General discussion
These are exciting times in multiple sclerosis (MS) research. Not only are we seeing great improvement in MS treatment with the development of novel therapeutics like Nataluzimab, BG12 and FTY720, but we now increasingly understand which genetic and environmental factors can contribute to disease initiation. Also, the importance of understanding neurodegenerative processes, next to inflammatory processes, is now widely recognised. Like in most neurodegenerative diseases, there is a crucial role for mitochondria in neurodegeneration in MS. The main aim of this thesis was to explore mitochondrial changes and underlying mechanisms in the central nervous system (CNS) of MS patients. Here we will summarize and discuss the results described in this thesis, and try to embed them in current knowledge of MS pathogenesis and neurodegeneration in general.

1. Mitochondrial dysfunction in MS white matter

White matter in MS patients is generally classified according to myelination and inflammation status. Normal-appearing white matter (NAWM) is often regarded as non-affected and reflects the white matter of healthy controls best. Microglial clustering in the absence of overt demyelination is frequently referred to as preactive lesions, and they are regarded by some as the initial step towards demyelination. Lesions with ongoing demyelination by macrophages and activated microglia are called active lesions, and they are believed to gradually evolve into chronic active lesion. Chronic active lesions are characterized by a completely demyelinated centre with ongoing demyelinating activity at the lesion edges. Eventually, inflammation resolves and the demyelinated lesion is now referred to as chronic inactive. A substantial proportion of demyelinated areas is remyelinated over the course of the disease, whereas non-remyelinated areas are filled with a gliotic scar. Importantly, both myelination status of axons and presence of inflammatory cells alter mitochondrial function in MS lesions in different ways.

Currently, studies looking into mitochondrial changes in NAWM and preactive lesions are lacking. Those studies are warranted to elucidate if mitochondria already react to the subtle changes occurring in NAWM and/or contribute to lesion initiation in preactive lesions. Intriguingly, nitration of mitochondrial proteins has been observed as early as 3 days after sensitisation in experimental autoimmune encephalomyelitis, a widely used MS animal model. These data suggest that mitochondrial dysfunction precedes leukocyte infiltration. Unfortunately, activation of microglia, which also occurs before influx of leukocytes in EAE, was not assessed and therefore the likely relation between mitochondrial protein nitration and microglial activation remains unresolved.

1.1 Mitochondria in inflammatory lesions

Most studies have focussed on mitochondrial (dys)function in demyelinated MS lesions and its animal models, among which are the studies described in chapter 2, 3 and 4 of this thesis. In (chronic) active MS lesions, blood-borne macrophages and activated microglia destroy myelin, oligodendrocytes and a substantial proportion of axons, as evidenced by amyloid precursor protein (APP)-positive axonal bulbs. In this process, macrophages and microglia produce substantial amounts of nitric oxide and reactive oxygen species (ROS), which are able to damage mitochondrial components and inhibiting mitochondrial function. Not surprisingly, we and others have described changes in activity of several oxidative phosphorylation (OxPhos) complexes, signs of mitochondrial oxidative stress
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and damage to mitochondrial macromolecules in (chronic) active MS lesions.\textsuperscript{14-16} The first study describing the occurrence of mitochondrial changes in MS brain tissue was published more than a decade ago. They observed reduced activity of complex I and a concomitant increase in mtDNA oxidation in chronic active MS lesions.\textsuperscript{14} Interestingly, complex IV activity was upregulated in these lesions. We, in chapter 2, and others corroborated this finding and located the increase in complex IV activity to astrocytes in inflammatory lesions.\textsuperscript{15} Next to an increase in activity of complex IV, mitochondrial content was significantly increased in hypertrophic astrocytes compared to astrocytes in NAWM. The presence of mitochondrial heat shock protein 70 (mtHSP70) in mitochondria in hypertrophic astrocytes revealed that mitochondria in astrocytes are under increased oxidative stress. However, mitochondrial changes in astrocytes in inflammatory MS lesions are not restricted to changes in oxidative phosphorylation. In chapter 3, we showed increased astrocytic expression of Parkin, an E3 ubiquitin ligase important in targeting specific mitochondria for mitophagy, indicating increased mitochondrial turnover and possibly mitochondrial dysfunction in astrocytes in MS lesions. Moreover, in chapter 4 we demonstrated increased expression of mitochondrial antioxidants peroxiredoxin3 (Prx3) and thioredoxin2 (Trx2) in hypertrophic astrocytes in inflammatory MS lesions. The enhanced expression of mitochondrial antioxidant enzymes is likely an intrinsic response to ongoing mitochondrial oxidative stress. Taken together, we showed that mitochondria in astrocytes respond to the invasion of leukocytes in the white matter of MS patients by upregulating proteins that protect themselves. It is conceivable that the increased activity and number of mitochondria in astrocytes lead to increased ROS production and thereby contribute to the degenerative processes going in active MS lesions. However, astrocytes are known to be crucial in keeping CNS homeostasis,\textsuperscript{17} and they promote survival of both oligodendrocytes and axons after or during various toxic insults.\textsuperscript{17-21} As such, we demonstrated in chapter 4 that the mere presence of astrocytes in a neuroblastoma culture severely decreased neuronal sensitivity to exogenous ROS. Moreover, astrocytic overexpression of Prx3 and Trx2 further decreased ROS-induced neuronal vulnerability, indicating that mitochondrial changes in astrocytes are perhaps contributing to neuronal/axonal survival in active MS lesions. Further studies are warranted to elucidate the involvement of mitochondrial changes in astrocytes during the early inflammatory stage of the disease.

Notably, mitochondrial alterations in axons in inflammatory MS lesions have received most attention as axonal degeneration is extensive in active demyelinating MS lesions and the principal cause of permanent clinical disability in MS patients.\textsuperscript{8,22,23} At this point, convincing evidence points towards a cardinal role of mitochondrial dysfunction in processes underlying axonal degeneration in CNS inflammation. Primary cause of mitochondrial dysfunction in active MS and EAE lesions are the mitochondrial toxins nitric oxide and ROS,\textsuperscript{10} which are excreted in large amounts by macrophages and activated microglia.\textsuperscript{9} Nitric oxide is mainly produced by inducible nitric oxide synthase and ROS by NADPH oxidases,\textsuperscript{24,25} and both are thought to facilitate phagocytosis of myelin and oligodendrocyte remnants.\textsuperscript{9} During this inflammatory process, ROS and nitric oxide are also thought to impair intra-axonal mitochondrial function as collateral damage. Recently, a very elegant EAE animal study demonstrated that inflammatory cells induce intra-axonal mitochondrial swelling and subsequent focal axonal degeneration.\textsuperscript{26} Importantly, local application of ROS scavengers blocked inflammation-induced mitochondrial
swelling and axonal degeneration, demonstrating that inflammation-derived ROS act as principal instigators of mitochondrial dysfunction and associated axonal degeneration. Mitochondrial targets of inflammation-derived ROS and nitric oxide are believed to be several complexes of the OxPhos chain.\textsuperscript{10,12,13} Previously, it has been shown that nitric oxide can substitute for oxygen as the final electron acceptor at complex IV.\textsuperscript{11,27} Moreover, complex I and III are known to be irreversibly inhibited by peroxynitrite,\textsuperscript{28,29} which is formed by the reaction of nitric oxide with superoxide. Together, this will lead to reduced ATP production, increased ROS production, opening of the mitochondrial permeability transition pore (PTP) and eventually mitochondrial swelling and axonal degeneration. The importance of mitochondrial dysfunction in axonal degeneration is further illustrated by markedly reduced axonal loss in cyclophilin D (CyPD) knock-out mice after EAE induction.\textsuperscript{30} CyPD is a key regulator of PTP opening, indicating that opening of the PTP is a critical step in inflammation-induced axonal degeneration. Moreover, overexpression of the mitochondrial antioxidant superoxide dismutase 2 (SOD2) significantly reduced degeneration of the optic nerve in EAE.\textsuperscript{31} However, all these studies have been performed in the EAE model, which only partially reflects MS pathology.\textsuperscript{32} But, evidence is strong that similar mechanisms occur in MS. For instance, the various stages in focal axonal degeneration in EAE, including mitochondrial swelling, can also be observed in inflammatory MS lesion in human brain biopsies.\textsuperscript{26} Moreover, decreased activity of complex IV in acutely demyelinated axons in fulminant MS has been observed, together with a loss of mitochondrial content.\textsuperscript{33} Fulminant MS is a rare form of MS where initial lesions exhibit hypoxia-like features.\textsuperscript{24} In these hypoxia-like MS lesions, a strong correlation exists between activated microglia/macrophage content and complex IV dysfunction,\textsuperscript{33} again indicating a strong causative role for inflammation-derived mediators in mitochondrial dysfunction in MS. In contrast, in brain tissue from primary and secondary progressive MS patients we found a global increase in complex IV activity in active lesions, as described in chapter 2. Although we did not specifically assess axonal complex IV activity, complex IV histochemical assays showed no signs of reduced complex IV activity in axons in inflammatory MS lesions. Rather, complex IV activity seemed upregulated in these axons, which coincided with increased axonal mitochondrial content. A possible explanation for this discrepancy between our findings and those of Mahad et al could be the difference between active lesions in fulminant MS, which appear more aggressive and hypoxic, and those in more common progressive MS.\textsuperscript{34,35} Although this difference might explain the observed differences, it remains intriguing that in fulminant MS cases inflammation inhibits axonal complex IV and decreases mitochondrial content, whereas in established MS inflammation increases complex IV activity and mitochondrial content. A better and more reasonable explanation lies in the subtle but substantial difference in definition of an active lesion. Active lesions were identified by Mahad et al as being actively demyelinating, as indicated by a preferential loss of specific myelin proteins and abundantly present myelin-positive macrophages,\textsuperscript{35} whereas active lesion classification in chapter 2 was less strict and solely based on abundance of macrophages. Reduced axonal complex IV activity and mitochondrial mass, as observed by Mahad et al, might represent the initial response to extensive inflammation and or demyelination, while our finding of increased complex IV activity and number of mitochondria in axons already reflects repair or a compensatory mechanism to the initial response. This is strengthened by in vivo imaging analysis where an initial drop in mitochondrial metabolism, as visualised with N-acetyl-aspartate (NAA)
magnetic resonance spectroscopy (MRS), in a newly formed lesion was quickly followed by a sustained upregulation of mitochondrial metabolism. Moreover, a larger increase in mitochondrial metabolism was associated with lower clinical impairment, indicating an important role for mitochondrial metabolism in functional repair.36

1.2 Mitochondria in chronic MS lesions

Axonal degeneration is not confined to inflammatory lesions. Post-mortem studies revealed presence of axonal degeneration in non-inflammatory chronic lesions, albeit to a lesser extent than seen in active lesions.8,37 But because chronic lesions, if not remyelinated, last for years or even decades, axonal loss in non-inflammatory MS lesions is substantial and likely surpasses inflammation-mediated axonal generation.38 Processes leading to degeneration of chronically demyelinated axons differ from those in active lesions, as there are no inflammatory mediators to induce axonal degeneration. Instead, the chronic loss of myelin and its trophic support are believed to cause degenerations in these axons.38,39 Demyelination of axons blocks their primary function, the conduction of signals. However, conduction can be restored by upregulation and redistribution of Na⁺ channels along the demyelinated parts of the axon.40,41 As a consequence, depolarization of demyelinated axons is accompanied by a greater influx of Na⁺. To facilitate quick depolarization Na⁺/K⁺ ATPase activity needs to be increased. Hence, demyelinated axons require more ATP for maintenance of axonal function, a state generally referred to as ‘virtual hypoxia’.38,42 To fulfill the increasing ATP demand, mitochondrial mass is increased, as we described in chapter 2. Moreover, we observed upregulation of complex IV activity in chronic MS lesions. Similarly, Mahad et al observed increased mitochondrial content and complex IV activity in a large proportion of chronically demyelinated axons.15 However, they also observed that, in chronic MS lesions, a subset of damaged axons, indicated by the accumulation of amyloid precursor protein (APP) or the presence of non-phosphorylated neurofilament (SMI32), harboured a complex IV defect. In SMI32-positive axons, this coincided with a marked loss of mitochondria. APP-immunopositive axons with complex IV defects contained normal mitochondrial numbers and were predominantly found in the active rim of chronic active lesions, indicating that most APP-positive axons in chronic lesions likely reflect inflammation-mediated axonal damage. SMI32-positive axons, on the other hand, might represent a late stage of degeneration of chronically demyelinated axons. As stated before, demyelinated axons require increased amounts of ATP for proper signal conduction. However, in the long run, mitochondria cannot cope with this amplified ATP demand leading to detrimental processes cumulating in axonal degeneration. First, axons that survived the inflammatory attack might harbour mitochondria that have been slightly damaged by inflammatory mediators. This could increase mitochondrial ROS production, which further damages intra-axonal mitochondria. Eventually, mitochondrial function is impaired to such an extent that it cannot fulfill the increased energy demand. Evidence for such a mechanism is supported by our findings described in chapter 2, where we showed increased mitochondrial oxidative stress in chronically demyelinated axons. Secondly, we and others have provided compelling evidence for severe defects in neuronal mitochondria in the cortex of MS patients as described in chapter 5 and 6.43,44 As neurons provide their axons with these defective mitochondria, intra-axonal mitochondria are probably incapable of producing the necessary energy in these axons. The moment that mitochondrial function is too impaired to provide the required amount
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of ATP to Na⁺/K⁺ ATPase, intra-axonal Na⁺ concentration starts rising. To remove the excess Na⁺, the axolemmal Na⁺/Ca²⁺ exchanger reverses, now pumping Na⁺ out and Ca²⁺ in, leading to steadily rising axoplasmic Ca²⁺ concentrations. Excessive Ca²⁺ will initiate a devastating series of events including dephosphorylation of neurofilaments (SMI32-positive), activation of destructive enzymes and further impairment of mitochondrial function which all culminate in axonal degeneration. The crucial role of mitochondria in the processes leading up to degeneration of chronically demyelinated axons is further strengthened by the relative sparing of large-diameter axons. These axons have a relatively low surface to volume ratio, which allows for more intra-axonal mitochondria per axolemmal surface or per Na⁺/K⁺ ATPase. Therefore, mitochondrial dysfunction can reach higher levels in large-diameter axons before the whole series of devastating events is initiated.

2. Mitochondrial dysfunction in MS grey matter

Although it has long been neglected, damage to grey matter structures occurs early in disease and is extensive in many MS patients. Cortical demyelination can reach up to 75% of the total cortex and even exceeds white matter demyelination in a subset of patients. Post-mortem studies revealed that cortical lesions are different than those in white matter, containing no significant infiltration of leukocytes and an intact blood-brain barrier. As cortical demyelination is most extensive in later stages of MS, mechanisms other than inflammation are thought to mediate demyelination of the cortex. Therefore, cortical lesion classification is based on location rather than on inflammatory status. However, a recent study in biopsies taken from patients with early fulminant MS reported that a large proportion of grey matter lesions in these patients exhibited significant levels of leukocyte infiltration, similar to active white matter lesions. Though this was most pronounced in leukocortical lesions, pure grey matter lesions did also contain some leukocytes, though very much less than white matter lesions. Furthermore, it remains to be determined if this is a specific feature of fulminant MS, or also occurs in the more common forms of early MS. If the latter is true, this indicates that in early stages of MS cortical demyelination might be mediated by infiltrating leukocytes, and cortical demyelination in chronic MS is mediated by other mechanisms. Such mechanisms could be mild but continuous microglial activation in the cortex or toxic molecules derived from meningeal infiltrates lying on top of the cortex may be responsible.

Besides demyelination, neurodegeneration is also common in the cortex of MS patients. Imaging studies revealed that cortical atrophy is detectable early in the disease but with higher rates in progressive MS. Several studies have now established cortical atrophy as the most predicting pathological substrate of clinical disability. A thorough histopathological study revealed that cortical thinning is the result of glial cell loss, neuronal loss and reduced synaptic density, which were most prevalent in cortical lesions. Specific neuronal subsets in cortical lesions appear more vulnerable, including pyramidal cells in layer 3 and 5 of the cortex. However, neuronal injury and loss can also be extensive in normal-appearing grey matter (NAGM). One study showed substantial loss of interneuronal processes in normally myelinated motor cortex. Whereas another study found a predominant loss of parvalbumin positive, but not calretinin positive interneurons in layer 2 of NAGM. Thus, neurodegeneration is at least partly independent of local demyelination. Processes proposed to contribute to this type of neurodegeneration
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are (distant) axonal changes, local meningeal inflammation\textsuperscript{58,64} the presence of (chronic) microglial activation\textsuperscript{55,53,57} and mitochondrial dysfunction.\textsuperscript{66} In \textbf{chapter 5} and 6, we described reduced expression of genes and proteins, essential in maintaining proper mitochondrial function, in normally myelinated cortex of MS patients. Furthermore, we provide strong evidence that PPARY coactivator-1α (PGC-1α) provides a molecular basis for not only the mitochondrial changes we observed in chapter 5 and 6, but also for most of the previously described mitochondrial changes in MS cortex.\textsuperscript{43,44,67} In 2006, a microarray analysis of non-demyelinated motor cortex revealed decreased expression of 26 nuclear-encoded transcripts of subunits of the OxPhos chain.\textsuperscript{44} These reduced transcripts encoded subunits of all 5 OxPhos complexes with the exception of complex II, and were associated with a significant reduction in OxPhos protein expression per mitochondrion and complex I and III activity. \textit{In situ} hybridization revealed a neuronal specific decrease in OxPhos transcripts, a strong indication that mitochondrial dysfunction in MS cortex predominantly occurs in neurons. Later, another study described a profound increase in the number of complex IV negative neurons, showing both decreased expression of a complex IV catalytic subunit and reduced activity of complex IV.\textsuperscript{43} These so-called respiratory-deficient neurons were present in both NAGM and cortical lesions and harboured many clonally expanded mtDNA deletions. Interestingly, neurons in NAGM have higher mtDNA copy numbers, possibly as a reaction to mtDNA damage or reduced OxPhos activity.\textsuperscript{68} The damage to mtDNA in neurons in MS might partly explain the reduced activities of complex I, III and IV, as mtDNA encodes for critical subunits of all three OxPhos complexes. However, it does not explain the reduction of many nuclear-encoded OxPhos subunits in MS cortex as found by Dutta \textit{et al.}\textsuperscript{44} In a follow up to this seminal paper, no change in the expression of nuclear respiratory factor 2 (NRF2), a transcription factor for many nuclear-encoded OxPhos subunits,\textsuperscript{69,70} was observed in MS motor cortex compared to control cortex. However, the ability of NRF-2 to bind NRF-2 transcription factor binding site was decreased in MS cortex as compared to controls.\textsuperscript{44} As binding to DNA is essential for NRF2 activity, they proposed impaired binding capacity of NRF-2 as a cause for reduced OxPhos subunit expression. However, NRF2 is not the only transcription factor involved in expression of nuclear-encoded OxPhos genes,\textsuperscript{69,70} and therefore we set out to analyze expression of all known regulators of nuclear-encoded OxPhos subunit expression in MS cortex, as described in \textbf{chapter 5}. We clearly showed that PGC-1α, a transcriptional cofactor that binds and activates key mitochondrial transcription factors\textsuperscript{69-71}, is reduced in cortex of MS patients, whereas no changes were observed in the expression of well-known mitochondrial transcription factors.\textsuperscript{69-71} PGC-1α silencing in neuroblastoma cells led to reduced expression of nuclear-encoded OxPhos subunits. Among the target transcription factors of PGC-1α are all transcription factors involved in OxPhos subunit transcription, including NRF2. Therefore, reduced PGC-1α also provides an explanation for impaired NRF2 transcription activity in MS cortex, as described previously.\textsuperscript{57} PGC-1α is often regarded as the main regulator of genes involved in oxidative metabolism and mitochondrial function.\textsuperscript{71} Among these genes, are genes encoding proteins important in mitochondrial protection against ROS.\textsuperscript{72} In \textbf{chapter 5}, we revealed that several key mitochondrial antioxidants, which detoxify various ROS in mitochondria, and uncoupling proteins (UCP), which lower mitochondrial ROS production, are reduced in MS cortex. Thus, the decrease in PGC-1α expression in MS cortex does not only reduce OxPhos subunit expression, but in addition increases mitochondrial ROS production and vulnerability to oxidative
damage. In chapter 5, we showed in vitro that reduced neuronal expression of PGC-1α increases both ROS-mediated cell death and neuronal ROS production. Thus, reduced neuronal PGC-1α is likely to contribute to the extensive and continuous oxidative stress in MS cortex. However, the main contributor to oxidative stress in MS cortex is likely the low grade but continuous microglial activation. Microglia upon activation produce nitric oxide and ROS and thereby contribute to oxidative damage in neurons and subsequent neuronal loss. We postulate that reduced neuronal PGC-1α, which plays an important role in the mitochondrial redox balance, contributes to oxidative mtDNA damage and augment neuronal loss in MS cortex. In chapter 6 we have shown decreased expression of fission and fusion genes. Mitochondrial fission and fusion are essential mechanisms to maintain a healthy mitochondrial population. Decreased fission and/or fusion results in mtDNA mutations, augmented mitochondrial ROS production and decreased ATP production, and thus further contributes to neurodegeneration. Taken together, we propose a mechanism that in chronic MS continuous microglial activation and reduced neuronal PGC-1α expression in the cortex synergistically lead to dysfunctional mitochondria and subsequent neuronal injury.

Interestingly, reduced neuronal PGC-1α expression and chronic microglial activation occur in most other chronic neurodegenerative disorders. Loss of PGC-1α has been described in striatal neurons in Huntington’s disease (HD), in dopaminergic neurons in Parkinson’s disease (PD) and in cortical and hippocampal neurons in Alzheimer’s disease (AD). Not surprisingly, in these three diseases mitochondrial dysfunction and oxidative stress play important roles in the neurodegenerative process. Genetic overexpression of PGC-1α has proven to be neuroprotective in animal models of HD and PD and increase lifespan. Microglial activation is also widespread in striatum, substantia nigra and hippocampus in HD, PD and AD, respectively. Microglial activation is an early and persistent phenomenon in these neurodegenerative diseases and is probably a response to aberrant protein aggregation. Like in MS, microglial activation is suspected to severely contribute to local oxidative and nitrosative stress and thereby inducing mitochondrial dysfunction and neurodegeneration. At this point, there is no evidence linking microglial activation to reduced expression of PGC-1α. Rather, current evidence suggests that microglial activation is more likely to upregulate PGC-1α expression, as exogenous ROS and nitric oxide are known inducers of PGC-1α. However, these

Figure 1 (next page). Mitochondrial dysfunction mediates neurodegeneration in MS via three distinct mechanisms. (A) In inflammatory MS lesions, macrophages and activated microglia produce vast amounts of reactive oxygen species (ROS) and nitric oxide (NO), which subsequently induce mitochondrial dysfunction in both myelinated and demyelinated axons. In tissue, this axonal mitochondrial dysfunction is reflected by swelling of intra-axonal mitochondria in intact axons. A substantial proportion of axons that harbour this mitochondrial defect will eventually degenerate. (B) Axons in chronic MS lesions have increased and redistributed their axonemal Na+ channels, leading to increased intra-axonal Na+ concentrations. As a result, more ATP is required for the removal of excess Na+ by Na+/K+ ATPase. To produce all this ATP, mitochondrial content is increased in chronically demyelinated axons. However, due to transcriptional changes in the cortex, mitochondria transported from the neuronal cell body into the chronically demyelinated axons are not fully functional. Over time, mitochondrial dysfunction accumulates to such an extent that ATP production becomes too low for Na+/K+ ATPase to remove all the excess intra-axonal Na+. The rising Na+ concentrations inside axons lead to reversal of the axoemal Na+/Ca2+ exchanger, which now pumps Na+ out and Ca2+ in. In turn, this leads to rising axoemal Ca2+ concentrations, which, when above a certain threshold, sets of several deleterious events, including further destabilization of intra-axonal mitochondria, which then contribute to axonal degeneration. One is the production of ROS and NO by the continuously activated microglia in MS cortex. This will lead to inhibition of several oxidative phosphorylation (OxPhos) complexes and damage to mitochondrial DNA in neuronal mitochondria. The second is the decreased expression of neuronal PGC-1α, a transcriptional co-activator, leading to reduced transcription of OxPhos subunits, mitochondrial antioxidants, uncoupling proteins and fission and fusion proteins. As
a consequence, neuronal mitochondria are less capable of producing ATP and more vulnerable to oxidative stress. We propose that the chronic, but low-grade microglial activation present in MS cortex eventually leads to reduced neuronal PGC-1α expression. Eventually, mitochondrial dysfunction in neurons in MS cortex will contribute to neuronal loss, as neurons heavily depend on oxidative metabolism.
studies focussed on PGC-1α expression after a relatively short incubation with ROS or NO. We speculate that chronic oxidative and nitrosative stress caused by microglial activation, which can last for decades, has an opposite effect on PGC-1α expression. On the other hand, reduced neuronal PGC-1α can augment microglial activation by increasing neuronal ROS production and subsequent oxidative damage to which microglia react. In HD, huntingtin aggregates are now thought to directly inhibit PGC-1α gene transcription, whereas in familial PD caused by parkin mutations, the parkin interacting substrate PARIS represses PGC-1α transcription. However, such clearly pathogenetic causes for reduced PGC-1α expression are unlikely to occur in MS. We therefore propose a mechanism whereby a mild long-lasting inflammatory milieu induces inhibition of PGC-1α transcription in cortical neurons.

3. Conclusions and future perspectives

The studies described in this thesis have made significant contributions to our understanding of the role of mitochondria in MS. Over the last four years, during which the studies described in this thesis were executed, the MS research community has shown an ever increasing interest in mitochondria, and, nowadays, the importance of mitochondrial dysfunction is widely acknowledged. We here argue that mitochondrial dysfunction and concomitant oxidative stress mediate neurodegeneration in MS via three distinct but interrelated mechanisms. 1) In acute, inflammatory MS lesions intra-axonal mitochondria are damaged by inflammation-derived ROS and/or nitric oxide, leading to severe mitochondrial dysfunction and subsequent axonal injury. 2) In chronically demyelinated axons, energy demand is increased several fold compared to normally myelinated axons. At a certain point, intra-axonal mitochondria cannot meet the energy required due to accumulating mitochondrial dysfunction setting of a cascade of deleterious events leading to axonal degeneration and loss. 3) Reduced expression of PGC-1α in cortical neuronal contributes to mitochondrial dysfunction, enhanced ROS production and impaired turnover of dysfunctional mitochondria. Moreover, loss of PGC-1α increases neuronal mitochondrial vulnerability to nitric oxide and ROS produced by activated microglia. As neurons are highly dependant on mitochondrial ATP, microglial activation and loss of neuronal PGC-1α will synergistically add to the neurodegenerative process in MS cortex. Thus far, the cause for reduced neuronal PGC-1α in MS cortex is an enigma, but we propose that chronic, but mild, inflammation in MS cortex might lead to diminished transcription of PGC-1α.

Although we and others have now provided evidence for an important role of mitochondria in different stages of MS, much remains to be uncovered. First, a thorough study of mitochondrial function in NAWM and preactive lesions is warranted to elucidate if, in analogy to EAE, mitochondrial alterations precede the influx of leukocytes. Moreover, levels of microglial inflammation in NAWM and NAGM can differ greatly between and in patients. A study looking at the relation between of the extent of microglial activation and mitochondrial (dys)function and/or PGC-1α levels in NAWM and NAGM might shed light on the here proposed causative role of activated microglia in mediating mitochondrial injury. Then there is still much to learn about what effect the changes observed in the cortex in MS patients have on mitochondria in axons in white matter. We and others have described an increased number of mitochondria in chronically demyelinated axons, whereas reduced PGC-1α and downstream mitochondrial fission and fusion are likely
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to decrease axonal mitochondrial mass. A thorough study on rates of fission, fusion and 
transport of mitochondria in the neuronal cell body and axons in MS brains might provide 
an explanation for this apparent discrepancy.

Traditionally, MS researchers make extensive use of different types of EAE, however the 
main drawback of EAE models is that they fail to mimic the progressive phase of MS.\textsuperscript{104,105} 
Nonetheless, EAE models are well-suited to study mitochondria metabolism during the 
acute, inflammatory phase of the disease.\textsuperscript{5,26} Moreover, EAE is an appropriate model to 
elucidate the role of astrocytes in inflammatory MS lesions. Do they protect axons by 
upregulation of mitochondrial antioxidant enzymes as we discussed in \textcolor{blue}{chapter 4}? Or do 
they contribute to the oxidative stress with their increased mitochondrial number and 
activity, as observed in \textcolor{blue}{chapter 2}? 

For studying mitochondria in the context of chronic demyelination or low grade 
inflammation we have to resort to other animal models, such as the cuprizone model. In this 
model, chronic demyelination is induced by feeding animals with a cuprizone-enriched 
diet.\textsuperscript{106} Analysis of the cortex of these animals might give us more clues on what distant 
demyelination of an axon does to their neuronal cell body in the cortex. Do transcriptional 
changes similar to MS occur in cuprizone cortex? Is mitochondrial functioning in the 
neuronal cell body altered due to such a distant adverse process? Although the cuprizone 
model might provide us with answers to these intriguing questions, researchers need 
to firmly realize that cuprizone-induced demyelination is completely different than 
demyelination in MS. Thus, a cuprizone-fed animal as a model for MS has its own set of 
limitations.

The ultimate proof of the importance of mitochondrial dysfunction in MS pathogenesis 
lies in the success of therapeutics aimed at alleviating mitochondrial dysfunction. But 
before we can test such therapeutics in MS patients there is a long way to go. The first 
step should be inducing an MS-like disease in animals that have genetically modified 
mitochondrial function. Previously, it has been shown that EAE in animals that have a 
defect in their mitochondrial PTP leads to less axonal degeneration.\textsuperscript{30} In line with \textcolor{blue}{chapter 5} 
and \textcolor{blue}{6}, it would be very interesting to assess neuronal and axonal degeneration in 
animals with altered (neuronal) PGC-1α expression after EAE induction or cuprizone 
treatment. The second step involves testing compounds that improve mitochondrial 
function in MS animal models. Again, PGC-1α is an interesting therapeutic candidate, 
as boosting its expression and/or activity should have such a broad range of beneficial 
effects on mitochondrial function and protection. Thus far, several compounds have 
been shown to enhance PGC-1α activity and thereby increase neuronal survival in animal 
models for various neurodegenerative disorders.\textsuperscript{107-109} However, these compounds, 
including resveratrol and thialozidines, have many other targets and therefore the specific 
effect of increased PGC-1α activity is hard to establish. Oxidative or nitrosative damage 
to mitochondria significantly contributes to mitochondrial dysfunction in MS. Hence, 
administration of mitochondrial targeted antioxidants could be an attractive therapeutic 
strategy.\textsuperscript{110,111} Moreover, therapies aimed at boosting total antioxidant protection in brains 
of MS patients might prove to be beneficial. Interestingly, BG-12, a MS therapeutic now 
in phase III trial, targets Nrf2 and is therefore believed to increase antioxidant capacity 
and improve clinical outcome.\textsuperscript{112,113} Taken together, we strongly believe that the several 
potential therapeutic strategies aimed at improving mitochondrial function in MS are 
worthwhile to pursue but warrant additional research.
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Reference List


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