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GENERAL INTRODUCTION



MULTIPLE SCLEROSIS

Demographics and disease course

In his famous “Histologie de la sclérose en plaques”,¹ professor Jean-Martin Charcot was the first to extensively describe the clinical and neuropathological characteristics of what he called sclérose en plaques, i.e. Multiple Sclerosis (MS). The name ‘sclérose en plaques’ refers to his observation that in the central nervous system (CNS) several areas containing scar tissue were observed. MS is a chronic inflammatory demyelinating disease of the CNS affecting more than 2 million individuals worldwide and approximately 16.000 in the Netherlands. Usually the onset lies between 25-45 years of age and it occurs two times more frequently in women than men.²

It is generally accepted that symptoms of MS are the consequence of destroyed myelin and oligodendrocytes resulting in blocked or impaired nerve conduction in areas of demyelination. Myelin is composed of a lipid-protein membrane produced by oligodendrocytes and is enwrapped around axons to form an insulating sheath needed for efficient neural signal transduction and to protect the axon from potentially harmful exogenous factors.³⁻⁵ Depending on the location of the lesions, the loss of myelin results in a multitude of neurological impairments⁶ e.g. disturbed vision,^{7,8} bladder dysfunction,⁹ sensory problems,^{10,11} motor problems,¹²⁻¹⁶ sleep disorders,^{17,18} depression and anxiety¹⁹ and/or cognitive dysfunction.²⁰ The disease course is variable among affected subjects, and about 85% of MS patients have a biphasic disease course, i.e. relapsing-remitting MS (RR-MS), during which patients experience alternating episodes of symptoms and complete or incomplete remission of symptoms.²¹ The majority of RR-MS patients transform into a secondary progressive disease course (SP-MS) which is characterized by gradual worsening of the neurological symptoms.²² Only 15% of MS patients present with primary progressive MS (PP-MS), during which the disease course is progressive from the onset without periods of remission.^{23,24} Progressive-relapsing MS (PR-MS) is a rare clinical course of the disease characterized by a progressive course from the onset, with clear acute relapses, with or without full recovery. Periods of remission are characterized by continuing progression.²¹

Etiology and treatment

Although the cause of MS is still unknown, both genetic and environmental factors have been associated with an increased risk of MS. Genetic involvement has been demonstrated in numerous family studies, e.g. twin studies showed that the risk to develop MS is higher in monozygotic twins compared to dizygotic twins with MS.²⁵ Several genes have been identified using genome wide association studies that are related to an increased risk of MS. The identified genes are mostly related to inflammatory pathways, with the human leukocyte antigen class II molecules, specifically the HLA-DRB1*1501 region, having the strongest association with MS.²⁶ Recently, it was shown that the HLA-DRB1*15 gene significantly enhances the extent of demyelination in spinal cord tissue, possibly by regulating the inflammatory response.²⁷

However, identical twins show a concordance rate of 25-30 %, ²⁸ which is relatively low, suggesting that the development of MS cannot entirely be attributed to genetics. In addition, the geographical distribution of MS is uneven, i.e. MS seems to be relatively rare in countries near the equator. These findings imply that environmental factors should be considered as well. Several environmental factors have been studied. For example, high sun exposure and vitamin D levels were shown to correlate with a lower susceptibility for MS. ^{29,30} In addition, oligoclonal immunoglobulin (IgG) bands have been demonstrated in MS lesions and cerebrospinal fluid (CSF) of MS patients, suggesting the involvement of an infectious agent to which antibodies have been developed. Although evidence on this matter is still inconclusive, the Epstein-Barr virus has been put forward as one of the leading candidates. ³¹

As inflammatory processes seem to importantly contribute to the pathogenesis of MS, current therapies for MS target mainly immune components. Patients suffering from exacerbations are acutely treated with corticosteroids for a short period of time. ³² The most widely prescribed immunomodulating treatments for RRMS are interferon (IFN)- β and glatiramer acetate, resulting in lower relapse rates and lower transition rates to SPMS. ³³⁻³⁶ In addition, relatively new immunomodulating treatments for RRMS have become available, i.e. dimethylfumarate and teriflunomide. ³⁶ These treatments are so-called first-line treatments characterized by moderate effectiveness but high safety. However, when treatment response is insufficient or tolerability to these first-line treatments occurs, a switch to more potent, but potentially less safe second-line treatment is sometimes recommended. ³⁷ For example, natalizumab and fingolimod are available treatments targeted at the prevention of immune cell infiltration through the blood-brain barrier (BBB). Although natalizumab and fingolimod seem to be more effective than IFN- β and glatiramer in reducing relapses and disease severity, severe side effects can be a serious problem. ³⁸⁻⁴⁰ Recently, alemtuzumab has become available, which showed higher efficacy compared to IFN- β , but has a higher chance of evoking adverse side-effects. ⁴¹ Two newly emerging promising therapies are ocrelizumab and daclizumab. Phase II trials of ocrelizumab and daclizumab lead to the expectation that these therapies will be as effective as natalizumab, but having a better safety profile. ^{42,43} However, all existing treatments are only effective in the early stages of the disease and a significant proportion of MS patients do not respond to these treatments. Specifically for MS patients in the progressive phase of the disease these treatments are of limited benefit. ⁴⁴

Neuropathological characteristics of MS lesions

MS is characterized neuropathologically by areas of demyelination containing or surrounded by infiltrated immune cells and activated resident immune-competent cells i.e. glial cells. One of the prevailing hypotheses states that MS is the result of an outside-in process that starts with an inflammatory response directed against components of oligodendrocytes and/or myelin, thereby resulting in degeneration of oligodendrocytes and axons. Alternatively, the inside-out hypothesis states that demyelination is the result of degeneration of oligodendrocytes and axons, accompanied by a secondary inflammatory response. ⁴⁵ Lesions can occur in the

white matter (WM) and grey matter (GM) of the CNS. For a long time, grey matter lesions (GML) have received little attention, due to the fact that conventional histochemical staining, i.e. Luxol fast blue and Klüver-Barrera, and magnetic resonance imaging (MRI) techniques were not, or only to a limited extent, able to visualize GML. However, since the introduction of advanced MRI techniques, i.e. double inversion recovery (DIR)-MRI,⁴⁶ GML can be demonstrated *in vivo*. In addition, immunohistochemical staining for myelin basic protein (MBP) or proteolipid protein (PLP) were found to be able to visualize GML in *post mortem* brain tissue.^{47,48} White matter lesions (WML) and GML are characterized by the loss of myelin, but their immunopathological features differ. During active demyelination, WML present with infiltrating leukocytes, while these immune cells are significantly lower in number in GML.^{47,49} Characteristics of WML and GML, the immunopathology of WML and GML and differences between these two types of lesions are described more in detail in chapter 2.

MICROGLIA AND ASTROCYTES

For a long time, glial cells were considered to function only as 'brain glue', just to keep the neurons together. Nowadays, they are recognized as cells that have a fundamental role in regulating homeostasis throughout the CNS. Moreover, the activation of microglia and astrocytes is a crucial and early event in the pathogenesis of both WML and GML.⁴⁹⁻⁵³

Microglia

Microglia are parenchymal tissue macrophages of the CNS and the primary responding cells when the homeostasis of the brain is challenged by infection or injury. **Astrocytes**

Astrocytes are the most abundant glial cell population within the CNS and have a number of important physiological properties related to CNS homeostasis and neuronal functioning. Astrocytes contribute to neuronal signaling and synaptic pruning at the tripartite synapse. The term tripartite synapse refers to the structure that comprises an astrocytic process, and presynaptic and postsynaptic terminals, where bi-directional communication between astrocytes and neurons occurs. Astrocytes communicate with each other via calcium (Ca^{2+})-waves, a propagation of intracellular elevation of Ca^{2+} . Interestingly, this rise in Ca^{2+} can be triggered by activation of neurotransmitter receptors present on astrocytes following neuronal release of neurotransmitters. In reverse, astrocytes regulate neurotransmitter levels in the synaptic cleft, e.g. by taking up or secreting glutamate.^{58,59} Another important feature of astrocytes is that they contribute heavily to the formation of the BBB, by encapsulating the brain vasculature with their endfeet⁶⁰ and inducing the formation of tight junctions between endothelial cells.⁶¹ In addition to their ability to provide metabolic support for neurons, regulating neurotransmission and maintaining the BBB astrocytes have also been described as immunocompetent cells. Similar to microglia, astrocytes are able to secrete neurotoxic and neurotrophic factors. The immunological characteristics of astrocytes and their role in MS are described in chapter 2.

Cytokines and chemokines

Cytokines and chemokines are secreted proteins that regulate and contribute to the immune response during MS. Increased levels of various cytokines and chemokines have been described in MS, e.g. interleukin-1 β (IL-1 β), tumor-necrosis factor- α (TNF- α), interferon- γ (IFN- γ), monocyte chemotactic protein-1 (MCP-1) and fractalkine. Two factors that have been implicated especially in the pathogenesis of WML in MS are IL-1 β and MCP-1, also known as CCL2. IL-1 β has gained attention owing to its propensity to induce the production of inflammatory factors,⁶²⁻⁶⁴ proliferation of macrophages,^{65,66} up-regulate cellular adhesion molecules⁶⁷ and induce leukocyte migration.⁶⁸ Furthermore, accumulating evidence suggests IL-1 β is a potential early mediator in WML formation.⁶⁹⁻⁷² CCL2 has been reported to play a crucial role in the pathogenesis of WML in MS,^{73,74} by inducing the migration of leukocytes into the CNS.⁷⁵⁻⁷⁷ In addition, CCL2 has been shown to induce migration,^{78,79} proliferation⁸⁰ and activation^{80,81} of microglia in vitro. Thus, IL-1 β and CCL2 both have been extensively described concerning their involvement in WML formation. However, much less is known about their role in GML. Therefore, in the next paragraphs, we focus on these two important inflammatory factors, within the context of our studies into understanding WML and GML pathology.

Interleukin-1 β

The pro-inflammatory cytokine IL-1 β is part of the family of interleukins, and is the most extensively studied of all cytokines.⁶²⁻⁶⁴ In the healthy brain, endogenous IL-1 β expression is very low.⁸² However, IL-1 β synthesis and expression is upregulated in response to e.g. systemic inflammation,⁸³⁻⁸⁶ excitotoxic and ischemic brain damage,^{63,87,88} brain trauma and infections.⁸⁹ IL-1 β signaling, and thereby IL-1 β actions, can be inhibited by the endogenous and competitive IL-1 receptor antagonist (IL-1ra), an anti-inflammatory cytokine that can be produced in inflamed tissues.⁶⁴

IL-1 β is produced by many immunological cell types, i.e. monocytes, lymphocytes, macrophages, dendritic cells and natural killer cells. In the CNS, IL-1 β production has been ascribed to glial cells.^{63,64,87} Initially, IL-1 β is present intracellularly in a biologically inactive form, known as pro-IL-1 β , that does not bind the IL-1 receptor. For activation, pro-IL-1 β has to be cleaved by intracellular caspase-1 into a mature and biologically active form.⁹⁰ Caspase-1 is activated by a complex of proteins, called the inflammasome.⁹¹ This multiprotein complex is activated by inflammatory factors, adenosine triphosphate (ATP) or signals from dying cells. After cleavage of pro-IL-1 β , it is released as an active cytokine into the extracellular space.⁹² When mature IL-1 β is released, it exerts its effect by binding to its receptor, IL-1R. Two types of this receptor exist, the type I and type II receptor. The type I receptor (IL-1RI) is a signaling receptor, while IL-1 receptor type II (IL-1RII) acts as a decoy receptor and is mainly expressed by IL-1 expressing cells to prevent excessive autocrine signaling.⁶³ IL-1 β needs to form a complex with IL-1RI and the IL-1 accessory protein (IL-1RAcP) to allow intracellular signaling. The ensuing signaling pathway includes the activation of myeloid

differentiation primary response protein (MYD88), TNFR-associated factor 6 (TRAF6) and IL-1R-associated kinase 4 (IRAK4), whose downstream signaling activates nuclear factor- κ B (NF- κ B) and mitogen activated protein kinase (MAPK) pathways.⁹³ IL-1ra binds to type I and type II receptors but fails to recruit IL-1RAcP, which is needed for IL-1 induced intracellular signal transduction. Moreover, IL-1ra inhibits the binding of IL-1 to IL-1RI by occupying the receptor resulting in inhibition of most, if not all of IL-1 mediated effects.^{64,93} After IL-1R1 activation by IL-1 β , antibody production is increased, the activation of a range of different cytokines and chemokines is induced, and it augments the T-cell response.^{64,94} Furthermore, IL-1 β is implicated in proliferation of macrophages^{65,66}, up-regulation of cellular adhesion molecules⁶⁷ and leukocyte migration.⁶⁸ This inflammatory response is considered to be involved in the pathogenesis of MS, resulting in various pathological characteristics of MS pathology ranging from increased BBB permeability⁹⁵, increased astrogliosis⁹⁶ to oligodendrocyte degeneration.⁹⁷ A schematic representation of IL-1 β signaling is depicted in figure 1.

CCL2

Chemokines are cytokines that have chemoattractant effects on monocytes, neutrophils and lymphocytes. Four subfamilies of chemokines have been described, based on the number and location of cysteine residues in the N-terminus of the protein. The two main subfamilies are the CXC- and the CC-chemokine subfamilies.^{74,75} CCL2 is the first and best characterized CC-chemokine and has been found to be the most potent chemokine for inducing and directing monocyte migration.⁹⁸ CCL2 can be produced by different types of cells, e.g. astrocytes, microglia and neurons,⁹⁹⁻¹⁰¹ and is induced by pro-inflammatory cytokines, e.g. IL-1 β and TNF- α ¹⁰², or growth factors like transforming growth factor- β (TGF- β).¹⁰³

CCL2 exerts its effect by binding to the seven-transmembrane G-protein coupled receptor CCR2. Several chemokines are able to bind CCR2, including CCL2, CCL7, CCL8, CCL12, CCL13 and CCL16. However, CCL2 is the most potent in directing the migration of CCR2 expressing cells.⁹⁸ CCR2 is mainly expressed by infiltrating T-cells and microglia and/or macrophages,¹⁰⁴⁻¹⁰⁶ but also endothelial cells¹⁰⁷ and neurons¹⁰⁸ have been described to express CCR2. When CCL2 binds to CCR2, a wide range of intracellular pathways is activated, including the activation of phosphatidylinositol 3-Kinase (PI3K), extracellular signal-regulated kinases (ERK) and protein kinase C. Consequently, CCR2 expressing cells rearrange their cytoskeleton and extracellular matrix (ECM) environment allowing their migration towards high local concentrations of CCL2.¹⁰⁹ This migration occurs along a chemical gradient of ligand, the chemokine gradient.¹¹⁰ Thus, CCL2-CCR2 signaling eventually results in increased influx of leukocytes after injury,⁷⁶ which is characteristic for WML in MS (see figure 2). In addition, CCL2 has been described to be involved in increased BBB permeability,^{111,112} but also in the activation and proliferation of microglia,^{80,81} which are also considered to contribute to the formation of WML. Studies in animal models of MS underline the involvement of CCL2 in MS, e.g. genetic ablation of CCL2¹¹³ or CCR2¹¹⁴ in experimental autoimmune encephalomyelitis (EAE) reduced the influx of macrophages and

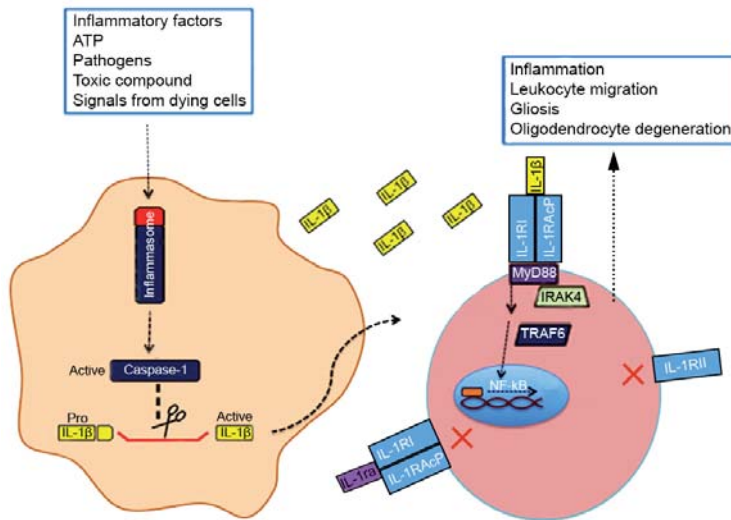


Figure 1 Inflammatory factors, ATP or signals from dying cells activate the inflammasome, a multiprotein complex. The inflammasome activates caspase-1, which cleaves the pro-form of IL-1 β , is required for the secretion and activation of IL-1 β . Biologically active IL-1 β that is released into the extracellular space binds to the IL-1R. IL-1 β needs to form a complex with IL-1RI and IL-1RAcP to allow intracellular signaling. Activated IL-1RI results in the recruitment of MyD88 and IRAK4, which, via TRAF6 expression, results in the activation of NF- κ B and MAPK pathways, which induce an inflammatory response. When IL-1 β binds the IL-1RII receptor or when IL-1ra binds the IL-1RI receptor, the intracellular signalling cascade is not activated, as indicated by the red cross. (Adapted from: 204)

ameliorated progression of EAE. In addition, the extent of demyelination, clinical deficits and axonal damage in EAE was diminished after conditional deletion of astroglial CCL2.⁷⁷

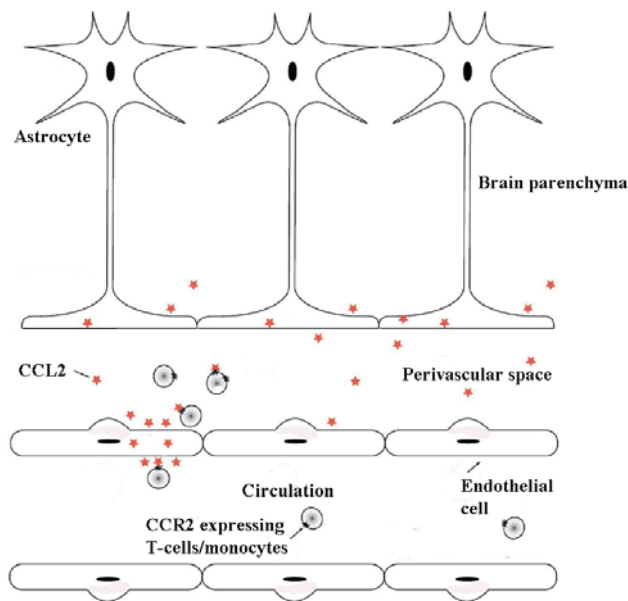


Figure 2 Inflammatory factors or ATP are able to induce CCL2 protein (*) by astrocytes. When CCL2 is released it binds to its receptor CCR2 expressed by T-cells or monocytes. This induces the migration of these immune cells across the BBB, which is characteristic for WML in MS. (Adapted from 73).

ANIMAL MODELS OF MS

To unravel the underlying mechanisms contributing to MS pathology, animal models are highly useful tools. Several models exist, each mimicking different aspects of MS pathology. Three main categories of animal models can be distinguished. The first category comprises one of the most extensively studied animal models of MS, EAE. EAE mimics the inflammatory response considered to underlie the pathogenesis of MS. The second category includes virally induced chronic demyelination models, including Theiler's Murine Encephalomyelitis Virus (TMEV), used to study the pathogenesis of virally induced inflammation. Finally, the third category consists of toxin induced models of demyelination, including cuprizone and lysophosphatidyl choline induced demyelination. Toxin induced models are useful in the study of de- and remyelination processes in the absence of infiltrating immune cells.^{115,116}

Experimental autoimmune encephalomyelitis

EAE is considered to be a mainly T-cell driven disease and is in line with the hypothesis that MS starts with T-cell driven autoimmunity against myelin antigens. EAE can be induced in non-human primates and rodents by immunization with a myelin antigen, usually MBP, PLP, or myelin oligodendroglial glycoprotein (MOG).¹¹⁷ The myelin antigens are emulsified in complete or incomplete Freund's adjuvant (CFA or IFA, respectively), which activates the innate immune response.^{118,119} When animals are injected with myelin antigens, it is called actively-induced EAE, as opposed to adoptive-transfer EAE, during which naïve animals are injected with T-cells from animals with EAE or *in vitrosensitized* T-cells.¹¹⁷

Neuropathological characteristics of EAE mimic aspects of the pathology observed in MS patients. When animals are immunized, activated myelin-specific T-cells enter the bloodstream and penetrate the BBB via interaction with cytokines, chemokines and integrins.¹²⁰ Upon entering the CNS, T-cells encounter their cognate myelin antigens and get reactivated by antigen presenting cells (APC's).¹²⁰ A mutually reinforcing process during which T cells can amplify the immune response by attracting more leukocytes into the CNS, secreting pro-inflammatory cytokines, which in its turn induces the activation of microglia results in an augmentation of the immune response. This leads to an increase in e.g. the production of pro-inflammatory cytokines, reactive oxygen species and matrix metalloproteinases (MMP's), resulting in myelin breakdown and phagocytosis, followed by axonal damage and neurological impairment.¹²¹ Clinically, motor impairments range from loss of tonus to paralysis of the hind limbs,¹²² i.e. only partially reflecting the range of clinical symptoms in MS patients. In addition, all though far less studied than motor impairments, increased sensitivity to pain¹²³ and cognitive problems^{124,125} have been demonstrated in EAE.

Since the description of EAE in 1933¹²⁶, a variety of EAE models, each characterized by its own clinical course, has been described, using different species, strains and immunization protocols.¹¹⁷ In some EAE models, the disease follows a monophasic, non relapsing course, e.g. when it is induced in Lewis rat by MBP¹²⁷, or in C57BL/6 mice by MOG.¹²⁸ Although this model is useful to study the acute phase of EAE characterized by CNS inflammation, it is limited by the absence of relapses. A model that mimics aspects of RRMS is chronic relapsing EAE (cr-EAE). Cr-EAE can for example be induced in Dark Agouti rats or Biozzi mice using MOG and is characterized by spontaneous occurring relapses, demyelination and inflammatory infiltration,^{128,129} reflecting more extensively the most frequently occurring RRMS course of disease and concomitant pathology.

The cuprizone mouse model

Another extensively studied animal model for MS is the cuprizone mouse model. In contrast to EAE, this model is characterized by consistent and reproducible patterns of demyelination, which are not primarily immune-mediated. The BBB is not affected in the cuprizone mouse model and no infiltration of leukocytes is observed in this model, which makes it suitable to study the process of de- and remyelination without the influence of infiltrating immune cells.

Cuprizone (bis-cyclo-hexanone oxaldihydrazone) is a copper chelator, which induces severe oligodendrocyte death and consequent demyelination when fed, using a diet containing 0.2-0.3% cuprizone, to 6-9 week old mice. Acute demyelination is induced when animals are fed with the cuprizone diet for 5 weeks, after which the corpus callosum is largely demyelinated. If cuprizone treatment is terminated after 5 or 6 weeks, spontaneous and almost complete remyelination occurs. However, if the cuprizone treatment is continued for 12 or 13 weeks, chronic demyelination is induced, after which no spontaneous remyelination occurs.^{115,130,131} Demyelination is accompanied with increased microglia activation and astrogliosis. Both cell

types increase in number within the lesions as demyelination progresses.¹³²

The most extensively studied and described demyelinated brain region after cuprizone treatment is the corpus callosum. However, cortical¹³³, cerebellar¹³⁴ and hippocampal¹³⁵ demyelination have also been described. The extent of demyelination and the brain regions affected and the dose of cuprizone required to induce demyelination can vary depending on the strain, gender and age of mice used.¹³⁶

The exact mechanism by which cuprizone induces the selective damage and death of oligodendrocytes is still unknown. Since administration of copper failed to prevent or reduce the effects of cuprizone, copper deficiency does not seem to be causing demyelination.¹³⁷ It has been hypothesized that cuprizone treatment leads to deficits in mitochondrial function, as indicated by enlarged mitochondria observed in oligodendrocytes¹³⁸ and the reduced activity of the mitochondrial enzymes cytochrome oxidase and monoamine oxidase.¹³⁹ This might lead to disturbed energy metabolism and functioning of highly metabolic active oligodendrocytes. Furthermore, the increase in microglia and the subsequent release of pro-inflammatory factors was found to be crucial in the process of oligodendrocyte death and consequent demyelination after cuprizone treatment.¹⁴⁰

HIPPOCAMPAL PATHOLOGY IN MS

Demyelinating lesions within the hippocampus of MS patients have been shown in *post mortem* tissue^{141–144} as well as using MRI.^{145,146} Significant volume loss of the hippocampus was observed both in RRMS as well as PPMS patients.¹⁴⁷ A significant decrease in CA1 volume in the hippocampus of MS patient has been associated with cognitive dysfunction.¹⁴⁸ Moreover, hippocampal functioning in MS patients is changed, i.e. cognitively impaired MS patients showed significantly less activation of the hippocampus during a memory encoding task, compared to healthy controls and cognitively preserved MS patients.¹⁴⁹ Lesions within the hippocampal GM areas are devoid of infiltrating immune cells, although increased activated microglia are observed surrounding the lesion.¹⁴² The fact that the hippocampus is composed of WM tracts in addition to GM, makes this brain structure well suited to study pathological differences between WML and GML. Moreover, its crucial role in learning and memory makes it an important target to study, since lesions within the hippocampus may be linked to dysfunction of learning and memory processes implicated in MS pathophysiology.

Hippocampal anatomy

The hippocampus is a subcortical GM structure located in the medial temporal lobe and is essential in the process of learning and memory. The hippocampus plays a crucial role in visual and spatial memory, which are amongst the most affected cognitive domains in MS patients.²⁰

The hippocampus is composed of WM tracts and GM areas. WM tracts include the stratum radiatum and the alveus. GM areas within the hippocampus are the dentate gyrus (DG), the subiculum, and the cornu ammonis (CA)1, CA2, CA3 and CA4 (see figure 3A).¹⁵⁰ A

mechanism instrumental to learning and memory formation is termed long-term potentiation (LTP). LTP is a phenomenon that describes the increased synaptic strength and transmission between afferent fibres and pyramidal cells after repeated synaptic stimulation.^{151,152} Several anatomical pathways are involved in LTP, e.g. the perforant pathway, the mossy fibre pathway and the Schaffer collateral pathway. The perforant pathway comprises connections between the hippocampus and the entorhinal cortex (EC), which supplies information from the cortex to the DG. The mossy fibre pathway propagates the information from the DG to the CA3 and, finally, information reaches the CA1 pyramidal cells through the Schaffer collaterals, which send the information back to the EC, either directly or via the subiculum (see figure 3B).^{153,154}

The hippocampus is anatomically connected to other brain regions involved in learning and memory, acting as a central node, receiving input from the cortex needed for memory acquisition, and sending projections back to the cortex needed for memory consolidation. The hippocampus receives input and sends projections to the perirhinal, the entorhinal, the parahippocampal and the prefrontal cortex.^{155–157} Input from the perirhinal and parahippocampal cortex to the hippocampus mostly goes through the EC, while projection from the hippocampus to the prefrontal cortex and the EC goes through the subiculum.¹⁵⁷ In addition, the hippocampus sends direct projections to the striatum and amygdala. Thus, demyelination within the hippocampus can disturb signal transduction from the hippocampus to other brain areas, thereby affecting the process of learning and memory.

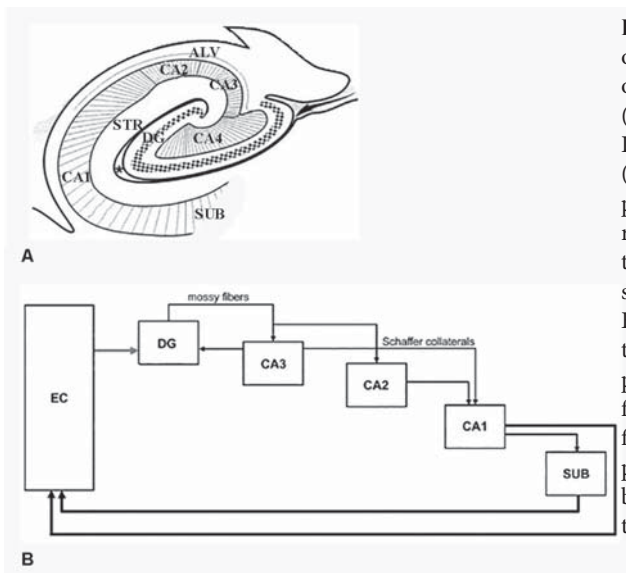


Figure 3 A) The hippocampus consists of WM and GM. The WM is composed of the alveus (ALV) and the striatum (STR). The grey matter is composed of DG, the CA1-CA4 and the subiculum. (Adapted from: 150). B) An important pathway by which the hippocampus receives and delivers information is the perforant pathway. Information is sent to the hippocampus through the EC, which supplies information from the cortex to the DG. The mossy fibre pathway propagates the information from the DG to the CA3 and, finally, information reaches the CA1 pyramidal cells. In turn, CA1 projects back to the EC, either directly or via the subiculum (Adapted from: 205).

Neurotransmitter systems involved in learning and memory

Within the hippocampus, several neurotransmitters and receptors are involved in learning and memory formation. Signaling of glutamate, the major excitatory neurotransmitter in the CNS, is crucial in successfully establishing a memory trace. Although research has focused mainly on glutamate signalling in the context of learning and memory, other neurotransmitters are also important in learning and memory. Glutamate, acetylcholine (ACh) and γ -aminobutyric acid (GABA) are highly interdependent and both ACh and GABA are known for their potentiating and inhibiting effect, respectively, on glutamatergic neurotransmission.^{158,159} The major neurotransmitters involved in learning and memory, their actions and receptors are summarized in table 1.

Although data on neurotransmitters in the hippocampus of MS patients is scarce, it has recently been shown that in MS patients glutamate levels in the right hippocampus were lower compared with healthy controls, which correlated with deficits in visuospatial memory.¹⁶⁰ In addition, mRNA encoding glutamate receptors, i.e. α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and N-methyl-D-aspartate (NMDA) receptors, was significantly reduced in demyelinated hippocampi.¹⁴¹ Moreover, GABA receptor expression was found to be increased in demyelinated hippocampi.¹⁴¹ This indicates that changes in multiple neurotransmitter systems are observed in MS hippocampus and might be involved in memory deficits occurring in this patient group.

Glutamate

Glutamate is the principal excitatory neurotransmitter in the mammalian CNS and is synthesized from glutamine or from glucose metabolites. In neurons, glutamate is produced from glutamine by phosphate-activated glutaminase (PAG).¹⁶¹ In addition, astrocytes are able to take up extracellular glutamate and convert it into glutamine by glutamine synthetase.¹⁶² The glutamine is then released and taken up by neurons which use it as a substrate for glutamate production. High concentrations of extracellular glutamate can be cleared from the synaptic cleft by astrocytes, thereby preventing excitotoxicity.¹⁶³ Two glutamate receptor families exist, i.e. metabotropic and ionotropic glutamate receptors (mGluR and iGluR, respectively). The iGluR family includes the NMDA and AMPA receptor subtypes.¹⁶⁴ For LTP to occur, a cytoplasmic increase in calcium has to be induced, which is often mediated by the activation of AMPA and NMDA receptors by glutamate.¹⁶⁵

Acetylcholine

Another key neurotransmitter in the process of learning and memory is ACh. Cholinergic neurons located in the basal forebrain, including, amongst other nuclei, the nucleus basalis of Meynert, the diagonal band of Broca and the medial septal nucleus, are the primary source of ACh in the cerebral cortex. The hippocampus receives the majority of its cholinergic projections from the medial septal nucleus, via the fimbria-fornix, and the vertical limb of the diagonal band,¹⁶⁶ although intrinsic cholinergic interneurons have also been described within the hippocampus.^{167,168}

Ach is synthesized from choline and acetyl coenzyme A by the cytosolic enzyme choline acetyltransferase (ChaT). This enzyme is crucial in the process of acetylcholine synthesis and is exclusively expressed in cholinergic neurons.^{169,170} When Ach is released into the synaptic cleft it binds to G-protein coupled muscarinic and ligand-gated nicotinic Ach receptors (mAChR and nAChR, respectively) on postsynaptic excitatory and inhibitory neurons.¹⁷¹⁻¹⁷³ Five subtypes of mAChR exist (M1-M5), of which the M1 and M2 types are the major postsynaptic mAChR in the hippocampus.¹⁶⁶ nAChR are composed of several subunits including α , β , σ and ϵ , of which the $\alpha 4\beta 2$ and $\alpha 7$ -containing nAChRs are the most common subtypes in the human brain.¹⁷⁴ In addition to mAChR and nAChR, Ach binds to the enzyme acetylcholinesterase (AChE), which is present in the synaptic cleft. AChE regulates the concentration of acetylcholine in the synaptic cleft by the inactivation of Ach by rapid hydrolysis.¹⁷⁵

Ach contributes to learning and memory by the stimulation of the synchronization of neuronal firing and is thus essential in the process of LTP. Binding of Ach to nAChR and/or mAChR potentiates the activation of excitatory or inhibitory neurons and is therefore a major modulating neurotransmitter.^{159,176,177} The crucial role of Ach in learning and memory is best illustrated in Alzheimer's Disease (AD), during which cholinergic neurons in the basal forebrain, their projections to the hippocampus and the number of AChR receptors are decreased, which is accompanied by severe learning and memory deficits.^{178,179}

γ -aminobutyric acid

The inhibitory neurotransmitter GABA is released by many cells in the CNS, primarily by interneurons. Various types of GABAergic interneurons can be distinguished, based on their differential staining for the calcium binding proteins parvalbumin (PV), calretinin (CR) and/or calbindin (CB).¹⁸⁰ GABA is released in response to action potentials, thereby modulating glutamatergic signaling. Since one GABAergic interneuron is able to project to large numbers of excitatory cells, they play a key role in the regulation of hippocampal excitability.¹⁸¹

GABA is produced in GABAergic interneurons by the enzyme glutamic acid decarboxylase (GAD). GAD converts glutamate into GABA by interacting with the co-factor pyridoxal phosphate. Two isoforms of GAD have been identified, i.e. GAD65 and GAD67. GAD65 is mainly localized in synaptic terminals, while GAD67 is present in both terminals and the cell body. In the hippocampus, GAD67 is the most prominently expressed isotype.^{182,183} GABA is released in response to action potentials and GABA exerts its effects by activating GABA-receptors. Two types of receptors exist, i.e. GABA-A and GABA-B receptors. The ionotropic GABA-A receptor consists of several subunits, including two α , two β -subunits, and one additional subunit, usually a ϵ , γ , δ or π -subunit. Together, these subunits form a chloride channel in the cell membrane.¹⁸⁴ In contrast, metabotropic GABA-B receptors are G-protein coupled receptors.¹⁸⁵ Activation of GABAergic receptors leads to hyperpolarization and inhibition of postsynaptic neurons. Furthermore, inhibition of GABAergic neurotransmission appears to be involved in the increase of LTP.^{186,187}

Cognitive deficits in MS

Nearly half of all patients diagnosed with MS suffer from cognitive dysfunction,^{20,188-190} of which memory impairment is one of the most frequently reported. Memory deficits tend to worsen over time¹⁹¹ and have a strong impact on the quality of life.¹⁹² Memory problems are already evident in patients recently diagnosed with clinically isolated syndrome (CIS), considered to be a very early phase of MS,¹⁹³ and in the early stage of MS including patients with a maximum disease duration of two years.¹⁹⁴ Types of memory that are affected during MS are diverse, ranging from explicit, conscious memory,¹⁹⁵ e.g. autobiographical memory¹⁹⁶ to verbal and visual memory.^{20,197} It has been suggested that problems during the acquisition or encoding phase of learning are responsible for deficits in long-term memory of MS patients,^{198,199} while deficits in recalling information have also been shown to be involved, although the latter was specifically found in patients with primary progressive MS.²⁰⁰ Moreover, memory deficits are more common during the progressive phase of MS compared to patients with RRMS.¹⁸⁹ No effective treatment for impaired cognition in MS has yet been developed. Symptomatic treatment can be in the form of pharmacological treatment, behavioral intervention or cognitive rehabilitation. Two types of pharmacological treatment exist, that is disease-modifying treatment (DMT), e.g. interferon- β and glatiramer acetate, and symptomatic treatment, e.g. acetylcholinesterase inhibitors like donepezil that specifically targets cognitive disturbances. A comparison between DMT and symptomatic treatment did not reveal significant differences in efficacy. However, until now the use of acetylcholinesterase inhibitors seems the most promising.²⁰¹ The cause of memory impairment in MS is currently unknown, but an increasing number of studies suggest that hippocampal pathology is involved.

Table 1. Major neurotransmitters involved in learning and memory

Neurotransmitter	Actions	Receptors
Glutamate	Excitatory	<ul style="list-style-type: none"> • Ionotropic receptors (AMPA and NMDA) • Metabotropic receptors
Acetylcholine (Ach)	Modulatory (Excitatory or inhibitory)	<ul style="list-style-type: none"> • Muscarinic Ach receptors • Nicotinic Ach receptors
γ -aminobutyric acid (GABA)	Inhibitory	<ul style="list-style-type: none"> • Ionotropic GABA-A receptors • Metabotropic GABA-B receptors

AIMS AND OUTLINE OF THIS THESIS

MS is neuropathologically characterised by inflammatory, demyelinating lesions. Besides WML, GML are often found in MS. WM consists mainly of axons enwrapped with myelin from oligodendrocytes surrounded by glial cells and ECM. WML can give rise to a variety of neurological symptoms, e.g. bowel and bladder dysfunction,²⁰² and motor and sensory deficits.^{14,203} In contrast to WM, GM is characterized by the presence of neurons surrounded by an extracellular environment consisting of glial cells and ECM. In the healthy brain, all these cell types and the matrix act together, providing an optimal environment for effective neurotransmission and neuronal survival. In MS, GML can disturb this environment leading to less accurate or even loss of communication between neurons, thereby contributing to neurological, neuropsychological and psychiatric symptoms of MS patients.

It is of interest that a remarkable pathological difference can be observed between WML and GML. Thus, GML in general show a lower number of activated microglia and less infiltration of immune cells compared to WML. We put forward that this may result from local differences in glial cell responsiveness. The hippocampus, consisting of both WM and GM, is very well suited to study differences between WML and GML in MS. In addition, the hippocampus is one of the key structures within the brain involved in learning and memory. Lesions therein are thought to contribute to cognitive disturbances seen in more than 50% of MS patients. Effective neurotransmission in the hippocampus is necessary for optimal memory function. In the hippocampus, several neurotransmitters control the process of learning and memory formation, in particular glutamate, Ach and GABA. Alterations in glutamate levels in cortex and hippocampus of MS patients have already been described in MS patients, and were found to correlate with memory deficits in these patients. However, no information is available on the status of the Ach and GABAergic neurotransmitter systems in the brain of MS patients.

Therefore, the two aims of the studies described in this thesis were

- 1) **to determine the glial cell responsiveness in GM and WM of MS patients.**
- 2) **to identify changes in cholinergic and GABAergic neurotransmitter systems in the hippocampus of MS patients.**

The first aim is addressed in chapters 2-4. First, in **chapter 2** we reviewed the inflammatory aspects and glial cell activity in MS WML versus GML, and present explanations for the observed differences. In **chapters 3** and **4** we focussed on two well characterized inflammatory mediators, i.e. IL-1 β and CCL2, which may play an important role in the differential pathology of WML and GML in MS. In this context, we addressed the following research questions:

- 1) Are IL-1 β and its endogenous receptor antagonist IL-1ra present in GML?
- 2) Is there a differential expression of CCL2 and its receptor CCR2 in WML versus GML?

The first question is addressed in **chapter 3**, which describes the presence of IL-1 β and IL-1ra in WML and GML in an experimental MS model, i.e. chronic relapsing EAE (cr-EAE) rats. The brain and spinal cord of cr-EAE rats were examined for IL-1 β and IL-1ra expression at

three different time-points during the early phase of cr-EAE.

To answer the second question we examined the presence of CCL2 and CCR2 in WML and GML using post-mortem hippocampal material of MS patients and control subjects. Furthermore, *in vitro* studies were performed with glial cells derived from WM or GM from rat brains to determine whether functional differences can be found. The results of these experiments are presented in **chapter 4**.

The second aim of the studies described in this thesis, i.e. the identification of changes in cholinergic and GABAergic neurotransmitter systems in the hippocampus of MS, are presented in **chapter 5** and **6**. To this end, we used post-mortem hippocampal material of MS patients and control subjects and studied ChaT and AchE as markers of the cholinergic system (**chapter 5**), and PV and GAD67 as markers of the GABAergic system (**chapter 6**).

Finally, in **chapter 7** the results of the previous chapters are summarised and discussed, and suggestions for future research are put forward.

