most studied of these processes is the selective removal of damaged mitochondria, termed mitophagy, which produces excess ROS that may promote mtDNA damage.

Intriguingly, mitophagy is a molecular mechanism that is frequently observed in neurons and is responsible for quality control and clearance of mitochondria damaged by oxidative stress. In addition to energy production, neuronal mitochondria play a role in Ca^{2+} buffering, and because neurons exhibit very limited proliferation activity, it is clear that their mitochondria are sensitive to the accumulation of oxidative stress and damage. For instance, mitophagy is an important neuronal mechanism for minimizing oxidative damage to the cell as well as for quality control and clearance of damaged mitochondria. Recent findings have suggested a possible role of altered mitophagic processes in several human neurological diseases, such as PD and AD (Ashrafi et al., 2014; Ye et al., 2015; Palikaras and Tavernarakis, 2012).

Emerging evidence suggests that this process could play a role in MS as well (Lassmann and van Horssen, 2016; Mahad et al., 2015; Rone et al., 2016). This is likely, considering that the levels of ROS are known to be high in MS. Although mitophagy is a physiological process that occurs routinely in cells, its excessive activation leads to cell death. It is tempting to speculate that mitophagy is one of the mechanisms underlying neurodegeneration in MS.

Mitophagy is regulated by the PINK1-Parkin axis (Patergnani and Pinton, 2015). Briefly, when a subset of mitochondria suffers an important mitochondrial injury, PINK1 and Parkin cooperate to remove the damaged mitochondria. Under physiological conditions, PINK1 is imported and cleaved by mitochondrial proteases. With the accumulation of mitochondrial damage, these mechanisms are lost, and PINK1 accumulates on the outer mitochondrial surface, where it recruits Parkin from the cytosol to mitochondria. Finally, Parkin mediates the recruitment of damaged mitochondria to the autophagosome (Narendra et al., 2010; Rimessi et al., 2013).

Interestingly, elevated levels of Parkin were found in both active and chronic lesions, and elevated levels of PINK1 were found in active MS lesions (Wilhelmus et al., 2011) **(**Fig. 6**)**.

Further studies are needed to distinguish the molecular pathways underlying the cytoprotective versus the cytotoxic roles of these clearance processes (UPS and autophagy/mitophagy). However, accumulating evidence indicates that impaired function of these pathways may be a critical determinant of MS progression.

6. MITOCHONDRIA AS A POTENTIAL THERAPEUTIC TARGET FOR MS

As previously mentioned, all current therapies for MS are immunomodulatory agents that have little or no effect against the progression of neurodegeneration. In addition, not all patients respond well to these treatments. Hence, novel therapeutic targets and drugs are needed.

It is now clear that the mitochondrial compartment is an important factor in MS and mitochondria-directed therapies appear feasible and attractive.

The primary approach may be to counteract the excessive ROS levels in MS. Promising results have been obtained using MS animal models. Specifically, endogenous intake of antioxidants (such as flavonoids and alpha-lipoic acid) has been shown to reduce clinical symptoms and disability scores in treated animals (Hendriks et al., 2004; Kean et al., 2000; Moriya et al., 2008; van Horssen et al., 2011).

Unfortunately, antioxidant therapy has several disadvantages, including the difficulty in crossing the BBB, and consequently, large quantities of these compounds are required to achieve protective effects.

As described earlier, MS lesions are characterized by the impairment of endogenous antioxidant enzymes, particularly SOD, catalase, and glutathione peroxidase. Interestingly, administration of catalase or upregulation of its expression was found to reduce the severity of clinical signs in preclinical studies (Ruuls et al., 1995). Similarly, studies focusing on glutathione peroxidase showed that increased activity of this antioxidant enzyme is sufficient to ameliorate BBB integrity and neurological symptoms (Tajouri et al., 2003; Guy et al., 1989).

Heme oxygenase-1 (HO-1) is involved in iron homeostasis, and alterations in its expression were found in MS lesions (Hung et al., 2008; Takata et al., 2002). Upregulation of HO-1 has been correlated with improvements

in the disability score in an EAE model (Chora et al., 2007). The effect of HO-1 could also be mediated by its role in heme metabolism. HO-1 degrades heme into biliverdin, which is converted by biliverdin reductase into bilirubin, a potent antioxidant. Notably, treatment with bilirubin and/or biliverdin reductase protected oligodendrocytes against ROS-mediated cell death and reduced oxidative damage in an MS animal model (Liu et al., 2003, 2006).

Ion channels and ionic imbalance are also attractive candidates for novel therapeutic strategies. The most promising approach is to counteract mPTP opening, which was suggested by studies using CyPD-deficient mice that showed improvements in mitochondrial function accompanied by significantly reduced axonal damage and clinical scores after EAE induction. Interestingly, the CyPD inhibitor cyclosporin A (CsA) and its derivatives provided protection in neuronal cultures grown under adverse conditions, such as oxidative and hypoxic stress. Furthermore, by silencing another mPTP regulator, $p66^{Shc}$, it was possible to reduce the severity of clinical impairment and paralysis in EAE-induced animals (Su et al., 2012). Notably, compounds targeting the mPTP are currently available and have been used in several clinical trials. Dysregulation of Ca^{2+} signaling is certainly another important target for neuroprotective therapies. In fact, it has been shown that specific Na^{+} channel blockers (tetrodotoxin, phenytoin, and flecainide) exert a protective effect on axons, preserve ATP levels, and protect white-matter axons from NO-mediated damage (Waxman, 2008).

Enhancing the expression of PGC-1α, the master regulator of mitochondrial biogenesis, may lead to improvement in mitochondrial function and attenuation of redox stress. Preclinical studies identified potential compounds that promote neuronal survival through upregulation of PGC-1α (Nijland et al., 2014).

An alternative approach to treat MS may be the use of antiapoptotic therapies. In this approach, antisense-mediated knockdown of essential proapoptotic factors reversed the clinical score and disability in an animal model (Hebb et al., 2008). Furthermore, as we noted earlier, autophagy and mitophagy remain poorly understood processes that are emerging as critical contributors to neurodegeneration in MS. Several autophagy-regulating compounds have been tested for the treatment of different pathologies, including neurodegenerative disorders. Some of these compounds function by activating the catabolic process and others act by inhibiting autophagy. Further examination of the exact role of the autophagic/mitophagic processes in MS is warranted before moving to translational studies.

7. MS MODELS

In summary, several different mitochondria-related pathways have been explored to develop novel targets and therapies aimed at counteracting the progressive disability and degeneration caused by MS. Some of these pathways have already been extensively studied, but others have only begun to be understood.

A major hurdle in the MS field is the lack of an ideal experimental model. MS is a human disorder that does not spontaneously affect rodents or other animals commonly used in biomedical research, but human cells are scarcely available and are only obtainable through highly invasive procedures. Several experimental models have been developed over the years to overcome this issue, and they will be described in subsequent sections. We should emphasize that although none of these models alone can recapitulate the pathogenesis of MS, the combination of different and complementary approaches can very likely provide a better understanding of MS, leading to the development of more efficient drugs.

7.1 In Vitro Model

In vitro studies of CNS cells have frequently been performed using cell lines. However, these models have several limitations. First, they are generated by immortalization, a process that irreversibly modifies the normal physiology and genetics of the cell. Thus, the use of primary CNS cultures is preferable. To fully understand the pathogenic mechanisms underlying MS, all CNS cells (oligodendrocytes, neurons, and astrocytes) should be analyzed.

7.1.1 Oligodendrocytes

Oligodendrocytes are the myelin-forming cells of the CNS. Oligodendrocyte differentiation is a complex and precisely timed sequence of events that ultimately gives rise to mature oligodendrocytes, which wrap axonal fibers with their myelin sheets (Boulanger and Messier, 2014).

Each of these stages can be recapitulated in vitro and be detected based on the expression of specific molecular markers. For example, spinal cord oligodendrocytes originate from precursor cells expressing OLIG2 and NKX2.2, which mature into SOX10+ early oligodendrocyte progenitor cells (OPCs) and then into PDGFRA+ late OPCS, O4+ immature oligodendrocytes and, finally, MBP+ mature oligodendrocytes (Baumann and Pham-Dinh, 2001). Primary oligodendrocyte cultures can be obtained from

different rodent species typically originating from early progenitors, which can be expanded or induced to differentiate and undergo final maturation by varying culture conditions as appropriate (Trotter, 1993; Chen et al., 2007; Itoh, 2002; Medina-Rodriguez et al., 2013). Primary human oligodendrocytes are, of course, much more difficult to obtain, but they can be derived from brain biopsies of postmortem samples (Medina-Rodriguez et al., 2013). The culture conditions for human cells remain suboptimal, and it is not possible to expand the pool of OPCs in vitro. Human umbilical cord blood cells have shown some potential to differentiate into oligodendrocytes (Tracy et al., 2011); however, these data are still controversial.

7.1.2 Neurons

We noted that neuronal damage is one hallmark of MS. Indeed, axonal loss is extensive in the brain and spinal cord of MS patients (Itoh, 2002). It has been estimated that in chronic lesions, axonal density is reduced by approximately 60–70% compared to normal white matter (Mews et al., 1998). Current treatments cannot reverse axonal damage, and therefore, neuroprotective strategies are required to prevent neurodegeneration beginning from disease onset. Studies of the role of ion channels, hypoxia, and oxidative stress in neurons will certainly shed light on the pathogenesis of axonal degeneration and will help in the development of new treatments.

Primary neuronal cells are available, especially from mice and rats (Kitazawa and Shimizu, 2005; Forsby et al., 2009; Zhang et al., 1994; Itoh et al., 1992). However, mature postmitotic neurons cannot be expanded and stored, and therefore, new batches of progenitor cells must be isolated for each experiment. Similarly to oligodendrocytes, primary human neurons are scarcely available (Brewer et al., 2001). However, there are many cell lines that can differentiate into neuronal cells under specific culture conditions. For example, neuroblastoma (SH-SY-5Y) and NT2 cell lines can differentiate into neurons when exposed to retinoic acid and brain-derived neurotrophic factor, respectively (Agholme et al., 2010; Gordon et al., 2013; Pleasure et al., 1992). However, as mentioned earlier, immortalized cell lines often differ with respect to many molecular pathways due to genetic alterations. Thus, the findings obtained using these cells do not necessarily reflect what occurs in primary neurons in the brain.

7.1.3 Astrocytes

Astrocytes are the most abundant cell type in the CNS and are morphologically characterized by numerous long, star-like projections (He and Sun, 2007). Under normal conditions, astrocytes maintain brain homeostasis and

preserve BBB integrity (Barnett and Linington, 2013). Animal studies suggest that reactive astrocytes play a dual role in MS. These cells can both support neuronal survival by controlling the levels of extracellular glutamate and release factors that induce inflammation and active demyelination of neurons. For example, astrocytes release IL-6, TNF-α, and IL-1β, which increase BBB permeability by acting on endothelial cells and tight junctions (Nair et al., 2008). During repair, reactive astrocytes can either inhibit or promote remyelination and are crucial in regulating gliosis or glial scar formation after neuronal destruction. Thus, these cells represent an ideal target to address both demyelination and neurodegeneration. Primary cultures of astrocytes are easy to obtain using rodents (Foo, 2013; Schildge et al., 2013; Skaper et al., 2012). In contrast to oligodendrocytes and neurons, astrocytes can be cryopreserved and can be treated using different transfection methods. Several protocols have been developed to isolate human astrocytes from postmortem fetal or adult tissues or biopsies, but as discussed earlier, these sources are very limited (De Groot et al., 1997; Sharif and Prevot, 2012).

7.1.4 Cocultures

Although studies of a single cell population may be highly informative, the biggest disadvantage of such studies is that they cannot investigate pathogenic mechanisms related to communication between different cell types. This is particularly true when studying brain cells, as the brain is composed of an intricate network of neurons, astrocytes, and oligodendrocytes (Wang et al., 2008). Coculture systems may therefore be preferable.

Several coculture models have been used in the past. The most sophisticated models consist of OPCs and purified sensory neurons (dorsal root ganglion neurons or retinal ganglion neurons) (Chan et al., 2004; Watkins et al., 2008). These cultures are difficult to obtain and require expensive reagents. Alternatively, cocultures consisting of mixed cortical neurons and oligodendrocytes have been described. One disadvantage of these cocultures is that the different types of cells are mixed together (Lubetzki et al., 1993).

Cocultures of OPCs and hippocampal neurons are relatively easy to obtain, do not require purification, and permit the study of both neuronal and oligodendrocyte populations as well as their interactions. However, these cultures still lack the astrocyte component. To overcome this limitation, alternative protocols have been developed to simultaneously isolate all three cell types. Primary neurons are typically seeded on a layer of astrocytes, and oligodendrocytes are added in a second step (Itoh, 2002). Even if this

system presents several advantages, it is not commonly used because it requires technical expertise and is expensive and time consuming.

7.1.5 Human Stem Cell–Derived Neural Cells

Over the past decade, studies using rodent cells have been further and further integrated with studies using human pluripotent stem cell–derived cells. Stem cell technologies and the advent of induced pluripotent stem cells (iPSCs) are certainly revolutionizing in vitro disease modeling, as unprecedented numbers of patient-specific iPSCs can be generated and guided to differentiate into respective disease-associated cell types. In fact, based on studies of spatiotemporal expression of morphogens in fetal brain development, protocols for generating human iPSC-derived neurons, astrocytes, and oligodendrocytes have been described (Boulting et al., 2011; Chambers et al., 2009; Cho et al., 2008; Czepiel et al., 2011; Dimos et al., 2008; Douvaras and Fossati, 2015; Douvaras et al., 2014; Goldman and Kuypers, 2015; Hargus et al., 2010; Jiang et al., 2013; Krencik and Zhang, 2011; Lee et al., 2007; Li et al., 2005; Merkle et al., 2015; Shaltouki et al., 2013; Shi et al., 2012; Smith et al., 2008; Wang et al., 2015). Since gliogenesis begins late in the neurogenic process, extensive work was undertaken by researchers to develop these protocols. Pioneering work in mouse and human embryonic stem cells (ESCs) (described in Goldman and Kuypers, 2015) was followed by a study of human iPSCs by Czepiel and coworkers (Czepiel et al., 2011). Our lab has significantly accelerated the lengthy process of generating oligodendrocytes from stem cells and shown that MS patient-derived oligodendrocytes can myelinate in vivo (Douvaras et al., 2014; Douvaras and Fossati, 2015). This exciting work will lead to the analysis of specific defects in patient-derived cells. Going forward, the field must be cautious in addressing the heterogeneity of neural and glial cell types obtained from the aforementioned protocols as well as the potentially conflicting results due to the differential patterning along the neuroaxis. Despite these concerns, iPSCs have potential to reveal the causes of diseases, whether genetic or environmental, as both can be modeled in vitro in a relevant human cell context.

7.1.6 Brain Slices

The closest model of a brain is probably represented by organotypic brain slice cultures. The first efforts to generate these cultures were reported in 1941 (van der Star et al., 2012). Since then, protocols have been optimized, and it is currently possible to obtain organotypic cultures from different areas of the brain. Interestingly, these cultures have been adapted from a wide

range of mammalian species, particularly mice and rats (Gogolla et al., 2006, Drexler et al., 2010; Mi et al., 2009). Notably, human organotypic samples may be obtained from human fetal brains (Verwer et al., 2002).

Brain slices have been reported to be viable for up to 4–6 months in culture and appear to be easy to transfect using viral infection, single-cell electroporation, a gene gun, and Lipofectamine. Therefore, their research applications are numerous. Demyelination/remyelination events may be observed and long-term investigations of the mechanisms of neurodegeneration and cell death processes may be performed. Treatments with several drugs, stimulated ischemia, or bacterial insults are easy to apply. Moreover, electrophysiology, molecular biology, and immunohistochemistry can be performed to study physiological or pathological processes. The primary application of organotypic cultures in MS is the study of the remyelination process.

7.2 In Vivo Model

In vivo studies are needed to understand the complex interplay between different cells and dissect the pathogenic mechanisms related to disease initiation and progression. This is particularly important in the case of MS, in which many cell types of both the immune system and the CNS are involved. Animal studies are also necessary in the preclinical phase of drug discovery to test the toxicity and efficacy of novel drugs. Mice and rats have largely been used to address demyelination and axonal degeneration, despite the evidence that they are not affected by MS because the disease is exclusively human. Therefore, models mimicking the inflammatory cascade or demyelinating insults in the CNS have been developed to recapitulate the major pathogenic mechanisms occurring in the bona fide disease. Here, we will discuss two widely used models of MS, the EAE model and the cuprizone model.

7.2.1 EAE

The EAE model involves T cell-driven inflammatory demyelination that recapitulates the major pathological features of MS (Constantinescu et al., 2011). Inflammation is induced in animals following immunization with myelin peptides (e.g., from MOG or PLP) in the presence of an adjuvant, typically from mycobacteria, to enhance the immune response.

Clearly, the major differences between MS and EAE are that in humans, T cells are not artificially activated and, most importantly, no specific autoantigen triggering a T lymphocyte response in patients has ever been identified (Fitzgerald et al., 2007).

Despite these limitations, EAE models enabled the exploration of several molecular mechanisms underlying MS pathogenesis and supported the development of several currently available therapies, such as IFNs and natalizumab, a monoclonal antibody against integrin 4 that prevents leukocyte infiltration into the CNS (Farooqi et al., 2010).

The EAE model is most commonly used in mice; however, it can be applied to different species and strains, including, rats, guinea pigs, and Rhesus macaques, as well as to transgenic animals. Notably, the clinical course of induced EAE depends on different factors, including the species, strain, and gender of the animal used, the immunizing antigen, and the immunization protocol. These variations have been employed to develop different models that distinguish between the acute inflammation, relapsing-remitting, and progressive courses of MS (Constantinescu et al., 2011). Generally, the first signs of EAE, including weight loss and motor impairment, are observed between 12 and 18 days after immunization. A clinical score from 0 (clinically normal) to 5 (total paralysis and death) has been created to quantify the severity of the disease (McCarthy et al., 2012).

Overall, the EAE model is relatively easy to use and is a valuable tool to study inflammatory demyelination in MS. Although not all the findings derived from this model have been translated into successful therapies in humans, EAE studies through the years have been critical for dissecting the immunopathogenic mechanisms in MS and have contributed preclinical data supporting the development of the most recently approved drugs.

7.2.2 Cuprizone Model

Cuprizone [oxalic acid bis(cyclohexylidene hydrazide)] is a selective and sensitive copper-chelating agent. Cuprizone is used to produce toxic demyelination that resembles the demyelination that occurs in MS (Torkildsen et al., 2008). In this very simple and useful model, young mice are fed a diet containing 0.2–0.3% cuprizone for 5–6 weeks, which leads to progressive demyelination in several brain regions. However, if mice are switched to a normal diet, the demyelination process is reversed due to spontaneous remyelination by endogenous OPCs (van der Star et al., 2012). Administration of cuprizone induces severe oligodendrocyte damage with concomitant microglial activation and severe astrocytosis (Pasquini et al., 2007).

These effects have also been attributed to the mitochondrial compartment. Cuprizone is a potent chelator of copper, which is a crucial component of respiratory chain complex IV—cytochrome *c* oxidase in oligodendrocytes (Matsushima and Morell, 2001). Thus, it has been

supposed that administration of cuprizone significantly reduces the mitochondrial activity and metabolism of oligodendrocytes concomitant with activation of oxidative stress and ROS production. These events trigger microglial/macrophage recruitment and, subsequently, the secretion of proinflammatory cytokines, which ultimately promote oligodendrocyte death or inhibit differentiation. Mice are the preferred species for the cuprizone model. Notably, there is strain-dependent susceptibility to cuprizone (Kipp et al., 2009). Differences in demyelinated foci have been found between C_{57}BL/6 and BALB/cJ mice (Skripuletz et al., 2008). The age of the animals may also affect the toxicity of cuprizone. Therefore, in aged animals, the cuprizone concentration in food is usually increased (Kipp et al., 2009).

From a research perspective, cuprizone treatment is a reliable model for inducing and examining demyelination/remyelination events. In contrast to the EAE model, the cuprizone model is highly suitable for examining the remyelination process in the absence of continued demyelination. In addition, this model is valuable for screening and identifying remyelinating compounds. Finally, because C_{57}BL/6 mice are the strain most commonly used to create transgenic animals, the application of the cuprizone model to specifically knockdown or overexpress genes associated with myelination allows researchers to fully elucidate the role of these genes and select targets for therapeutic interventions.

8. CONCLUSIONS AND PERSPECTIVES

Mitochondria are highly complex structures with roles in bioenergetics as well as cell stress responses and apoptotic fate determination, and it is clear from specific examples of genetic mutations that mitochondrial dysfunction results in devastating neurological conditions. Given the unique energy expenditure of neurons, it is perhaps not surprising that mitochondria are involved in myriad neurological conditions. Indeed, the mitochondrion might be the Achilles heel of the nervous system.

The pathology of MS is characterized by inflammation and neurodegeneration, yet the root cause of this disorder remains a mystery. It is apparent that the more progressive form of MS has an underlying neurodegenerative component, and the role of mitochondria in this disorder remains to be determined. Mutations in mtDNA have been linked to an increased risk of developing MS, and studies using rodent models of MS have also implicated

its function in the biology of the cell types underlying MS, including neurons and oligodendrocytes. Clearly, studies using rodent models have provided great advances, and the tools developed in these studies are primed for use in potentially more relevant human models. Due to the continuous advancements in iPSC differentiation technology, researchers are poised to apply the methods perfected in rodent cells to tackle genetics, oxidative stress, ionic imbalances, environmental toxicity, autophagy, and mitophagy in one relevant human cell milieu. The additional power of combinatorial modeling by coculturing human iPSC-derived neurons, astrocytes, and oligodendrocytes in one dish, provides enormous potential to unravel the quagmire that plagues the MS field.

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