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1. Multiple sclerosis

1.1 Clinical features and diagnosis
Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS), affecting over one million people worldwide. It is the most prevalent neurological disorder in young adults with an incidence of one per 1000 in Europe and Northern America with its onset generally between 20 and 40 years of age. The disease affects women more frequently than men, in a ratio of about 2:1. In MS, infiltrated immune cells in the CNS cause damage to myelin sheaths surrounding the axons, which results in impaired axonal conductance and neuronal dysfunction.

Clinical symptoms of MS vary depending on lesion number, size and location and include motor deficits, like muscle weakness, tremor, spasms, and paralysis, and progressive sensory deficits, such as impaired vision due to optic neuritis. Depending on clinical features four main subtypes of MS can be distinguished. The most common form (~70%) is relapsing-remitting MS (RR-MS), which is characterized by episodes of clinical illness, followed by periods of clinical improvement. In time, the majority of RR-MS patients develop secondary progressive MS (SP-MS), with progressive neurological deterioration. Approximately 15-20% of MS patients suffer from progressive disease without relapses from the onset of symptoms, termed primary progressive MS (PP-MS). Less than 5% of the MS patients experience progressive relapsing-MS (PR-MS), which is characterized by a continuous progressive disease course from the onset with occasional relapses.

The diagnosis of MS is primarily based on clinical history and neurological examination and is established after two independent episodes of clinical symptoms, separate in time and affected brain region. Magnetic resonance imaging (MRI) of the brain and spinal cord is used to confirm the diagnosis, to determine the number and size of MS lesions, and to distinguish between active inflammation and blood-brain barrier (BBB) leakage. Furthermore, abnormalities in the cerebrospinal fluid (CSF) occur in about 70% of the MS patients at some time during the disease course. These CSF abnormalities include the presence of elevated IgG levels and the identification of two or more unique oligoclonal bands.

1.2 Etiology
To date, the cause of MS is unknown, but it is commonly believed that MS is an autoimmune disorder that is etiologically heterogeneous and has a multifactorial background. Both environmental and genetic factors may contribute to disease susceptibility as well as disease outcome. Sibling and twin studies have demonstrated that the incidence of MS is higher in monozygotic twins (25-30%) than in dizygotic twins (2-5%). Furthermore, first, second and third-degree relatives of MS patients are more likely to develop the disease than non-relatives, and the recurrence risk correlates with relatedness, suggesting that there is a genetic background to MS susceptibility. A high number of genetic polymorphisms, mostly related to the immune system, have been linked to susceptibility to MS, but up until now no single susceptibility gene has been...
identified. Thus far, the strongest association was found with the major histocompatibility complex (MHC) class II allele human leukocyte antigen (HLA)-DR2\textsuperscript{17,18}. Associations with genes encoding for T-cell-receptor α\textsuperscript{19}, intercellular adhesion molecule-1 (ICAM-1)\textsuperscript{20,21}, and cytotoxic T-lymphocyte-associated protein 4 (CTLA4)\textsuperscript{22-24} have also been described. Polymorphisms in the gene family of glutathione-S-transferase, which is involved in redox regulation and free radical scavenging, have been associated with a more severe disease course\textsuperscript{25}. Likely, a combination of genes influences susceptibility and clinical outcome of MS.

Environmental factors also contribute to the risk of developing MS\textsuperscript{26}. MS incidence is dependent on geographical distance from the equator and is most prevalent in Northern Europe and North America\textsuperscript{27}. This may be related to exposure to sunlight (vitamin D), diet, or viral infections\textsuperscript{28,29}. Exposure to viral antigens that have structural similarities with myelin antigens may be a priming event for the development of cross-reactive T cells or antibodies (molecular mimicry) and may lead to the development of MS\textsuperscript{30,31}. Viruses that have been associated with MS pathology include human herpes virus 6\textsuperscript{32-35}, measles virus\textsuperscript{36,37}, chlamydia pneumoniae\textsuperscript{38,39}, and Eppstein Barr virus\textsuperscript{40-42}. However, thus far evidence for direct association between one single virus and MS is lacking.

1.3 Pathology

Neuropathologically, MS is characterized by the presence of multiple focal lesions, scattered throughout the CNS. Preferential locations of these lesions include periventricular areas, optic nerves, brain stem, and spinal cord. Pathological features of MS plaques are destruction of myelin sheaths, oligodendrocyte death, axonal damage, glial scar formation, and the presence of inflammatory infiltrates that mainly consist of lymphocytes and macrophages. In particular monocyte-derived macrophages are thought to play a central role in demyelination and axonal damage, two characteristic hallmarks of MS pathology\textsuperscript{43}. Macrophages produce a variety of inflammatory mediators like reactive oxygen species (ROS), nitric oxide, and proinflammatory cytokines, which all contribute to neuroinflammation and disease progression\textsuperscript{44-46}.

MS lesions can be classified as (p)reactive, active, chronic active, and chronic inactive lesions. This classification is based on the degree of myelin loss, the presence of inflammatory cells, and HLA-DR expression on leukocytes and resident microglial cells\textsuperscript{47,48}. Preactive lesions are marked by clusters of activated microglial cells that have increased HLA-DR expression. Occasionally, perivascular leukocyte infiltrates are present, but there is no apparent loss of myelin. Active demyelinating lesions are characterized by a demyelinated area with perivascular and parenchymal macrophages. In addition, CD4-positive and CD8-positive T cells and some B cells are present within the perivascular space of blood vessels. GFAP positive reactive astrocytes with long processes are evenly distributed throughout the demyelinated areas. In these lesions macrophages and microglial cells appear as foamy macrophages containing myelin degradation products, including myelin proteins and lipids. Chronic active MS lesions are characterized by a hypocellular demyelinated center and a hypercellular rim with high numbers of parenchymal foamy macrophages. Reactive astrocytes are predominantly
localized at the edge of the lesion center and within the hypercellular rim. Chronic inactive lesions are hypocellular and demyelinated lesions containing widened extracellular spaces occupied by gliotic scar tissue. Relatively small numbers of macrophages and lymphocytes are present in the brain parenchyma and perivascular spaces. Within demyelinated MS lesions, Lucchinetti and colleagues identified four different patterns of demyelination, based on myelin protein loss, geography and extension of plaques, the patterns of oligodendrocyte destruction, and complement activation. In some MS patients, remyelination of demyelinated areas occurs. These remyelinated areas can be restricted to the lesion edge, but they can also extend through the entire lesion and are then called shadow-plaques. Demyelinated MS lesions are found in both white and grey matter of the CNS. However, in grey matter lesions leukocyte infiltration and inflammation are uncommon.

It is assumed that in MS sequential degradation of myelin proteins takes place. This is reflected by the presence of different myelin proteins in foamy macrophages in time and defines different stages in demyelination. In early active MS lesions, foamy macrophages are filled with various myelin degradation products, including myelin oligodendrocyte glycoprotein (MOG) and cyclic nucleotide phosphodiesterase, whereas in later stages only myelin basic protein (MBP) and proteolipid protein (PLP) can be detected. This sequential degradation of myelin in macrophages was confirmed in vitro.

It is now evident that axonal damage is also an important hallmark of MS pathology. Axonal damage is found in and around inflammatory MS lesions and can occur independently of demyelination. Axonal damage has been suggested to determine the neurological deterioration of progressive MS.

1.4 Treatment

MS has not been curable thus far and current therapies consist of lifelong disease and symptom management. Most current therapies of MS are based on the hypothesis that MS is an autoimmune disease and are anti-inflammatory, immunosuppressive or immunomodulatory. The most widely used drugs during a relapse are corticosteroids, like prednisone or methylprednisolone. Corticosteroids reduce the duration of a relapse and accelerate recovery. However, it is unknown whether corticosteroids are effective in reducing long-term course of MS. The exact mechanisms by which corticosteroids exert their effects are unknown, but corticosteroids are probably both immune suppressive and anti-inflammatory. Corticosteroids reduce the expression of adhesion molecules on both endothelial cells and monocytes. In addition, corticosteroids decrease the production of proinflammatory cytokines and matrix metalloproteinases, thereby preventing leukocyte diapedesis through brain endothelium and increasing BBB integrity.

In the long-term, RR-MS patients are often treated with interferon beta (IFN-β) or glatiramer acetate. Two forms of IFN-β, IFN-β1a and IFN-β1b, are currently used in the clinic. In clinical trials, IFN-β treatment has been shown to reduce both exacerbation frequency and severity. Possible mechanisms of action of IFN-β include causing a
shift in the cytokine profile towards an anti-inflammatory phenotype, increasing BBB integrity, reducing T cell migration\textsuperscript{80}, and preventing monocyte migration across the BBB\textsuperscript{81}. Glatiramer acetate (GA) (Copaxone\textsuperscript{©}) is a mixture of random synthetic polypeptides that is cross-reactive with MBP. GA reduces the relapse rate of MS patients and improves neurological disability\textsuperscript{82}. MRI showed that GA treatment leads to a reduction of active lesions\textsuperscript{83;84}. Possibly, GA acts via tolerance induction of MBP-specific T cells and via the induction of GA-reactive TH2 regulatory cells\textsuperscript{85}.

Other disease-modifying treatments for MS are intravenous immunoglobulin, azathioprine and methotrexate\textsuperscript{86}. In addition, new therapies are being developed, aiming to improve efficacy of MS treatment with less adverse effects\textsuperscript{97}. One of these new drugs that seems very promising is natalizumab (Tysabri\textsuperscript{©}). Natalizumab is a recombinant humanized monoclonal antibody directed against the α4 chain of α\textsubscript{4}β\textsubscript{1} integrin (very late antigen 4, VLA-4) that is expressed on activated lymphocytes and monocytes and is involved in transendothelial migration\textsuperscript{81;88}. In a study where natalizumab was administered to RR or SP-MS patients every 28 days for six months, the treatment significantly reduced the number of new MRI lesions and clinical relapses\textsuperscript{89}, possibly by blocking leukocyte migration into the CNS. Another clinical trial revealed that administration of a single dose of natalizumab after the onset of a relapse did not accelerate clinical recovery, but decreased the volume of MRI lesions\textsuperscript{90}. In addition, a two-year phase 3 trial in patients with RR-MS showed reduced progression of disability and rate of clinical relapse\textsuperscript{91}. However, clinical trials were ended instantly, due to three cases of progressive multifocal leukoencephalopathy (PML) caused by the reactivation of the polyomavirus JC\textsuperscript{92-94}. Recently, the use of natalizumab was reconsidered by the regulatory authorities and is now allowed in clinical trials under very strict conditions. Another monoclonal antibody used to treat MS patients is alemtuzumab. Alemtuzumab is humanized monoclonal antibody directed against the leukocyte antigen CD52. In SP-MS patients, alemtuzumab reduced cerebral inflammation and the number of relapses. However, again there were serious side effects, since about 30% of the patients developed Graves’ disease\textsuperscript{95}.

Statins are promising candidates for future treatment of MS. Statins are 3-hydroxy-3-methylglutaryl Coenzyme A reductase inhibitors that reduce cholesterol synthesis and recently their anti-inflammatory effect was revealed. Statins are effective in reducing the severity of experimental encephalomyelitis (EAE), the animal model of MS\textsuperscript{96-99}. In vitro, statins increase BBB integrity and reduce transendothelial migration of leukocytes\textsuperscript{99;100}. The first clinical trials using simvastatin or lovastatin in small groups of MS patients were promising; patients treated with simvastatin or lovastatin had a significant decrease in the number and volume of new MRI lesion\textsuperscript{101;102}. Fingolimod (FTY720) is another immunomodulatory agent that is currently under investigation for the treatment of MS. Fingolimod is a sphingosine-1-phosphate receptor agonist that sequesters circulating lymphocytes in peripheral lymph nodes, thereby strongly reducing peripheral lymphocyte counts\textsuperscript{103-106}. Fingolimod treatment significantly reduced the severity of EAE\textsuperscript{103;107;108}. The first clinical trial with fingolimod in MS patients revealed a decrease in the number of MRI lesions and reduced clinical disease activity\textsuperscript{109}. Since
MS patients experience less relapses during pregnancy, estrogens are also under evaluation as new therapy for MS\textsuperscript{110}. The first clinical trials using oestriol revealed a reduced number and volume of gadolinium-enhanced lesions, which was accompanied by a decreased production of proinflammatory cytokines\textsuperscript{111;112}. Since there is evidence that axonal damage plays a role in progression of MS, new drugs are developed to prevent neuronal damage and improve neuronal conduction, such as neurotrophic factors\textsuperscript{113;114} cannabinoids\textsuperscript{115;116} or 4-aminopyridine\textsuperscript{117;118}. These compounds may also act as anti-inflammatory agents. Nerve growth factor, for instance, limits the transendothelial migration of monocytes across the BBB\textsuperscript{119}. Further study is needed to investigate efficacy and safety of these new therapies.

1.5 Animal model: experimental allergic encephalomyelitis

EAE is a widely used animal model to study underlying mechanisms of MS pathology. EAE is an experimentally induced neuroinflammatory and demyelinating disease. In most models EAE is induced in rodents or non-human primates by active immunization with whole myelin or myelin components (e.g. MOG, MBP, PLP, or peptides derived from these proteins) together with a strong adjuvant, such as complete Freund’s adjuvant (CFA)\textsuperscript{120}. In addition, CNS inflammation can also be induced by viruses, such as Theiler's murine encephalomyelitis virus (TMEV)\textsuperscript{121;122}. Depending on the immunization protocol, EAE can be either monophasic (acute EAE) or biphasic (chronic EAE).

Acute EAE is usually induced in the Lewis rat via injection of MBP together with CFA in the footpad resulting in ascending clinical signs\textsuperscript{123}. The disease is characterized by a highly reproducible monophasic and transient disease course with a mean peak of disease at day 14 after immunization and spontaneous recovery after 17 days. First signs of disease are loss of tail tonus progressing to hind-limb paralysis. This may proceed into paralysis of the front limbs and even in death due to EAE, although this is rare. After recovery the animals are resistant to reinduction of the disease, due to the development of suppressor cells\textsuperscript{124;125}. Acute EAE is characterized by the presence of high numbers of infiltrated T cells and monocyte-derived macrophages in the CNS, whereas demyelination and axonal damage are not apparent. These features make acute EAE a suitable \textit{in vivo} model to study cellular infiltration into the CNS and BBB damage and to evaluate immunotherapeutic strategies. Alternative methods to provoke acute EAE include the injection of activated CD4\textsuperscript{+} T cells isolated from immunized EAE animals into naïve recipients. This will induce so-called adoptive transfer EAE and clinical signs and disease course of this model are similar to that of actively induced acute EAE\textsuperscript{123;126}. In addition, EAE can be induced using encephalitogenic T cell lines\textsuperscript{127}.

To study demyelination and axonal damage chronic EAE models are more appropriate. Chronic EAE can be induced in mice strains like C57BL/6 or SJL, rat strains like Dark Agouti, and certain non-human primates\textsuperscript{128-132}. Initially, the course of chronic EAE is similar to that of acute EAE. However, after recovery from the acute phase, animals will experience a relapse with increased severity of clinical signs.

Although EAE is not similar to MS, the model has been proven to be very useful to study underlying mechanisms of MS pathology and to investigate possible therapies.
2. Cellular infiltration into the CNS

Active MS lesions are characterized by the presence of high numbers of inflammatory cells, mainly T cells and monocyte-derived macrophages, in the CNS. It is unknown what factor initiates the infiltration of immune cells into the CNS. Based on EAE studies, it is hypothesized that myelin-specific CD4\(^+\) T cells are primed in secondary lymphoid organs. During immune surveillance of the CNS these myelin-specific T cells encounter myelin antigens presented by antigen presenting cells, leading to T cell activation\(^{133}\). T cell activation initiates local secretion of chemokines, including MCP-1 (Monocyte Chemotactic Protein, CCL2), RANTES (Regulation upon Activation, Normal T-lymphocyte Expressed and Secreted, CCL5), MIP1\(\alpha\) (Macrophage Inflammatory Protein \(\alpha\), CCL3) or MIP1\(\beta\) (CCL4)\(^{134-137}\). Chemokines activate brain endothelial cells (ECs) and attract other lymphocytes and monocytes, which migrate across the BBB and accumulate in the CNS, where they cause damage to myelin and axons. Recently, it was suggested that oligodendrocyte apoptosis may be a primary event in MS lesion formation, rather than T cell activation\(^{138}\). Apoptotic oligodendrocytes may initiate an inflammatory response, leading to the formation of new MS lesions. Whether the initial event is primary T cell activation or oligodendrocyte apoptosis, the infiltration of monocytes into the CNS is considered a critical step in the formation and persistence of MS lesions. This is supported by studies in EAE, where depletion of blood monocytes suppressed the development of clinical signs\(^{139,140}\).

2.1 Blood-brain barrier

To enter the CNS and exert their damaging effects, monocytes have to cross the BBB. The BBB forms an anatomical and physiological barrier between the CNS and the systemic circulation and impedes the entrance of circulating molecules and immune cells into the CNS. The BBB is essential for maintenance and regulation of the neuroparenchymal environment and optimal neuronal functioning. Neuroinflammatory diseases like MS\(^{141-143}\), HIV-associated dementia\(^{144}\) and encephalitis\(^{145,146}\), stroke and brain trauma\(^{147}\) are associated with BBB disruption and infiltration of inflammatory cells into the CNS.

2.1.1 BBB morphology

The BBB is primarily formed by specialized brain endothelial cells (ECs) lining the microvascular system that are connected via tight junctions (TJ) and surrounded by basement membranes (Figure 1)\(^{148}\). Brain endothelium differs in numerous ways from peripheral endothelia. Firstly, brain ECs have more complex organized TJs then ECs derived from peripheral organs. The TJs form an impermeable seal between brain ECs and limit paracellular diffusion of solutes, even of ions such as Na\(^+\) and Cl\(^-\). This results in an extremely high transendothelial electrical resistance (TEER) of 1000-1500 \(\Omega\).cm\(^2\), compared to 2-20 \(\Omega\).cm\(^2\) in peripheral capillaries\(^{149}\). In addition, brain ECs lack transendothelial fenestrae that are present in peripheral endothelium. Furthermore, brain ECs contain high numbers of mitochondria in the cytosol that provide energy for specific
transport systems and enzymes that contribute to maintaining the barrier function of the BBB. Brain ECs express specialized enzymes to export or degrade potentially harmful compounds. Multidrug transporters, such as P-glycoprotein, multidrug-resistance protein 1 (MRP1) and breast cancer resistance protein (BCRP), actively export cytotoxic products and drugs\textsuperscript{150-152}. Enzymes, including monoamine oxidase A and B, catechol O-methyltransferases, pseudocholinesterase, angiotensine converting enzyme, DOPA decarboxylase, γ-glutamyl transpeptidase, and alkaline phosphatase contribute to the barrier function by the degradation of compounds that have entered the brain ECs and neurotransmitters released in the CNS\textsuperscript{153-157}. In addition, passive fluid uptake in brain ECs is limited due to extremely low pinocytotic activity\textsuperscript{158;159}. To maintain brain homeostasis, brain ECs express specific transport systems that regulate the uptake of small hydrophilic molecules like nutrients and metabolites\textsuperscript{160}. Larger molecules are taken up via specific receptor-mediated transcytosis\textsuperscript{161}

Astrocytes are important for the induction and maintenance of BBB properties and contribute to the BBB by projecting their endfeet tightly to the basement membrane\textsuperscript{162}. This close interaction is essential for the formation of BBB phenotype of ECs, as was shown in grafting experiments\textsuperscript{163-165}. The perivascular endfeet of astrocytes show several characteristic features, including the presence of a high density of orthogonal arrays of particles (OAPs), which contain the Kir4.1 K\(^+\) channel and the water channel aquaporin 4. These channels are involved in water homeostasis of the brain\textsuperscript{166}. In vitro studies revealed that astrocytes are involved in the induction of many features of the BBB, including the formation of TJs\textsuperscript{167;168}, the expression and polarized localization of transporter proteins, such as P-glycoprotein and GLUT1\textsuperscript{150;169}, and enzymes involved in the metabolic barrier function of the BBB\textsuperscript{170-173}. Astrocytes secrete a range of factors that are involved in the induction of the BBB phenotype, including transforming growth factor-β, glial-derived neurotrophic factor, basic fibroblast growth factor, and angiopoietin 1\textsuperscript{170;174-177}. Conversely, endothelial cells influence growth and differentiation of astrocytes\textsuperscript{178;179}. In addition, coculture of astrocytes with brain endothelium enhances the expression of antioxidant enzymes in both endothelial cells and astrocytes\textsuperscript{180}. Thus, communication between the two cell types is required for BBB function.

Other cells located at the BBB and involved in BBB function are pericytes and perivascular macrophages. Pericytes are contractile smooth muscle-like cells located within the vascular basal lamina, which play a regulatory role in BBB differentiation and TJ junction formation\textsuperscript{181}. Furthermore, pericytes contribute to the vasodynamic capacity and structural stability of brain microvessels\textsuperscript{182}. Perivascular macrophages are a subset of CNS macrophages that lie between the endothelial and glial basement membranes of cerebral blood vessels\textsuperscript{183}. Perivascular macrophages occupy a strategic location at the BBB to encounter pathogens and are involved in scavenging of pathogens and macromolecules\textsuperscript{184-187}. Perivascular macrophages express both antigen presenting and costimulatory molecules and are thought to play a role in antigen presentation\textsuperscript{188;189}. Recently, both pericytes and perivascular macrophages have been shown to contribute to BBB integrity\textsuperscript{190-194}.  

\begin{table}
\centering
\begin{tabular}{|c|c|c|}
\hline
Gene & Function & Expression Level \\
\hline
P-glycoprotein & Export of cytotoxic compounds & High \\
MRP1 & Export of cytotoxic compounds & Moderate \\
BCRP & Export of cytotoxic compounds & Low \\
Monoamine oxidase & Degradation of compounds & Moderate \\
Pseudocholinesterase & Degradation of compounds & High \\
Angiotensine converting enzyme & Degradation of compounds & Moderate \\
DOPA decarboxylase & Degradation of compounds & Low \\
γ-glutamyl transpeptidase & Degradation of compounds & High \\
Alkaline phosphatase & Degradation of compounds & Moderate \\
Kir4.1 & Water channel & High \\
Aquaporin 4 & Water channel & Moderate \\
P-glycoprotein & Antigen presenting & Low \\
GLUT1 & Antigen presenting & Moderate \\
basic fibroblast growth factor & Antigen presenting & High \\
angiopoietin 1 & Antigen presenting & Moderate \\
transforming growth factor-β & Antigen presenting & Low \\
glial-derived neurotrophic factor & Antigen presenting & High \\
TJ junction formation & Antigen presenting & Moderate \\
endothelial cells & Antigen presenting & High \\
pericytes & Antigen presenting & Moderate \\
perivascular macrophages & Antigen presenting & Low \\
\hline
\end{tabular}
\caption{List of important genes and their functions in BBB properties.}
\end{table}
Endothelial cells of the BBB are enclosed by a double basement membrane consisting of an endothelial and an astroglial basement membrane. The basement membranes consists of extracellular matrix components that are produced by endothelium, astrocytes and microglia and create a mechanical barrier for cells and macromolecules. 

Figure 1. Cellular constituents of the blood-brain barrier. The BBB is primarily formed by endothelial cells that are connected by tight junctions and surrounded by basement membranes. Other cell types localized at the BBB are pericytes and perivascular macrophages. Astrocytes contribute to the BBB by projecting their endfeet to the basement membrane.

Various studies suggest that the BBB is disturbed in MS lesions. In MS and EAE lesions, BBB leakage has been detected by gadolinium-DTPA enhanced MRI and immunohistochemistry. In addition, MS lesions are associated with abnormal expression of TJ molecules in brain ECs and enhanced expression of aquaporin-4 in astrocyte endfeet. It has been suggested that increased brain endothelial expression of dysferlin, a muscle protein involved in cell membrane repair, is a marker for BBB dysfunction in MS lesions. Studies performed in MS patients and animals suffering from EAE suggest that increased BBB permeability is an early event in lesion formation that precedes cellular infiltration into the CNS.

In MS lesions, basement membrane alterations are observed as well. Under normal conditions the endothelial and astroglial basement membrane lie closely together and appear as one basement membrane. However, during MS and EAE the two membranes become separated by accumulation of infiltrating immune cells, creating a perivascular cuff. Recent observations from our group demonstrated the presence of fiber-like depositions of extracellular matrix components within inflammatory cuffs. Interestingly, myelin-laden macrophages in the perivascular cuffs were located close to the basement membrane molecules. These data suggest that basement membrane proteins within inflammatory cuffs may serve as a conduit network that facilitates the transport of myelin-containing macrophages out of the CNS towards peripheral lymph nodes.
2.1.2 Tight junctions

Endothelial cells of the BBB are connected by TJs. TJs are continuous strands located at the apical site of the endothelium that are formed by transmembrane and cytoplasmic proteins associated with the actin cytoskeleton (Figure 2). The major transmembrane molecules mediating the cellular interaction between brain ECs are occludin and the claudins. Occludin was the first tight junctional transmembrane molecule discovered. It is a 65 kDa phosphoprotein that spans the plasma membrane four times with intracellular location of both the amino and the carboxy termini\textsuperscript{202,203}. Occludin is exclusively localized at TJs and its expression is associated with increased electrical resistance across the endothelium and decreased paracellular permeability\textsuperscript{204-206}. The family of claudins consists of at least 20 members of which claudin-1, claudin-3, claudin-5 and possibly claudin 12 are expressed in brain endothelium\textsuperscript{207}. Claudins contain two extracellular loops and four transmembrane domains and interact in a homophilic and heterophilic manner with claudins on adjacent endothelial cells\textsuperscript{208,209}. Claudins are believed to be the major transmembrane proteins of TJs as claudin knockout mice are non-viable\textsuperscript{210}, whereas occludin knockout mice are still able to form functional junctions\textsuperscript{211}. The claudins are believed to be responsible for permeability restriction and high TEER in brain endothelium\textsuperscript{166,203,212}.

The carboxyterminal tails of TJ transmembrane proteins can interact with a number of cytoplasmic assessor proteins, including the zona occludens (ZO) family\textsuperscript{213-215}. These proteins belong to the family of membrane associated guanylate kinase (MAGUK) proteins and can interact with other cytoplasmic molecules like cingulin\textsuperscript{216,217} and 7H6 antigen\textsuperscript{218}. ZO-molecules ZO-1, ZO-2, and ZO-3 link transmembrane TJ proteins to the actin cytoskeleton and have both structural and signaling roles\textsuperscript{219,220}.

Adhesion molecules like the family of junctional adhesion molecules (JAM), endothelial cell-selective adhesion molecule (ESAM) and coxsackievirus and adenovirus receptor (CAR) also localize at TJs. These proteins belong to the immunoglobulin superfamily and consist of extracellular variable (V-type) and constant (C2-type) immunoglobulin domains, a single transmembrane region and a cytoplasmic tail. Thus far, four members of the JAM family have been identified, JAM-A, -B, -C, and -D and they are expressed at the apical region of the junction\textsuperscript{221-223}. JAM molecules play a role in junction assembly and stabilization\textsuperscript{223}. ESAM and CAR are also localized within the junctional region\textsuperscript{224}. The cytoplasmic domain of ESAM is homologues to CAR, but longer than the cytoplasmic domain of JAM molecules\textsuperscript{225}. The role and function of ESAM and CAR in the BBB remain to be established.

Other proteins also associate with brain endothelial junctions, including platelet endothelial cell adhesion molecule (PECAM-1 or CD31) and CD99. PECAM-1 is a member of the immunoglobulin superfamily that is located at the apical domain of the intercellular junction\textsuperscript{226}. PECAM-1 is involved in cell-cell adhesion through either homophilic\textsuperscript{227,228} or heterophilic interactions\textsuperscript{229,230}. CD99 is a heavily glycosylated transmembrane molecule located at the apical site of the intercellular junction. The exact function of PECAM-1 and CD99 in the BBB is not clear, but both molecules were shown to play a role during leukocyte transendothelial migration\textsuperscript{81,231}. 
Besides TJ, adherens junctions (AJ) are important building blocks of the junctional complex. AJs interact with TJs along the entire length of the junctional area, but are located more at the basal site. AJs are formed by transmembrane glycoproteins of the cadherin superfamily, which bind to cadherins at adjacent cells in a homophilic and Ca\(^{2+}\) dependent manner. The cytoplasmic tail of cadherins interacts with the actin cytoskeleton and other signaling molecules via intracellular catenins. Although TJs control the main barrier function of the BBB, AJs are needed for the initial interaction between endothelial cells and are supposed to be required for proper TJ function, assembly, and organization.

**Figure 2. Schematic overview of the molecular composition of tight and adherens junctions.** Transmembrane proteins occludin, claudins, and JAMs seal the paracellular space between brain ECs. The carboxyterminal tails of TJ transmembrane proteins are connected to the actin cytoskeleton and signaling molecules via scaffolding proteins ZO-1, ZO-2, and ZO-3. Cadherins stabilize adhesion between brain ECs at adherens junctions and interact with the cytoskeleton via catenins.

### 2.2 Monocyte migration into the CNS

The BBB prevents the entry of circulating molecules and immune cells into the CNS. The brain has long been considered to be an immune privileged organ, but it has become clear that constant immune surveillance takes place, although lower than in peripheral organs. Under physiological conditions only few activated T cells enter the CNS through an intact BBB. However, neuroinflammatory diseases, such as MS, HIV-associated dementia and stroke, are accompanied by massive cellular migration across the BBB. Migration of leukocytes across endothelial barriers is mediated by various sets of adhesion molecules and occurs according to the multistep model of leukocyte extravasation (Figure 3). The first step is rolling and tethering of leukocytes on the endothelium to reduce the velocity of circulating leukocytes. Next, chemokines, displayed or released by endothelial cells, activate the leukocytes via G-protein coupled chemokine receptors, which leads to integrin activation and arrest of leukocytes onto the endothelium. The third step is firm adhesion of leukocytes to the endothelium, followed by diapedesis or transmigration through the endothelial layer.
2.2.1 Rolling

The first step of leukocyte transendothelial migration is rolling and tethering of leukocytes on the endothelium to reduce the velocity of circulating cells and to allow leukocytes to closely interact with signaling molecules, such as chemokines expressed on the endothelium. This step is mediated by (relatively weak) interactions between selectins and carbohydrate ligands. The family of selectins consists of three structurally related membrane glycoproteins: L (leukocyte)-selectin, P (platelet)-selectin, and E (endothelial)-selectin. Selectins are expressed on activated endothelial cells (E- and P-selectin), activated platelets (P-selectin), and leukocytes (L-selectin). Data about the involvement of selectins in the pathogenesis of MS are contradictory. Engelhardt and coworkers described that expression of E- or P-selectin is not induced on brain endothelium during EAE, whereas Kerfoot and Kubes described that P-selectin expression was increased in EAE brains and spinal cords. In addition, antibodies directed against selectins or a ligand of P-selectin did not affect the course of EAE in some studies, while others demonstrate that anti-L-selectin antibodies suppress clinical signs and cellular infiltration in EAE. In addition, antibodies directed against P- and E-selectin blocked rolling of autoreactive T-cells in vivo. In MS brains, expression of E-selectin is increased. Furthermore, elevated levels of soluble E- and L-selectin are found in serum and CSF of MS patients, suggesting that selectins play a role in MS pathology. Besides selectins, the $\alpha_4\beta_1$ integrin (Very Late Antigen-4,
VLA-4) can also mediate leukocyte rolling on endothelium through its interaction with vascular adhesion molecule-1 (VCAM-1). In the CNS, VLA-4 mediated rolling may be dominant over selectin-mediated rolling, although this may depend on the type of leukocyte and the location in the CNS.

### 2.2.2 Firm adhesion

Rolling allows leukocytes to closely interact with signaling molecules, including chemokines, which are presented on and released by endothelial cells. Chemokines activate G-protein coupled receptors on leukocytes, which increases integrin activation and enhances binding of integrins to their counter receptors on endothelium, leading to firm adhesion of leukocytes to endothelium. Integrins are heterodimeric membrane proteins, formed by a combination of α and β subunits. Integrins are expressed on leukocytes in their inactive form, but they are rapidly activated by intracellular stimuli via a process called inside-out signaling. Two mechanisms have been described for integrin activation. The first suggests that increased affinity involves conformational changes (affinity modulation), whereas the second suggests that the avidity of integrins is enhanced through increased integrin clustering at the cell surface (avidity modulation). Central integrins involved in leukocyte transendothelial migration are \( \alpha_4\beta_1 \) (VLA-4), \( \alpha_1\beta_2 \) (Lymphocyte Function Antigen-1, LFA-1), and \( \alpha_M\beta_2 \) (Mac-1). Ligands for integrins on endothelium involved in leukocyte migration are cellular adhesion molecules (CAMs) that belong to the immunoglobulin (Ig)-superfamily, including VCAM-1 and ICAM-1. ICAM-1 interacts with LFA-1 via the first Ig-like domain and Mac-1 via the third Ig domain. VCAM-1 is a ligand for VLA-4, but VCAM-1 also weakly binds to \( \alpha_4\beta_7 \) (LPAM-1). Under basal conditions, brain ECs express low levels of ICAM-1, both in vitro and in vivo, whereas VCAM-1 is absent. Generally, ICAM-1 and VCAM-1 are upregulated on inflamed endothelium by cytokines such as TNF-α, IFN-γ, and IL-1β. PECAM-1 (CD31) interactions also play a significant role during leukocyte migration. PECAM-1 is constitutively expressed on endothelium at intercellular junctions and, in contrast with ICAM-1 or VCAM-1, PECAM-1 expression is not regulated by proinflammatory cytokines. PECAM-1 is also expressed on platelets and subsets of leukocytes and it mediates cell-cell adhesion through either homophilic or heterophilic interactions.

In MS and EAE, increased expression of ICAM-1 and VCAM-1 was found on brain endothelium. In addition, LFA-1 and VLA-4 expression was observed on infiltrated inflammatory cells around blood vessels. Furthermore, elevated levels of soluble ICAM are found in serum and CSF of MS patients. These data suggest that integrin/CAM interactions play a role in MS pathology and therefore interference of these interactions may reduce cellular infiltration into the CNS. In vitro, adhesion of T lymphocytes to activated brain endothelium is mediated by LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions. Transendothelial migration of T lymphocytes is mainly regulated by ICAM-1 and partly by PECAM-1 and can be suppressed with antibodies blocking LFA-1/ICAM-1 interactions. For monocytes, it was demonstrated that adhesion and subsequent migration across brain endothelium are predominantly regulated by
VLA-4/VCAM-1 and PECAM-1. This is different from monocyte migration across peripheral endothelium, which is mainly controlled by LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions. In vivo, several studies have been conducted using blocking antibodies against the different adhesion molecules involved in leukocyte migration. Studies using blocking antibodies against ICAM-1 or LFA-1 in EAE were inconsistent. In contrast, both antibodies against VLA-4 and VLA-4 blocking peptides (CS-1) prevented leukocyte infiltration into the CNS and reduced clinical signs in EAE, suggesting that VLA-4/VCAM-1 interactions are essential for the development of EAE. These data imply that the interference of this interaction may be a good target to prevent MS lesion formation. Indeed, as described in section 1.4, clinical trials using recombinant antibodies directed against VLA-4 significantly reduced lesion formation and clinical relapses in MS patients, but the therapy had lethal side effects in a small number of patients. Contradictory results were obtained in studies on the role of PECAM-1 in EAE. Treatment with anti-PECAM-1 antibodies did not affect cellular infiltration and clinical symptoms in EAE, suggesting that the presence of PECAM-1 is not required for cellular infiltration in the CNS. However, PECAM-1 knock-out mice exhibit an early onset of EAE, suggesting that the presence of PECAM-1 hampers cellular migration across brain endothelium. On the other hand, antibodies directed against PECAM-1 reduce migration of leukocytes across brain endothelium in vitro. Thus, the exact role of PECAM-1 in neuroinflammation remains to be elucidated.

2.2.3 Signaling to brain endothelium

Brain ECs play an active role during transendothelial migration of leukocytes. The interaction of leukocytes and brain ECs triggers the activation of various signaling pathways in brain ECs that lead to rearrangement of the cytoskeleton and TJs, thus facilitating transendothelial migration. Upon activation, leukocytes produce a range of inflammatory mediators, including vascular endothelial growth factor (VEGF), nitric oxide, reactive oxygen species (ROS), and cytokines that affect BBB integrity and enhance transendothelial migration of leukocytes. In addition, recent studies demonstrate that members of the Ig-superfamily, such as ICAM-1 and VCAM-1, may also act as signal transducers in ECs and that signals from these molecules are important for transendothelial migration. In brain ECs most research has focused on signaling properties of ICAM-1. ICAM-1 interacts with the cytoskeleton via a number of molecules, including filamentous actin. One of the signaling molecules downstream of ICAM-1 that is involved in cytoskeleton reorganization is the GTP-binding protein Rho. Adamson and coworkers showed that ICAM-1 engagement induced Rho-dependent reorganization of the endothelial actin cytoskeleton. In addition, pretreatment of brain ECs with a Rho-inhibitor blocked leukocyte migration in vitro, indicating that signaling via Rho is necessary during transendothelial migration. Less is known about VCAM-1 signaling in brain ECs. However, in peripheral endothelium VCAM-1 ligation activates various signaling cascades that are involved in the modulation of endothelial junctions during leukocyte extravasation. Similar pathways may be activated in brain ECs upon VCAM-1 engagement.
Rho signaling and cytoskeleton rearrangements have been implicated in the regulation of TJ integrity. It has been reported that cytoskeleton depolymerization causes redistribution of TJ molecules\textsuperscript{308,309}. In addition, RhoA activation leads to phosphorylation of occludin and claudin-5\textsuperscript{145} and TJ reorganization\textsuperscript{145,310,311}. Evidence suggests that the phosphorylation state of occludin is important in the regulation of TJ assembly and disassembly\textsuperscript{312,313}. Occludin can be phosphorylated on Ser, Thr and Tyr residues\textsuperscript{314} and number of kinases have been associated with occludin-phosphorylation, including extracellular signal related kinase (ERK), mitogen-activated protein (MAP) kinase, and phosphatidylinositol 3 (PI3) kinase\textsuperscript{315-317}. More recently, phosphorylation of claudins has been investigated. For brain ECs, phosphorylation of claudin-5 by protein kinase A (PKA)\textsuperscript{318}, protein kinase C (PKC)\textsuperscript{319}, and myosin light chain kinase (MLCK)\textsuperscript{320} has been reported and these studies suggested that phosphorylation of claudin-5 reduces the strength of intercellular junctions. ZO proteins play a central role in the regulation of TJ integrity. Several studies have shown that ZO-1 can be directly phosphorylated\textsuperscript{321,322}. PKC is considered a major regulator of phosphorylation of ZO proteins\textsuperscript{321}. ZO-1 contains 34 protein PKC phosphorylation consensus sequences, suggesting that ZO proteins serve as scaffolds for PKC signaling at intercellular junctions\textsuperscript{323}. Several other cytoplasmic signaling molecules are located at epithelial TJs and are involved in the regulation of TJ integrity\textsuperscript{324}, including Ca\textsuperscript{2+}\textsuperscript{325-327}, heterotrimeric G-proteins\textsuperscript{328-331}, and cyclic AMP\textsuperscript{332,333}. These signaling molecules may also be involved in the regulation of brain endothelial junctions\textsuperscript{207}.

2.2.4 Transmigration, paracellular vs. transcellular
Recently, the route of transmigration across brain endothelium has become a matter of debate. Peripherally, leukocytes can migrate across the endothelial layer either through the endothelial junctions (paracellular route) or through the endothelial cell body (transcellular route). \textit{In vitro} and \textit{in vivo} data have provided evidence for both pathways. In neuroinflammatory diseases like MS or HIV-associated dementia and encephalitis, CNS inflammation is associated with abnormal expression of TJ proteins\textsuperscript{141,142,144-146}, suggesting that TJ are disrupted during leukocyte extravasation and that cells migrate through junctions. In animal models for neuroinflammation, vascular loss of TJ proteins coincides with leukocyte extravasation\textsuperscript{334-336} and \textit{in vitro}, it was demonstrated that TJ proteins are redistributed or even degraded upon leukocyte transendothelial migration\textsuperscript{145,337}. The TJ molecule occludin contains a putative matrix metalloproteinase (MMP) cleavage site in its first extracellular loop\textsuperscript{338}, suggesting that MMPs are involved in transmigration via cleavage of TJ molecules. This is supported by \textit{in vitro} observations that, upon the interaction of monocytes with brain ECs, occludin disappears from the junctions and is degraded, which could be prevented by MMP inhibitors\textsuperscript{337}. These data imply that brain endothelial TJs are involved in leukocyte trafficking across the BBB. Other junction molecules are thought to play a role as well in leukocyte migration across endothelium by the paracellular route. \textit{In vitro}, leukocyte migration can be inhibited by antibodies directed against junction proteins PECAM-1\textsuperscript{81,231}, JAM-A\textsuperscript{339,340}, and CD99\textsuperscript{231}. In addition, antibodies directed against JAM-A prevent leukocyte migration into the CNS.
in EAE. It has been suggested that PECAM-1 and JAM-A are involved in directing leukocytes through the intercellular cleft. CD99 may have a similar role, but this remains to be investigated.

Ultrastructural studies in brains of mice suffering from EAE suggested that leukocytes migrate solely through EC bodies rather than through the TJs. Also in other vascular beds leukocyte migration through the endothelial cell body is observed, both in vivo and in vitro. Carman and coworkers demonstrated in vitro that transendothelial migration of leukocytes across peripheral endothelium occurs via both the transendothelial and paracellular pathway. In addition, they demonstrated that both paracellular and transcellular diapedesis occurs in the context of ICAM-1- and VCAM-1-enriched endothelial projections (cup-like “docking structures”) that are involved in the guidance of leukocytes through the endothelium. Conceivably, migrating leukocytes use both the paracellular and transcellular route, and which pathway is preferred may depend on the specific leukocyte or vascular bed involved.

2.2.5 Migration across the extracellular matrix
Monocytes and T cells that have crossed the brain endothelium accumulate in the perivascular space, which is lined by two distinct continuous basement membranes. Part of the leukocytes migrates across the basement membranes further into the brain parenchyma. For this purpose, infiltrating leukocytes secrete MMPs that degrade and remodel extracellular matrix components, such as fibronectin, collagen, and laminin. Alterations in the extracellular matrix composition have been observed in MS lesions, suggesting that the barrier function is disturbed. Besides degrading matrix components, MMPs are able to cleave certain cytokines, growth factors, membrane bound receptors, and myelin basic protein. In brain tissue of MS patients, increased production of various MMPs has been found in and around lesions, predominantly in macrophages. In addition, elevated levels of MMP-9 were detected in CSF and serum of MS patients. In vitro, leukocyte migration across brain endothelium is inhibited by MMP inhibitors, whereas in vivo MMP inhibitors reduce the severity of EAE, suggesting that MMP-inhibitors may be a tool to prevent lesion formation in MS. MMP inhibitors have been used in clinical trials for the treatment of various diseases, including rheumatoid arthritis, asthma and cancer. Therapy with MMP inhibitors was not very successful thus far, but new inhibitors are being developed that may be more selective and efficient for clinical use, and may be a tool for the treatment of MS.
3. Reactive oxygen species and antioxidants

3.1 Reactive oxygen species and free radicals

In various pathological processes underlying MS lesion formation and persistence, free radicals and reactive oxygen species are involved. Free radicals are molecules or atoms that contain one or more unpaired electrons. Free radicals absorb electrons from other molecules, a reaction called oxidation, and therefore free radicals are oxidizing agents. Superoxide (O$_2^-$), hydroxyl radical (OH$^-$) and nitric oxide (NO$^-$) are the most common cellular free radicals. Hydrogen peroxide (H$_2$O$_2$) and peroxynitrite (ONOO$^-$) are not considered free radicals, but are highly reactive and can lead to the formation of free radicals. Oxygen radicals and hydrogen peroxide together are called reactive oxygen species (ROS), whereas nitrogen radicals and related molecules are classified as reactive nitrogen species (RNS).

Cells contain multiple sources of ROS. ROS are generated both enzymatically by oxidoreductases and non-enzymatically as side products of the mitochondrial respiratory chain. Nitric oxide is enzymatically produced by nitric oxide synthases (NOS) through the oxidation of L-arginine. A source of superoxide is the NADPH oxidase complex, which is mainly expressed by phagocytes, but also by other cell types. The NADPH oxidase complex consists of several components. In non-stimulated cells, gp91phox and p22phox are located in the plasma membrane, while p47phox, p67phox, and p40phox are located in the cytosol as a complex. Upon stimulation, for instance during phagocytosis, the cytosolic components and the small GTP-ase Rac1/Rac2 translocate to the plasma membrane to form an active complex that generates superoxide. Other sources of ROS include xanthine oxidase, lipoxygenase, and cyclooxygenase.

Superoxide is generated via the one-electron reduction of oxygen. Superoxide can react with nitric oxide to produce peroxynitrite. In addition, superoxide can be dismutated, spontaneously or by superoxide dismutases (SOD), to hydrogen peroxide. In turn, hydrogen peroxide can be broken down by catalase, glutathione peroxidase, thioredoxin, or peroxiredoxins into water and oxygen. Alternatively, hydrogen peroxide can be used to generate hydroxyl radical via non-enzymatic reactions in the iron-catalyzed Fenton reaction (Figure 4). Hydrogen peroxide and superoxide are only moderately reactive with other biological molecules. Of the above-mentioned ROS, the hydroxyl radical is the most damaging, since it has a high reactivity and reacts with many macromolecules close to its site of formation.

3.2 Cellular antioxidant defense

Free radicals and ROS can cause extensive damage to macromolecules such as DNA, lipids, and proteins. Therefore, the control of the intracellular redox environment is crucial for proper cellular function. To protect themselves from oxidative damage, cells contain high concentrations of antioxidants. The antioxidant system can be divided into two major groups: enzymatic antioxidants and non-enzymatic or low-molecular-weight antioxidants. The enzymes include superoxide dismutases (SOD), catalase, glutathione peroxidase, and peroxiredoxin. The group of low-molecular-weight antioxidants consists...
of both endogenous molecules (e.g., glutathione and NADPH) and exogenous molecules. The majority of exogenous antioxidants are derived from dietary products, including ascorbic acid (vitamin C), α-tocopherol (vitamin E), α-lipoic acid, flavonoids, polyphenols, and carotenoids\textsuperscript{378-380}.

**Figure 4. Simplified schematic overview of ROS production and scavenging.** $O_2$, oxygen; \(O_2^-\), superoxide; $H_2O_2$, hydrogen peroxide; $OH^-$, hydroxyl radical; NO, nitric oxide; ONOO$^-$, peroxynitrite; NOS, nitric oxide synthase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GRd, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; Cat, catalase; Prx, peroxiredoxin. Adapted from Maher and Schubert, 2000\textsuperscript{377}.

The most abundant endogenous low-molecular-weight antioxidant is glutathione, a water-phase antioxidant and an essential cofactor for antioxidant enzymes, including glutathione peroxidase. Due to its high electron-donating capacity and intracellular concentration (between 2 and 10 mM), glutathione has great reducing power and is a potent antioxidant. Glutathione exists in two forms: the antioxidant form, a tripeptide (γ-glu-cys-gly), is usually called glutathione and abbreviated as GSH; the oxidized form is a sulfur-sulfur linked molecule, called glutathione disulfide or GSSG. Normally, GSSG represents less than 1% of the total cellular glutathione content. However, upon increased intracellular ROS concentration, a transient increase in GSSG occurs. Therefore, the GSSG/GSH ratio is considered a sensitive indicator of the intracellular redox status\textsuperscript{381,382}.

Compared to other organs, the brain has a high metabolism, which leads to the generation of large quantities of ROS. In addition, the brain is rich in lipids with unsaturated fatty acids that are targets of lipid peroxidation, making the brain vulnerable to oxidative stress. All areas of the brain express SODs, glutathione peroxidases and peroxiredoxins\textsuperscript{383,384}. In addition, the CNS is enriched in various low molecular weight antioxidants, including glutathione\textsuperscript{385,386}, ascorbic acid\textsuperscript{387}, and α-tocopherol\textsuperscript{388-390}. Nevertheless, free radicals and oxidative damage are thought to play a role in many neurological diseases. The role and regulation of ROS and endogenous antioxidant
enzymes in MS and other neuroinflammatory diseases will be discussed in more detail elsewhere (chapter 5 of this thesis).

### 3.3 ROS and signaling

In recent years it has become increasingly clear that ROS can act as second messengers. The activation of signaling pathways by ROS involves the direct modification of proteins that belong to these signaling pathways. Generally, redox regulation of proteins occurs primarily via a reaction of ROS with sulphydryl groups (RSH) on cysteine residues within the protein. The sulphydryl group is easily oxidized to form a disulphide bond (RSSR), sulphenic acid (RSOH), suphonic acid (RSO$_2$H), or sulphonic acid (RSO$_3$H)$^{376}$. Reactions with mild oxidizing ROS or low concentrations of ROS can lead to reversible modulations of protein activity, whereas reactions with strong oxidizing ROS can lead to permanent modification of enzymes, which can induce cell death. The majority of the cysteine residues is surrounded by a highly reducing environment and is protected against oxidation. Likely, only proteins with accessible sulphydryl groups are involved in redox signaling$^{391,392}$.

The activity of many signaling proteins, including receptors, enzymes, and transcription factors is dependent on protein phosphorylation. Phosphorylation of signaling proteins is regulated by two classes of enzymes; protein kinases cause phosphorylation of target proteins, whereas protein phosphatases are involved in the removal of phosphate$^{393-395}$. Cysteine-based phosphatases (CBP) are particularly sensitive to redox regulation$^{396}$. All CBPs, including protein tyrosine phosphatases, dual-specific phosphatases, and low-molecular-weight protein tyrosine phosphatases, contain a cysteine within their active site, which participates directly in the dephosphorylation reaction and is very susceptible to oxidation$^{397-399}$. Oxidation of CBPs results in the reversible conversion of the catalytic cysteine to a stabilized sulfenic acid intermediate, which causes structural rearrangements and inactivation of the phosphatase$^{398}$. Because the level of phosphorylation depends on the balance of kinase and phosphatases activity, inactivation of CBPs leads to enhanced phosphorylation. Both protein tyrosine kinases and serine/threonine kinases can be activated by ROS$^{376,400,401}$. Various protein kinases are regulated by phosphorylation and therefore it is not clear whether the activation of kinases by ROS is a direct effect or if this occurs via the inactivation of upstream phosphatases (Figure 5)$^{377}$. Downstream targets of ROS-sensitive kinases include mitogen-activated protein (MAP)-kinases, PI3-kinase, Ca$^{2+}$, and transcription factors$^{376,402}$. Antioxidants are also involved in redox signaling. They affect signal transduction pathways not only by controlling the redox balance, but possibly also via direct interactions with signaling proteins. This was demonstrated for thioredoxin that inactivates apoptosis signal-regulated kinase 1 via the formation of a complex under reducing conditions$^{403,404}$. In addition, SOD1 can interact with and regulate calcineurin activity$^{405}$.

One of the targets of signal transduction is gene transcription. The activation of gene transcription is regulated by transcription factors, which bind to specific DNA sequences and regulate RNA polymerase II activity. Several transcription factors have been shown
to be regulated by ROS, including activator protein-1 (AP-1), nuclear factor κB (NFκB), and nuclear factor-E2-related factor (Nrf2). The AP-1 transcription factors are c-Jun homodimers or heterodimers consisting of c-Fos and c-Jun proteins that are involved in the expression of cell growth mediators. The DNA-binding domains of c-Jun and c-Fos contain a conserved cysteine residue that needs to be reduced for DNA binding activity. NFκB plays a role in the expression of genes involved in the immune response, stress response, cell growth, and cell survival. The inactive form of NFκB exists as a trimer that consists of p65, p50, and IκBα subunits. Upon activation, the IκBα subunit dissociates, and the activated p65/p50 subunit migrates to the nucleus to bind DNA. The p50 subunit of NFκB contains a redox-sensitive cysteine that is essential for DNA-binding. Oxidation of this cysteine residue reduces DNA binding activity of NFκB. The activity of AP-1 and NFκB is also regulated by kinases. Oxidants may activate kinases, whereas oxidation of transcription factors leads to inactivation. Consequently, oxidizing agents have dual effects on transcription factor activity (Figure 5). It was suggested that ROS increase the expression of ICAM-1 through NFκB-activation, thus enhancing leukocyte adhesion to endothelium. Other transcription factors that may be controlled by redox regulation but will not be discussed here include the signal transducer and activator of transcription (STAT) factors, Myb, p53, PEBP2/AML, and nuclear factor Y. The activation of the Nrf2/ARE pathway by ROS will be introduced in more detail in chapter 5 of this thesis.

**Figure 5. Simplified schematic overview of redox signaling.** Cysteine-based phosphatases are sensitive to inactivation by ROS. Protein kinases can either become activated by reduced phosphatase activity, or via direct activation by ROS. Downstream targets of activated kinases include receptors, enzymes, and transcription factors. A number of transcription factors are inactivated by ROS. Therefore, oxidizing agents have dual effects on transcription factor activity.
4. Outline of the thesis

In MS, monocyte-derived macrophages migrate into the CNS where they cause extensive damage to myelin and axons. To enter the CNS, monocytes need to cross the BBB, which consists of highly specialized brain endothelial cells and their TJ complexes. Previous studies showed that during migration ROS play a crucial role. The studies described in this thesis aim to elucidate the mechanisms underlying ROS-induced BBB dysfunctioning and enhanced monocyte transendothelial migration.

In chapter 2 we investigated the effect of the antioxidant α-lipoic acid on monocyte migration across brain endothelium in vitro as well as in vivo. In addition, the effect of lipoic acid on superoxide-induced alterations in brain endothelium was studied. A number of signal transduction pathways are known to be involved in monocyte transendothelial migration and BBB dysfunction. In chapter 3, we studied the influence of ROS on the activation of signal transduction pathways in brain ECs. In addition, we studied pathways involved in ROS-induced cytoskeleton and TJ rearrangements. Long-term exposure to ROS may provoke adaptive responses to counteract the oxidative attack by the induction of protective proteins. In chapter 4, we investigated the effect of ROS on gene and protein expression in brain ECs. In addition, we studied functional effects of overexpression of one of the identified proteins, peroxiredoxin-1, in brain ECs on monocyte adhesion and diapedesis.

ROS are not only involved in MS lesion formation through enhancement of BBB dysfunction and monocyte migration, but they also play a role in lesion persistence. For instance, ROS are produced during myelin phagocytosis and can cause oligodendrocyte and axonal damage. When levels of ROS production exceed the antioxidant capacity of cells, this may lead to oxidative stress. Oxidative stress activates protection mechanisms, including the production of antioxidant enzymes. In chapter 5 the role of ROS and antioxidant enzymes in neuroinflammatory diseases is reviewed. In chapter 6, we studied the expression of redox related enzymes in the course of acute EAE in the cerebellum and brainstem. Microarray analysis and immunohistochemistry revealed enhanced expression of antioxidant enzymes at the peak of disease. Next, we studied the expression of antioxidant enzymes in MS lesions. In chapter 7 we describe the expression of the antioxidant enzyme NQO1 in different types of MS lesions. Besides NQO1, numerous other antioxidant enzymes are produced upon oxidative stress. In chapter 8 we studied protein expression of a number of endogenous antioxidant enzymes in various MS lesions. Finally, in chapter 9, the findings described in this thesis are summarized and discussed in the context of recent developments in MS research.
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General introduction


General introduction


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