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Axonal degeneration in multiple sclerosis: The mitochondrial hypothesis

Kimmy G. Su, B.A.,
Oregon Health & Science University Vollum Institute, L474 3181 SW Sam Jackson Park Road Portland, OR 97239 Phone: (503) 494-5455 FAX: (503) 494-4976 suk@ohsu.edu

Gary Banker, Ph.D.,
Oregon Health & Science University Jungers Center for Neurosciences Research 3181 SW Sam Jackson Park Road Portland, OR 97239 Phone: (503) 494-2306 FAX: (503) 494-3849 bankerg@ohsu.edu

Dennis Bourdette, M.D., and
Oregon Health & Science University Department of Neurology 3181 SW Sam Jackson Park Road Portland, OR 97239 Phone: (503) 494-7321 FAX: (503) 494-7242 bourdett@ohsu.edu

Michael Forte, Ph.D.
Oregon Health &Science University Vollum Institute, L474 3181 SW Sam Jackson Park Road Portland, OR 97239 Phone: (503) 494-5454 FAX: (503) 494-4976 forte@ohsu.edu

Abstract

Multiple sclerosis (MS) is a chronic disease of the central nervous system, affecting upwards of 2 million people worldwide. Traditionally considered an inflammatory demyelinating disease, recent evidence now points to axonal degeneration as crucial to the development of irreversible disability. Studies show that axonal degeneration occurs throughout the entire MS disease course. While the specific mechanisms causing axonal damage may differ at various stages, mitochondrial failure seems to be a common underlying theme. This review addresses the mitochondrial hypothesis for axonal degeneration in MS, highlighting the mechanisms by which mitochondrial dysfunction leads to axonal disruption in acute inflammatory lesions and the chronic axonopathy in progressive MS. Emphasis will be placed on Ca^{2+}, free radical production, and permeability transition pore opening as key players in mitochondrial failure, axonal transport impairment, and subsequent axonal degeneration. In addition, the role of mitochondria as therapeutic targets for neuroprotection in MS will be addressed.

Introduction

Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system affecting upwards of 2 million people worldwide. Patients present with a spectrum of clinical signs and symptoms, including weakness, vision loss, fatigue, and cognitive impairment. Around 85% of patients have a relapsing-remitting (RRMS) clinical course, characterized by periods of clinical stability punctuated by subacute attacks of clinical worsening after complete or partial recovery. Many of these patients eventually transition into secondary progressive MS, during which there is continuous neurological deterioration. A minority of patients (around 10%) present with primary progressive MS, characterized by unremitting decline of neurological function. The remaining 5% of patients experience a

Correspondence to: Kimmy G. Su.

clinical course termed progressive relapsing MS, during which there is a steady progressive neurological decline interspersed with acute attacks with or without recovery [1].

Traditionally, MS has been classified as an immunological, demyelinating disease elicited by endogenous myelin-associated antigens such as myelin oligodendrocyte glycoprotein, proteolipoprotein, and myelin basic protein [2]. Acute lesions are thought to result when activated T cells responsive to these and other potential antigens traffic to the CNS and trigger a cascade of inflammatory events. This includes activation of recruited monocytes and resident microglial cells to generate macrophages that are key mediators of tissue injury and are the most abundant inflammatory cells found in MS lesions [3]. These acute inflammatory lesions are often clinically silent but may result in clinical relapses, depending on the severity and location of the lesion. Currently, all available FDA-approved therapies for MS are anti-inflammatory in nature and have been successful at treating RRMS, but not progressive forms of MS [4].

While classically thought of as a demyelinating disease, it is now recognized that MS pathology is much more complex. Axonal injury occurs commonly in acute inflammatory lesions and these lesions present in both white and grey matter [5–7]. Widespread axonal degeneration and brain atrophy appear early in the disease course, and are prominent in progressive forms of MS [7,8]. Of importance to this review is the recognition that axonal loss plays a critical role in the irreversible disability that occurs in MS.

The mechanisms involved in MS axonal injury may vary depending on the stage of disease. First, acute axonal disruption is a prominent part of the acute inflammatory lesions of MS [9]. Second, there is clinical, pathologic and magnetic resonance evidence suggesting that widespread axonal degeneration occurs independent of acute inflammatory lesions, and appears most prevalent in progressive forms of MS [10]. While it is likely that the specific mechanisms of axonal injury and degeneration differ between these two stages of disease, there seems to be a convergence of the pathways that involves mitochondrial failure.

Evidence is evolving that mitochondria are key players in axonal degeneration in all stages of MS, playing crucial roles in energy metabolism and cell homeostasis. In particular, much evidence has been obtained from magnetic resonance spectroscopy of N-acetylaspartate (NAA) levels. Commonly considered an indirect marker of axonal integrity, NAA is produced by neuronal mitochondria, and therefore also reflects the integrity of mitochondrial function within axons [11–13]. In acute inflammatory lesions, NAA levels fall dramatically, and partially reverse as inflammation subsides [14,15]. The initial dramatic decline in NAA undoubtedly reflects reversible mitochondrial dysfunction in axons within these acute lesions. NAA levels have also been shown to be abnormally low both in chronic focal white matter lesions as well as in normal appearing white matter [16]. While generally taken as evidence of axonal loss, these findings may also reflect chronic mitochondrial dysfunction within axons. Other evidence linking mitochondrial dysfunction to axonal degeneration includes oxidative damage to mitochondrial DNA in active MS lesions, and a decrease in nuclear encoded DNA transcripts of mitochondrial proteins in non-lesional cortex [17,18].

This review focuses on the mechanisms of mitochondrial dysfunction leading to 1) axonal disruption in acute inflammatory lesions, and 2) the chronic axonopathy and axonal degeneration in progressive MS. In particular, the acute inflammatory attacks common in RRMS will be analyzed, and the potential effects of inflammatory components such as nitric oxide and glutamate will be addressed. In addition, the mechanisms by which axons degenerate during the progressive phases independent of acute inflammation will be discussed, emphasizing Ca^{2+}, free radicals, and opening of the permeability transition pore as key players in mitochondrial failure and subsequent axonal damage. Finally, the effect of mitochondrial

dysfunction on axonal transport, specifically with regards to mitochondria motility, will be summarized.

Mitochondria: background

The mitochondrion is the power plant of the cell, the site at which aerobic respiration and ATP synthesis take place. A cytoplasmic organelle with double membranes, the mitochondrion is divided into two main compartments, the matrix and the intermembrane space. The outer mitochondrial membrane enclosing the organelle contains a large number of porin channels, which allow free diffusion of molecules 5000 Daltons or less. Larger proteins can enter mitochondria through the translocase of the outer membrane, which shuttles them into the intermembrane space. The intermembrane space is located between the outer and inner mitochondrial membranes; the pro-apoptotic protein cytochrome C is located here. The inner mitochondrial membrane separates the intermembrane space from the matrix, and contains a wide range of proteins including the electron transport chain, ATP synthase, the translocase of the inner membrane, and the permeability transition pore. The electron transport chain and ATP synthase are involved in oxidative phosphorylation, which generates the mitochondrial membrane potential, the proton gradient, and of course, the ATP that is necessary for cell survival. The permeability transition pore (PTP) is a transient pore that when open allows solutes with molecular masses up to 1500 Daltons to enter the matrix [19]. More about the PTP will be addressed later in the review.

As evident by their structural components, mitochondria are crucial to cell survival, not only by producing ATP, but also functioning to maintain ion homeostasis and to regulate apoptosis. Therefore, it is not surprising that external factors altering mitochondrial function during the acute and progressive phases of MS have a profound downstream effect on axonal degeneration.

Axonal degeneration within acute inflammatory lesions

NO hypothesis

During an acute inflammatory attack in MS, activated T cells initiate the pro-inflammatory cascade in response to encountering antigen. This response produces interferon gamma, which activates macrophages to produce elevated levels of nitric oxide (NO) by upregulating inducible nitric oxide synthase [20]. The increase in NO inhibits mitochondrial respiration and reduces ATP synthesis [21].

The mechanism by which NO inhibits mitochondrial respiration involves cytochrome C oxidase, the terminal member of the electron transport chain situated in the inner mitochondrial membrane. Cytochrome C oxidase has a binding domain for O_2 and catalyzes the oxidation of cytochrome C and the reduction of O_2 to water. At elevated levels, NO can outcompete O_2 for the binding position, block electron flow, and disrupt mitochondrial respiration [22,23]. Since the pumping of protons from the mitochondrial matrix into the intermembrane space is coupled to the electron flow, ATP synthesis is subsequently hindered [21]. Inadequate ATP production prevents crucial ATPase pumps from working properly, and the downstream effects are detrimental on cell survival.

Besides inhibiting oxidative phosphorylation, NO also can affect mitochondrial function by increasing free radical production. For one, interruption of electron transfer at cytochrome C oxidase by NO significantly increases electron leakage from the respiratory system resulting in elevated levels of superoxide [24]. Superoxide levels are usually regulated by antioxidant systems, which convert superoxide into hydrogen peroxide, and subsequently, oxygen and water. Superoxide that evades conversion can cause significant cellular damage. It can also

combine with NO to form highly toxic peroxynitrite (ONOO-), which can react with and inactivate lipids, proteins, DNA, and carbohydrates [24, 25]. Peroxynitrite also has a profound direct effect on mitochondrial function, increasing the peroxidation of mitochondrial membrane lipids, disrupting nearly all components of the electron transport chain, opening the PTP, and inducing cytochrome C release from the intermembrane space leading to apoptosis. Interestingly, peroxynitrite is prominent in acute inflammatory lesions, but absent from chronic non-inflammatory lesions [26]. This suggests that peroxynitrite plays more of a role in axonal damage during acute inflammatory MS attacks.

Glutamate hypothesis

Excitotoxicity due to elevated glutamate release can also disrupt mitochondrial function. Glutamate is an essential excitatory neurotransmitter that acts on AMPA and NMDA receptors located on the post-synaptic membrane of neurons. Upon binding and activation of these receptors, ion channels open allowing various cations such as Na^+, K^+, and Ca^{2+} to enter the cell. Synaptic levels of glutamate are regulated by glutamate transporters present on astrocytes, oligodendrocytes, and microglia, which take up released glutamate and convert it into glutamine. Glutamine is then shuttled back to neurons and regenerated into glutamate by glutaminase [27].

Glutamate excitotoxicity occurs when there is elevated release of glutamate into the synapses, and inadequate reuptake by the transporters on supporting cells. For instance, during an active inflammatory attack in MS, large quantities of glutamate are produced by activated immune cells such as macrophages and microglia [27]. There may also be a decrease in glutamate transporter expression in surrounding oligodendrocytes and astrocytes, further enhancing the severity of excitotoxicity.

Overstimulation of glutamate receptors leads to dysregulation of ionic gradients, including Ca^{2+} homeostasis. The increase in intracellular Ca^{2+} activates several enzymes including phospholipases, endonucleases, and proteases, which damage DNA, disrupt the cytoskeleton, and alter membrane lipids [28]. Elevated intracellular Ca^{2+} levels also alter mitochondrial dynamics, promoting Ca^{2+} entry into the matrix, opening of the permeability transition pore, and release of cytochrome C into the cytosol.

Magnetic resonance spectrometry can be used to monitor glutamate levels at various stages of MS [29]. Studies show that glutamate levels are elevated in acute inflammatory lesions as well as in normal appearing white matter. In comparison, glutamate levels are not elevated in chronic demyelinated regions. These findings suggest that glutamate-mediated excitotoxicity plays more of a role in acute than in chronic stages of MS.

Mitochondrial damage: the converging pathway

It is important to note that while the proposed NO and glutamate mechanisms of axonal injury are separate and distinct, they both converge onto a common pathway leading towards mitochondrial dysfunction. Both mechanisms affect the electron transport chain, ATP synthesis, ionic homeostasis, PTP opening, and release of pro-apoptotic factors. This convergence is not exclusive to the acute inflammatory attacks of MS, and in fact is present in the progressive stages independent of acute inflammation as well, further emphasizing the importance of mitochondria in maintaining axonal integrity and survival.

Axonal degeneration during progressive stages independent of acute inflammation

Chronic demyelination promotes upregulation and reorganization of ionic channels

In the progressive stages of MS, there are fewer acute inflammatory attacks occurring within the CNS, suggesting that other mechanisms are involved in axonal degeneration. At this stage in disease, commonly used anti-inflammatory medications, such as interferon beta and glatiramer acetate, have minimal effect on delaying or inhibiting the neurodegenerative symptoms of MS.

One of the main structural changes during progressive MS is the loss of myelin. In a normal myelinated axon, voltage-gated sodium channels are highly concentrated at the nodes of Ranvier, and the myelin sheath insulates the internodal axon so that current "jumps" from node to node. Loss of myelin greatly impairs the efficiency of action potential propagation. In response to demyelination, sodium channels become redistributed all along the axon and synthesis is upregulated, including both Nav1.6 and Nav1.2 subtypes [30]. The Nav1.6 subtype, which is normally expressed at the nodes of Ranvier, tends to produce larger and more persistent currents in comparison to the Nav1.2 subtype, which is predominately expressed on premyelinated axons.

The reorganization of voltage-gated sodium channels and upregulation of channel expression along demyelinated axons leads to altered energy requirements. The demand for ATP exceeds the production capabilities of existing mitochondria, and the Na^+/K^+ ATPase pumps crucial to maintaining ionic gradients begin to fail. An excess of Na^+ ions accumulates intracellularly, and eventually reverses the Na^+/Ca^{2+} exchanger that normally moves Na^+ into the cell and Ca^{2+} from the cell [18,31]. Prolonged elevation of Ca^{2+} levels within the axoplasm can stimulate a multitude of downstream events that ultimately results in mitochondrial dysfunction and axonal damage.

Effect of elevated intracellular Ca^{2+} levels on mitochondrial function

As mentioned previously, mitochondria are composed of two membrane systems, an outer membrane that is freely permeable to most ions and an inner membrane that is more tightly regulated and surrounds the innermost mitochondrial matrix. During oxidative phosphorylation and ATP synthesis, electrons are transferred along the electron transport chain located in the inner mitochondrial membrane, which is coupled with the movement of H^+ ions from the matrix across the membrane into the intermembrane space. This ionic movement generates a transmembrane potential across the inner membrane (~-200 mV), and it is this voltage gradient that is subsequently used to synthesize ATP.

Beyond ATP synthesis, this inside-negative transmembrane potential also drives positively charged ions such as Ca^{2+} into the matrix. Consequently, mitochondria accumulate Ca^{2+} whenever local cytoplasmic levels rise above a critical set point, and then slowly release Ca^{2+} when cytoplasmic levels are restored [32].

The accumulation of Ca^{2+} ions within the mitochondrial matrix is dependent on the cytoplasmic concentration of Ca^{2+} as well as the affinity of two key mitochondrial transporters, the inward electrogenic uniporter and the Na^+ or H^+/Ca^{2+} antiporters that extrude Ca^{2+} ions from the matrix. As Ca^{2+} ions have a higher affinity for the inward uniporter, they tend to be shuttled into the mitochondrial matrix when cytoplasmic Ca^{2+} levels are elevated [33].

The accumulation of Ca^{2+} in the mitochondrial matrix is physiologically relevant in the stimulation of oxidative phosphorylation. Three important rate-limiting metabolic enzymes are

activated by matrix Ca^{2+}, including pyruvate dehydrogenase, alpha-ketoglutarate, and isocitrate dehydrogenase [33]. However, prolonged elevated Ca^{2+} levels can also induce opening of the PTP, leading to a cascade of events, including matrix swelling, rupture of the outer mitochondrial membrane, and release of cytochrome C, triggering the pro-apoptotic pathway.

The permeability transition pore revisited

The PTP, as mentioned before, is a pore in the inner mitochondrial membrane that opens during permeability transition activated by mitochondrial stress. During permeability transition, high conductance channels in the inner membrane of mitochondria open, allowing solutes with molecular masses up to 1500 Daltons to enter [19]. Persistent PTP opening leads to loss of mitochondrial membrane potential and equilibration of ionic gradients, which can prevent ATP synthesis, and promote mitochondrial matrix swelling and outer membrane rupture. In addition, damage to the mitochondrial membranes can release cytochrome C from the intermembrane space, activating pro-apoptotic factors and inducing cell death.

Again, it is important to note that both the acute and chronic pathways of axonal degeneration in MS converge on the mitochondria, and specifically, the pathological opening of the PTP. As the PTP is still fairly elusive in structure, it is therefore essential to investigate the properties of this pore in order to potentially develop MS treatments that regulate pore opening and prevent mitochondrial rupture and ensuing axonal damage.

Much work has been done in the recent years to investigate permeability transition, and the permeability transition pore that is involved. Some of the candidate proteins include ANT (adenine nucleotide translocator), VDAC (voltage-dependent anion channel), and cyclophilin D (a peptidyl-prolyl cis-trans isomerase) [19]. Whether these proteins are essential components of the PTP has been heavily debated for numerous years. There is convincing evidence from genetic studies that neither VDAC nor ANT are essential for PTP formation, but some experiments suggest that ANT may still play a regulatory role [19]. In contrast, increasing evidence indicates that cyclophilin D (CyPD) plays a crucial regulatory role in the PTP. Various pharmacologic and genetic techniques have been used to alter CyPD activity and expression, including cyclosporin A administration, and *Ppif* gene deletion. Cyclosporin A treatment has long been shown to inhibit PTP opening, and has been tested in several *ex vivo* and *in vivo* models of disease, including ischemic-reperfusion injury of the heart, and ischemic and traumatic brain injury [34,35]. CyPD has also been knocked out by *Ppif* gene deletion (CyPD-KO), resulting in viable animals that still can form the PTP [36,37]. Interestingly, the mitochondria in these knockout mice are able to retain about double the amount of Ca^{2+} as wild-type animals, demonstrating that CyPD is a significant regulator of PTP opening. Due to the altered PTP properties in CyPD-KO mice, they have been used in many studies addressing mitochondria dysfunction and disease pathology. For instance, CypD-KO mice were subjected to ischemia/reperfusion injury, and showed significant reduction in heart and brain infarct size in comparison to wild-type counterparts [38].

Recently, to address the mitochondrial hypothesis of axonal degeneration in MS, CyPD-KO mice were induced with experimental autoimmune encephalomyelitis (EAE) [39]. EAE is a well-recognized animal model for MS, and involves immunization with fragments of myelin proteins, including myelin oligodendrocyte glycoprotein (MOG) and proteolipoprotein (PLP). In this study, immunized CyPD-KO mice developed clinical symptoms of paralysis, but unlike the wild-type mice, eventually regained function. Furthermore, spinal cord sections from the CyPD-KO mice showed decreased levels of axonal damage and loss. These results suggest that regulation of PTP opening and mitochondria integrity has a significant effect on EAE disease progression and axonal survival. In the broader scheme of things, they also suggest

that pharmacologic blockers of the PTP might be beneficial in reducing or preventing axonal degeneration in MS.

Mitochondrial dysfunction and axonal transport in MS

Axonal transport is necessary for the normal function and survival of neurons, conveying newly synthesized proteins from the cell body to sites along the axon, and delivering trophic signaling complexes from synaptic terminals back to the cell body. Despite this, little research has been done on the effects of inflammation on axonal transport and nothing is known about dysfunction of axonal transport in MS. It seems likely that mitochondrial dysfunction in MS will lead to abnormalities of axonal transport, which in turn could contribute to axonal degeneration. What follows is a brief discussion of how axonal transport might fail in MS and contribute to axonal degeneration.

Axonal transport is mediated by motor proteins that walk along microtubules, carrying membranous organelles along for the ride. Because axonal microtubules are oriented with plus ends pointing away from the cell body, members of the kinesin family of plus-end directed motors mediate anterograde transport and cytoplasmic dyneins mediate retrograde transport. The motors that drive axonal transport require ATP produced locally by mitochondria all along the axon. At the same time, motor-driven transport is required to deliver mitochondria to appropriate sites along the axon. Thus deficits in mitochondrial ATP production can disrupt axonal transport, and disruptions in transport can interfere with mitochondrial trafficking.

The number and localization of mitochondria in axons is a function of mitochondrial fission, mitochondrial fusion, and long-range bidirectional transport along the axon. These same processes occur in all cells, but neurons are likely to be particularly susceptible to dysfunctions in mitochondrial trafficking due to their extended dimensions. In support of this idea, mutations in genes that regulate mitochondrial fusion, fission, and transport are responsible for forms of spastic paraplegia, Charcot-Marie-Tooth disease and optic atrophy [40]. Thus mitochondrial trafficking is essential for maintaining axonal integrity.

While mitochondria are present all along the axon, they are concentrated in areas with high metabolic demand, such as presynaptic terminals and in some cases, nodes of Ranvier [41]. Three recent studies have elucidated the molecular mechanisms by which neural activity and associated increases in cytoplasmic Ca^{2+} regulate mitochondrial transport [42–44]. The principal motor responsible for anterograde mitochondrial transport is Kinesin-1 [41]. Kinesin-1 is linked to the mitochondrion by the protein Milton, which binds to the kinesin tail domain and links it to Miro, a protein in the outer mitochondrial membrane. Miro, a GTPase with two Ca^{2+} binding domains, regulates the integrity of this complex and hence the efficiency of mitochondrial transport. Ca^{2+} binding to Miro inhibits mitochondrial transport, either by causing dissociation of the complex or by inhibiting the kinesin-microtubule interaction, causing mitochondria to accumulate at sites of increased Ca^{2+} levels. This mechanism, which is essential for correctly positioning mitochondria under normal circumstances, may enhance the susceptibility of mitochondria to damage under pathological conditions, as discussed below.

During an acute inflammatory attack, increases in free radicals lead to mitochondrial damage and decreased ATP production, which would be expected to inhibit axonal transport, including the transport of mitochondria [45]. Inflammation also activates signaling pathways that inhibit axonal transport. For example, TNF-alpha and NO inhibit the transport of mitochondria and synaptic vesicle proteins by activation of JNK kinase [46]. JNK kinase phosphorylates a serine in the kinesin-1 motor domain, which inhibits its translocation [47]. Elevated glutamate release during the acute inflammatory phase also leads to increased Ca^{2+} entry, Ca^{2+} binding to Miro, and inhibition of kinesin-mediated transport, causing mitochondria to accumulate in the

affected regions. While this response may temporarily enhance the buffering of axoplasmic Ca^{2+} and increase the availability of ATP to restore ionic concentrations, it also exposes the immobilized mitochondria to further oxidative damage, which would eventually exacerbate the depletion of ATP and its effects on axonal organelle transport.

Likewise, during the progressive phases of MS, intracellular Ca^{2+} levels are elevated by altered distribution of voltage-gated channels, increased metabolic requirements, and inability of ATPase pumps to regulate ionic gradients. Again, the elevated intracellular Ca^{2+} levels would inhibit mitochondrial transport, and retain the organelles in damaged areas. With time, the persistent elevation in Ca^{2+} levels may lead to opening of the PTP, mitochondrial rupture, and initiation of apoptotic events within the axon [48]. Genetic studies in mice show that mutations or deletions in specific myelin proteins lead to changes in the axonal cytoskeleton, including alterations in the phosphorylation and spacing of neurofilaments and microtubules, changes in the velocity of axonal transport, and mislocalization of mitochondria [49]. These changes can occur even in cases where myelin structure is largely intact. Thus chronic demyelination may also disrupt the signaling between oligodendrocytes and axons that is required to maintain the axonal transport machinery.

In summary, pathophysiologic changes observed in both acute and chronic stages of MS are likely to inhibit the transport of mitochondria, which may further contribute to axonal damage. The development of transgenic mouse lines that express mitochondrially targeted GFP offers an important new tool for imaging mitochondria *in vivo*, an approach that could be used to assess axonal transport in animal models of MS [50].

Conclusions

In the past, MS has largely been considered a chronic inflammatory and demyelinating disease, driving most of the research and treatment development towards targeting the immune system. As of now, disease modifying therapies for MS are limited to various anti-inflammatory agents that reduce acute inflammatory lesions, clinical relapses and disability progression in RRMS. These anti-inflammatory agents, however, do not completely prevent axonal injury and are largely ineffective in treating progressive MS.

The recent resurgence of MS research focused on axonal degeneration mechanisms has resulted in convincing experimental evidence and potential treatment targets. As reviewed above, mitochondrial function is crucial in preserving axonal integrity in both acute inflammatory and progressive stages of MS. Therefore, therapies that protect mitochondria and enhance their functioning warrant investigation. Such therapies include sodium channels blockers to reduce axoplasmic Ca^{2+}, anti-oxidants to neutralize free radical production, and PTP inhibitors to maintain mitochondrial integrity. With continued progress in the understanding of MS and the mechanisms that drive the disease, it is hopeful that a successful treatment regimen targeting both inflammation and axonal degeneration may soon be developed.

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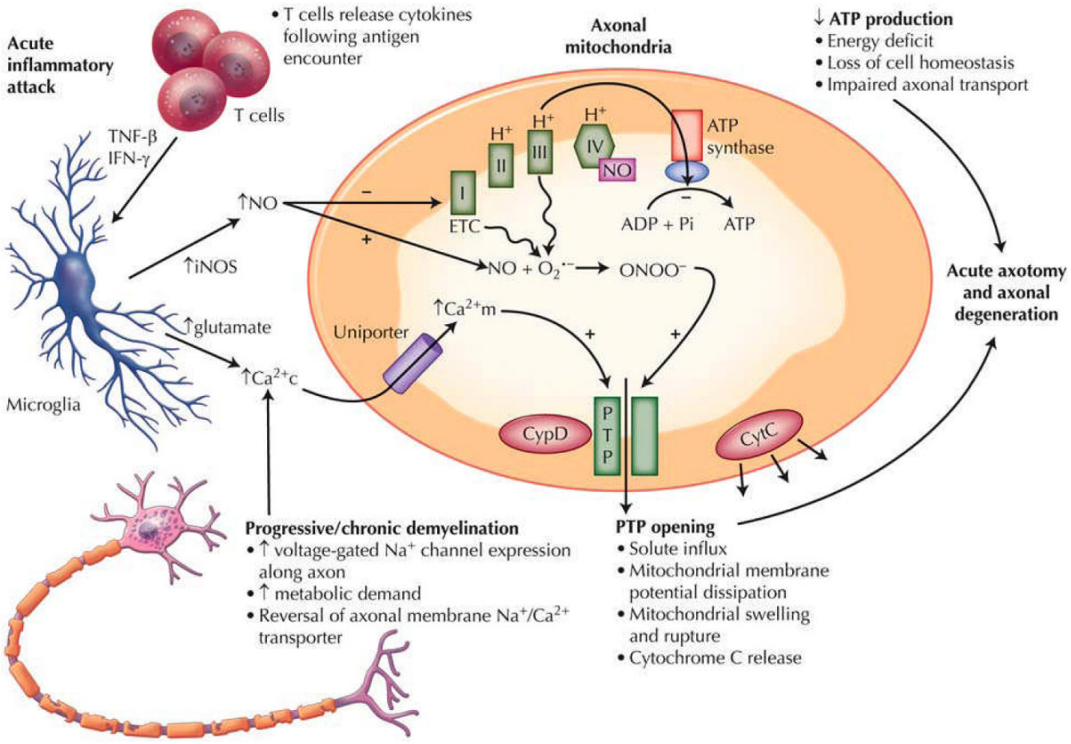

Figure 1.

Schematic representation of the mitochondrial hypothesis for axonal degeneration in multiple sclerosis. ATP—adenosine triphosphate (adenosine diphosphate plus a single phosphate [ADP + Pi]); Ca_2+c—cytoplasmic calcium; Ca_2+m—matrix calcium; CypD—cyclophilin D; CytC—cytochrome C; ETC—electron transport chain; IFN-γ—interferon-γ; iNOS—inducible nitric oxide synthase; NO—nitric oxide; O_2—superoxide; ONOO-—peroxynitrite; PTP—permeability transition pore;TNF-β—tumor necrosis factor-β.