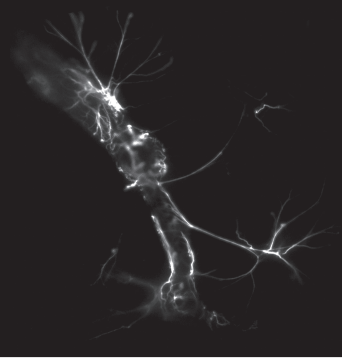
CHAPTER 6



General Discussion

The blood-brain barrier (BBB) is specialized to function as a barrier to protect the central nervous system (CNS) by restricting entry of unwanted molecules and immune cells into the brain and inversely, to prevent CNS-born agents from reaching the systemic circulation. The BBB endothelium, together with the CNS cells involved in its regulation form the neurovascular unit (NVU). BBB dysfunction is an important hallmark of early multiple sclerosis (MS) pathophysiology, leading to a consequent loss of the imperative CNS homeostasis. The unrestrained access of immune cells and blood-borne compounds into the CNS play a central role in demyelination and axonal damage, two major hallmarks of MS pathology underlying the clinical symptoms of patients. Since the aetiology of MS still remains an enigma, scientific advances that have led to new therapies have mainly brought forth disease dampening strategies. However, increasing scientific knowledge that leads to therapeutic means to halt the formation of new inflammatory lesions may improve the quality of life of patients with MS.

Although current therapeutic strategies for MS aim to dampen (systemic) inflammation and to prevent leukocytes from entering the CNS, no studies have been undertaken to address BBB repair or protection in an attempt to halt disease progression. This hiatus in neurological research might reflect the current knowledge on verified factors, whether endogenous or artificial, that can protect or repair the BBB *in vivo*. Recent literature and the work described in this thesis show that endogenous protect-and-repair strategies are induced in MS lesions following BBB disruption. Interestingly, both mechanisms derive from the developmental phase of the BBB. Future research might unravel novel ways to enhance the efficacy or temporal activation pattern of these endogenous protective pathways. Targets that restore the impaired function of the BBB are therefore a promising new therapeutic strategies, to be implemented alongside the existing immune-dampening therapies. In this chapter the results of the research described in this thesis will be summarized and discussed as a whole, including questions yet to be answered in this research field, followed by the future perspectives.

1. **Insights into the development and maintenance of the blood-brain barrier.**

The barrier between the CNS and the systemic circulation has been described over one hundred years ago, discovered by the observation that systemically injected dyes are excluded from the CNS, and vice versa. Since the discovery that this barrier was actually formed by endothelial cells, the obvious question was how these endothelial cells were instructed to form this highly specialized barrier. Transplantation studies showed that non-CNS vascular sprouts that invade embryonic neural tissue display BBB characteristics, and inversely, that neural tissue-derived vascular sprouts invading non-CNS tissue lose their BBB phenotype[1]. One of the specific characteristics of the brain endothelium is the absence of pan-endothelial marker plasmalemmal vesicle associated protein-1 (PLVAP). PLVAP is a transmembrane protein associated with transendothelial transport and the caveolae of the fenestrated microvasculature, and is developmentally silenced during BBB differentiation as described in **chapter 3** and by others[2]. The mechanism of PLVAP downregulation in endothelial cells during CNS development is not fully understood, and conflicting reports exist on the role of CNS pericytes in PLVAP regulation[3, 4].

Even though our current understanding of BBB development is far from complete, recent literature shows a rise in knowledge of CNS-specific cues that can drive BBB development.

Considering the overlap in guidance and differentiation cues of both the neuronal and vascular systems during CNS development[5, 6], and the involvement of radial glial cells in both processes, we investigated the effect of fetal astrocytes on brain endothelial cell (BEC) function. In **chapter 2** we describe the presence of retinoic acid (RA) in astrocyte-conditioned medium (ACM) derived from fetal astrocyte cultures. We furthermore show that the enzyme responsible for RA-production, retinaldehyde dehydrogenase (RALDH), is expressed by radial glial cells in the developing human CNS. The release of RA by radial glial cells has also been shown by others, as well as the dependence of neural progenitors on radial glia-derived RA as a differentiation factor[7]. However, our group is the first to report on the effect of radial glia-derived RA on BECs and BBB development, which is summarized in figure 1A. Combined with the recent report of sonic hedgehog (Shh)-induced barrier formation[8], these findings reflect the importance of astrocyte precursors in the induction of the BBB during CNS development. Another important consideration is the difference between human and mouse studies, when investigating developmental mechanisms. Most developmental studies incorporate the detailed investigation of the murine CNS, which does not always extrapolate well to the human situation, as exemplified by species-differences in CNS drug discovery[9]. We therefore also investigated the developing human CNS, in which microvascular responsiveness to RA was shown by the presence of endothelial RA receptor-β. Even though this is not final proof, it does indirectly show the involvement of RA signaling of BBB induction in the human CNS.
Further investigation of the effects of RA on human BECs *in vitro* showed a positive regulation of BBB-characteristics like high electrical barrier resistance (Rb) and increased expression of genes associated with high paracellular and transcellular resistance. However, *in vitro* studies using BECs and various culture conditions like cell-conditioned media, astrocyte and pericyte co-culture, or endogenous and exogenous ligands have led to a plethora of soluble molecules that are reported to increase BEC-function or barrier-related gene expression (see **chapter 1** §1). Whether all described soluble factors are involved in either development or maintenance of the BBB however, cannot be based on *in vitro* studies alone. We and others have shown the influence of RA, Shh[8], and Wnt/β-catenin[10, 11]-signaling systems on BBB development *in vivo* by investigating BBB function, morphology, immunohistochemistry, and gene expression. Furthermore, in vivo experiments have clearly established the importance of pericytes in BBB development[3, 4] and the importance of astrocytes in BBB maintenance[12, 13]. Continued efforts to understand the fundamental mechanisms behind BBB development and maintenance will widen our view on the possibilities to redefine or restore the barrier upon disruption, and is therefore a crucial part of disease-related BBB research.

2. Endothelial blood-brain barrier alterations in neuroinflammation.
The inflammatory changes that occur at the BBB in MS lesion formation are numerous and can vary depending on lesion stage and CNS location. The endothelial component of the BBB is in direct contact with the systemic circulation, which not only results in the recognition of CNS inflammatory sites by the peripheral immune system, but also results in altered communication of BECs with the systemic circulation via the shedding of microparticles[14]. Although the latter is a relatively young field in neuroinflammation and will require more investigation regarding its significance in the pathophysiology of MS. The classically described endothelial changes at the BBB during neuroinflammation include the expression of various

cell adhesion molecules (CAMs) such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin, under influence of pro-inflammatory cytokines. Activated leukocytes express the integrin counterparts for endothelial CAMs, ensuring a close interaction and eventually, transendothelial migration of leukocytes. The importance of CNS microvascular VCAM-1 expression in leukocyte transmigration in MS is clearly demonstrated by the ability to reduce disease progression by blocking VCAM-1-VLA-4 interaction with Natalizumab[15].

BECs do not only react to a pro-inflammatory environment by expressing adhesion molecules, but actively take part in the inflammatory process through the expression of pro-inflammatory cytokines (IL-6) and chemokines (CCL2), as described in **chapter 5**. Endothelial expression of CCL2 and other chemokines has also been confirmed in MS lesions[16]. The active involvement of brain endothelial CCL2 expression in neuroinflammation has further been demonstrated in the animal model for MS, experimental autoimmune encephalomyelitis (EAE), where the lack or suppression of endothelial CCL2 expression suppresses the clinical signs of EAE[17, 18]. A highly similar finding was reported in mice lacking IL-6 expression, where the lack of IL-6-induced upregulation of brain endothelial expression of ICAM-1 and VCAM-1 resulted in the inability to develop EAE symptoms.

BBB damage in neuroinflammation in MS also encompasses the loss of the structural and metabolic barrier, resulting in uncontrolled exchange of possibly harmful bloodstream components between the systemic circulation and the CNS parenchyma. **In chapter 4**, we show that the vascular expression of P-glycoprotein (P-gp), one of the four most described ATP binding cassette (ABC) transporters on BBB endothelium, is decreased in MS lesions compared to NAWM. Surprisingly, the expression of three other major ABC transporters remained unchanged, suggesting that endothelial P-gp expression is selectively affected by neuroinflammatory changes in MS. Previous work from our group already identified a role for activated CD4-positive T-cells in the regulation of endothelial P-gp expression[19]. To what extent the loss of P-gp results in the accumulation of harmful compounds in the brain is not clear and warrants further research, although it is likely that the efflux system of the BBB endothelium manages to cope due to a high level of ligand-redundancy between different ABC transporters[20]. Of note, the disruption of the solute barrier in EAE has been shown to precede the breakdown of the cellular barrier[21].

The loss of BBB integrity in MS can be assessed *in vivo* by the use of Gadolinium diethylenetriaminepentacetate (Gd-DTPA) as a contrast agent. Focal Gd-enhancement is often found to precede immune cell infiltration into the lesion[22], and lesions usually show transient Gd-enhancement for a time period of one month[23]. Since the return of BBB integrity, as measured by the disappearance of Gd-enhancement, does not coincide with a decrease of immune cell passage or clinical symptoms in MS patients[23, 24], regulation of barrier integrity and the facilitation of immune cell migration seem to be two separate systems in BECs that contribute to CNS damage during neuroinflammation.

Detecting BBB damage or loss of integrity with immunohistochemistry *ex vivo* often proves to be a more challenging task. The detection of serum proteins like fibrinogen or IgG in the CNS parenchyma can be used as an indicator of BBB disruption, but will reflect a relatively recent increase in permeability due to clearance of serum proteins by microglia. The inflamed BBB can furthermore be detected by the increased immunoreactivity for ICAM-1 or VCAM-1. We describe a new marker for the inflamed BBB in EAE in **chapter 3**, where

PLVAP expression is only found in the brain vasculature of EAE animals, in close association with inflammatory lesions. PLVAP expression in the CNS microvasculature has also been described as a marker for BBB disruption in acute brain ischemia, Alzheimer's disease, and malignant brain tumors in both human and mice studies[25-27]. Since normal PLVAP function involves promoting transendothelial transport, it is surprising that BECs respond to neuroinflammation by re-expressing this marker for the immature brain microvasculature and non-CNS endothelium. An interesting possible explanation for this observation is that BBB damage in neuroinflammation results in the loss of normal BBB-maintenance factors from the surrounding CNS, or the loss of endothelial receptivity for these factors. This would then lead to a state of de-differentiation and a consequent loss of BBB-specific properties in the affected endothelium. Although this explanation requires more supporting evidence, it could explain the protective effect of BBB developmental pathways in neuroinflammation, described in paragraph 3.2.

The exact mechanism leading to BBB integrity loss during neuroinflammation is still not fully understood. Interestingly, the lack of various pro-inflammatory mediators like TNFα or IFNγ in EAE animals alters the composition and amount of infiltrating immune cells in spinal cord lesions, but not the increase in BBB permeability[28]. Furthermore, this study shows that EAE disease severity is directly correlated with BBB permeability. This suggests that the loss of BBB integrity is a requisite for EAE development and can occur independently of classical pro-inflammatory mediators, possibly by the direct interaction with activated leukocytes. Immune-activated brain endothelium can furthermore promote barrier disruption by the expression of matrix metalloproteinase (MMP)-9[29], an extracellular matrix (ECM)-degrading enzyme that has been associated with the specific breakdown of the glia limitans during leukocyte infiltration in EAE[30]. Although MMPs expression during neuroinflammation is usually attributed to immune cells, BECs highly increase the expression of both MMP2 and MMP9 upon activation[31]. Blocking MMP-activity with fluoxetine after spinal cord injury resulted in the prevention of BBB disruption, as well as reduced infiltration of immune cells in vivo[32]. In the same study fluoxetine was also shown to decrease MMP9 expression in BECs. Endothelial BBB changes under inflammatory conditions further include the increased production of reactive oxygen species (ROS) described in **chapter 5**, which in turn can destabilize tight junction (TJ)-complexes and lead to loss of BBB integrity[33].

In **chapters 4** and **5** we mimic the human BBB *in vitro* and show that BECs in a pro-inflammatory environment reflect most of the changes in function as found in MS pathology, including increased permeability, decreased barrier integrity, the expression of CAMs and pro-inflammatory mediators, and the increased transmigration of leukocytes. This reflects the suitability of this model to investigate the protective potential of both endogenous and exogenous factors on BECs under neuroinflammatory circumstances.

3. Astrocyte-endothelial interaction alterations in neuroinflammation.

The role of astrocytes at the BBB during neuroinflammation has been investigated extensively due to the importance of astrocyte-endothelial interaction in the healthy CNS. If we consider the astrocyte as a product of its environment, a healthy CNS already provides numerous different microenvironments for astrocytes to react to. Besides their close interaction with the endothelial cells of the BBB described in this thesis, astrocytes are involved in most synaptic processes, communicate with surveilling microglia and myelinating

oligodendrocytes, and are in constant contact with neighbouring astrocytes through gap junctions. Furthermore, due to differences in cell composition and functional demands the grey and white matter regions display two distinct astrocyte phenotypes in rodents, ramified protoplasmic astrocytes in grey matter and fibrous astrocytes in the white matter[34]. In the human CNS, even more astrocyte subtypes are described, based on the specific location within the neocortex, and both fibrous and protoplasmic astrocytes are two to three times larger than their rodent counterparts[35]. This has led to the notion that the increased complexity and size of human astrocytes has paved the way for the increased functional competence of the human brain compared to other species. When the complexity and diversity of human astrocytes is combined with all described facets of neuroinflammation, documenting possible changes of astrocyte-endothelial interactions can become a daunting endeavour. The focus will therefore mainly lie on the description of neuroinflammatory changes occurring at the BBB in white matter MS lesions. Differences between white and grey matter pathology, in view of BBB disruption and astrocyte involvement will be further discussed in paragraph 4 of this discussion. A schematic overview of the altered astrocyte-endothelial interaction described in this thesis is depicted in figure 1B and C.

3.1 Reactive astrocytes actively contribute to neuroinflammation at the BBB.

Reactive astrocytes are described as active participants in neuroinflammation, and have the capacity to produce a wide range of pro-inflammatory cytokines and chemokines that can influence BBB function and contribute to the attraction of immune cells into MS lesions. Reactive astrocytes are a notable source for the pro-inflammatory chemokine CCL2[36], shown to be crucial in the recruitment and activation of myelin-degrading macrophages. The exact moment of astrocyte activation in MS is currently difficult to pinpoint, although astrocyte activation was observed before infiltration of leukocytes in EAE[37], possibly due to the early disruption of the BBB resulting in toxic molecule entry into the CNS[38].

As was previously described, the pathophysiology in MS lesions results in decreased microvascular P-gp expression. In the same study, described in **chapter 4,** where we investigated the expression patterns of the four major ABC-transporters we show that P-gp and MRP-1 expression is increased in reactive astrocytes in MS lesions. Initially thought to provide a possible complementary drug resistance barrier in areas of BBB disruption, *in vitro* experiments surprisingly revealed a different role for ABC-transporter expression by reactive astrocytes. The increased expression of P-gp and MRP-1 was found to mediate the release of CCL2 under inflammatory conditions. A likely mediating mechanism was proposed recently, wherein pro-inflammatory bio-active lipids transported by P-gp may induce the activation of CCL2 release in an autocrine fashion[39]. This was demonstrated by blocking P-gp and MRP-1 on reactive astrocytes *in vitro*, resulting in a decrease of CCL2 release and monocyte migration. The active involvement of astrocyte-derived CCL2 in immune cell infiltration has also been confirmed *in vivo*[40, 41]. Besides attracting leukocytes to the lesion, reactive astrocyte-derived CCL2 might also directly influence BECs by inducing TJ-complex degradation and a consequent disruption of barrier integrity[42, 43]. Further loss of BBB integrity might be the result of the previously mentioned activation of MMPs that can degrade the glia limitans during neuroinflammation. Reactive astrocytes increase MMP9 expression[44,45], further implicating reactive gliosis in the breakdown of glia limitans-specific ECM and reduced astrocyte endfeet anchoring to the basement membrane[30]. Once activated

leukocytes cross the glia limitans and invade the CNS parenchyma, reactive astrocytes are thought to facilitate their migration by interacting with leukocyte-integrins through the expression of VCAM-1 in EAE [46], and the expression of a splice variant of fibronectin, connectin segement-1 (CS-1), detectable in astrocytes at MS lesion rims[47]. Finally, astrocytes in neuroinflammatory foci in EAE showed massive loss of connexin 43 (Cx43) expression, which was hypothesized to result in decreased communication between neighbouring astrocytes, and linked to axonal dystrophy[48]. The astrocyte-endfeet specific potassium channel Kir4.1, normally highly expressed by perivascular endfeet, was unaffected in EAE, but was shown to be downregulated upon primary astrocyte activation by IL1β *in vitro*[49]. Since both Cx43 and Kir4.1 are essential in the homeostasis at the BBB, we were surprised to find a marked increase of both Kir4.1 and Cx43 immunoreactivity in active MS lesions, as well as increased gene expression in primary astrocytes upon activation with the toll-like receptor 3 agonist PolyI:C (**chapter 3**). The enhanced expression of Cx43 in MS lesions was recently confirmed by others[50], and raises the question whether increased Cx43 expression in reactive astrocytes in MS results in enhanced communication and whether enhanced communication has a beneficial or a detrimental role in MS pathophysiology. Besides the formation of gap junctions between astrocytes, Cx43 forms hemichannels resulting in enhanced exchange to the extracellular space[51]. Increased Cx43 hemichannel formation in this study was associated with promoting neuronal degeneration during NMDA-induced cytotoxicity The increased expression of Kir4.1 in active MS lesions was furthermore not reflected by EAE pathology.

Based on the alterations described for reactive astrocytes, it seems that these cells drive the neuroinflammatory damage of the BBB in MS lesions. As a consequence, dampening reactive gliosis in MS might halt BBB disruption as well as disease progression. However, in an animal model where reactive, proliferating astrocytes where specifically ablated during EAE pathogenesis, the protective and inflammation-regulatory role of reactive astrocytes was clearly demonstrated[52]. Animals that lacked an astroglial response to neuroinflammation in EAE showed increased disease progression and severity and increased numbers of CNS-infiltrated immune cells. Although the direct effect of reactive astrocytes or gliotic scar formation on BBB function was not addressed, it seems that astrocyte activation can both contribute to and dampen neuroinflammation in EAE and possibly MS.

3.2 Reactive astrocytes protect the BBB during neuroinflammation.

The astrocytic response to neuroinflammation is not restricted to detrimental effects on the surrounding cells, but also reflects protective aspects. Therefore, dampening the reactive state of astrocytes to reduce detrimental effects, might also result in the reduction of protective and anti-inflammatory effects, necessary for regeneration and repair. A better understanding of the inflammatory pathways resulting in the various astrocytic responses is therefore warranted to separate the detrimental and beneficial effects of the reactive phenotype on the BBB, as well as on other CNS cell types.

Protective or immune-dampening effects of reactive astrocytes have been described on various facets of neuroinflammation. Astrocytes at the BBB are known to induce T-cell apoptosis via the expression of death ligand CD95L on perivascular endfeet[53], the release of astrocyte-derived immune suppressor factor[54], and the generation of osteonectin-containing ECM[54]. Furthermore, the previously described (perivascular) scar formation by

reactive gliosis was proposed as a mechanism to limit immune cell infiltration and thereby ongoing neuroinflammation in MS lesions[55].
Interestingly, developmental pathways involved in BBB development are now emerging as possible protective mechanisms to reduce BBB damage in neuroinflammation, recently illustrated by the increased expression of Shh by reactive astrocytes in MS lesions[8]. Shh-signaling results in inflammation-dampening effects at the BBB, and also shows barrier-enhancing properties. In **chapter 5** we describe that RA synthesis by astrocytes re-emerges during neuroinflammation in MS pathology. RA has been shown to possess anti-inflammatory[56] and neuroprotective effects[57] in the CNS under neuroinflammatory conditions. Since we showed the involvement of glial-derived RA during BBB development, we focused on the possible protective effects of RA on the inflammation-induced disruption and activation of the BBB endothelium. Taken together, our data point towards RA as an anti-inflammatory signaling molecule released by reactive astrocytes in response to inflammation, capable of dampening endothelial immune activation *in vitro.* So far, we have described immune-dampening effects of RA on inflammation-induced expression of CAMs and pro-inflammatory cytokines and chemokines, inflammation-induced loss of the structural barrier, and the production of ROS. Although the relevance of endogenous RA-signaling *in vivo* remains to be investigated, the presence of the machinery needed for RA-synthesis by reactive astrocytes in MS pathology has been demonstrated. Furthermore, RA-signaling in the CNS represents an interesting endogenous protective mechanism with therapeutic potential. EAE clinical signs are inhibited by treatment with a synthetic RA analogue[58] and RA dampens the pro-inflammatory response of astrocytes and microglia. Furthermore, RA is neuroprotective and has been shown to enhance both remyelination[59], and neurite outgrowth[60] *in vivo*, after CNS injury. Interestingly, a recent report described an inverse correlation between serum retinol levels and newly formed Gd-enhanced lesions in relapsing-remitting MS patients, suggesting that increasing bio-availability of the precursor for RA might limit lesion formation.
The association of other pathways that have been associated with BBB development, the Wnt/β-catenin pathway[10, 11] and the early association of CNS pericytes with the developing BBB[3], with MS or EAE pathology remains to be investigated. Restarting developmental programs at the disrupted BBB might be an intrinsic mechanism to reinstate the barrier during or after neuroinflammation. Unraveling ways of boosting this self-regenerative capacity of the CNS to repair BBB disruption shows significant promise as a possible novel therapeutic avenue in MS.

4. Concluding remarks and future perspectives.
The BBB endothelium, together with all cell types involved in the NVU, ensures a tightly regulated CNS homeostasis that is crucial for normal brain function. A dysfunctional BBB is an early hallmark of MS lesion formation, and therefore represents an important target structure for the discovery of new disease modifying drugs for MS. A better understanding of the process leading to BBB dysfunction and the resulting alterations, as well insights in the mechanisms underlying BBB development and maintenance in the adult brain are therefore crucial to discover pharmaceutical targets to improve BBB function in MS.
The work described in this thesis has expanded our understanding of the regulation of the BBB during CNS development and during neuroinflammation, as occurs in MS. We have

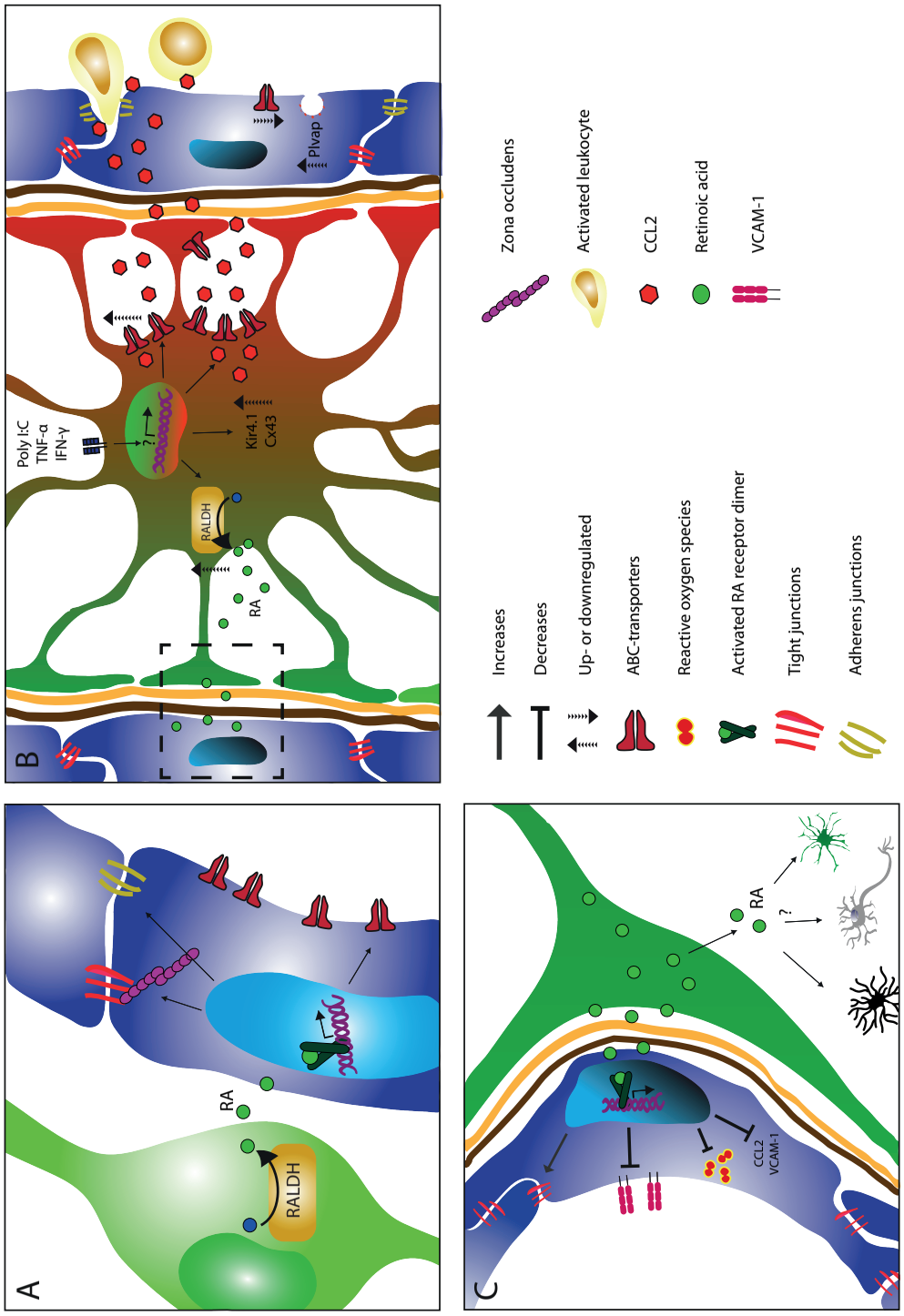


Figure 1. Schematic summary of the work described in this thesis.
A. During CNS embryogenesis, radial glial cells express RALDH, the key enzyme for RA-synthesis. Radial glial cell-derived RA activates RA receptors in immature brain endothelial cells, resulting in enhanced expression of ABC-transporters, tight and adherens junction complexes, and an overall increase in barrier intergrity. **B.** Reactive astrocytes contribute to the disruption (right hand side) as well as the protection (left hand side) of the blood-brain barrier. The increased expression of P-gp mediates the release of CCL2, which in turn attracts more leukocytes into the CNS. The increased expression of RALDH2 in reactive astrocytes results in the release of RA, able to activate RA receptors in brain endothelial cells. **C.** A detailed view of the boxed area in 1B depicts the effects of astrocyte-derived RA on the inflamed brain endothelium. RA activates RA receptors in brain endothelial cells, and counteracts inflammation-induced changes by increasing barrier function, downregulating inflammaotry genes, and increasing the anti-oxidant response. The effect of locally released RA on microglia, neurons, and astrocytes during neuroinflammation remains to be investigated.

uncovered a new BBB-inducing signal in the form of fetal astrocyte-derived RA, which we were able to confirm *in vivo* for murine BBB development. Furthermore, RA signaling seems to be conserved in human BBB development based on *ex vivo* investigation of RA receptor expression. Our investigation of the BBB and ABC-transporters in MS pathology has led to increased knowledge on the neuroinflammatory changes in ABC-transporter expression, and revealed a novel role for ABC-transporters in the release of the pro-inflammatory chemokines CCL2 by reactive astrocytes. The investigation of BBB damage and astrocyte-endothelial interaction in EAE has identified PLVAP expression by the BBB endothelium as a marker for neuroinflammatory damage, which could reflect an immature state of the damaged BBB. The same study also showed discrepancies between EAE and MS in the astrocytic response to neuroinflammation. Lastly, our investigation of the anti-inflammatory properties of RA has revealed a beneficial role for RA in inflammation-induced BBB damage and immune activation. Furthermore, we have described a CNS endogenous source of RA by showing that RA-synthesis is a direct effect of an inflammatory environment in human astrocytes, which was further corroborated by the marked increase of RALDH in MS lesion astrocytes.

To date, astrocyte-targeting therapies have not been designed for MS treatment. Interestingly, a number of existing and potential MS therapeutics including Interferon-β, the sphingosine-1-phosphate antagonist Fingolimod, and synthetic glucocorticoids are thought to exert their beneficial effects in part, through the modulation of astrocyte activation (reviewed in ref[61]). Considering the previously described exacerbating effect of reactive astrocytes in neuroinflammation, it is tempting to speculate that reducing or preventing astrocyte activation in MS might lead to the inhibition of lesion formation. However, as we and others have shown, the astrocytic response to an inflammatory environment not only encompasses detrimental effects, but also results in immune-dampening and BBB-restoring actions. Therefore, any pharmacological modulation of astrocyte activation would have to incorporate both the reversal of detrimental mechanisms at play, as well as enhance the beneficial effects of activation. The work described in this thesis also shows that a pro-inflammatory environment activates mechanisms in astrocytes that exacerbate neuroinflammation, but also induces protective pathways with inflammation-dampening effects. Because this apparent dual role of reactive astrocytes will become an important factor to consider when thinking of astrocytes as therapeutic targets, research within this field will have to focus on the simultaneous investigation of both sides of the inflammatory cascade. Furthermore, a clear consensus on what comprises a reactive astrocyte is needed to distinguish between the early response to inflammation and a possible late response

involved in lesion containment and scar formation. This especially holds true for *in vitro* studies of astrocyte behavior, where various inflammatory mediators lead to different reactive astrocyte phenotypes and their resulting impact on the BBB endothelium.
Another difference in astrocyte phenotypes that is often overlooked in *in vitro* investigation is the CNS location-specific phenotype. Protoplasmic (grey matter) and fibrous (white matter) astrocytes associate closely with the CNS microvasculature, but are distinct in cellular morphology and their functions in neuronal support. In recent years, grey matter pathology in MS was shown to be present in the early stages of disease and to correlate well with clinical symptoms. In contrast to white matter MS pathology, BBB disruption and immune cell infiltrates are thought to play minor roles in grey matter pathophysiology[62]. Nonetheless, in a chronic EAE model that involves subpial cortical demyelination, subtle TJ complex changes and leakage of FITC-dextran into the parenchyma was associated with areas of cortical demyelination, as was reactive astrogliosis and microgliosis[63]. In line with findings in grey matter MS lesions, parenchymal immune cell infiltration was not observed. The absence of leukocyte infiltration might reflect a more potent immune-suppressing environment in cortical neuroinflammation. The subtle changes in the BBB in cortical inflammation in EAE might contribute to astroglial and microglial activation, and might also play a role in cortical demyelination in MS. Although junction alterations have been described in normal appearing grey matter in secondary progressive MS patients[64], there is no knowledge of changes in solute barrier function and thus more effort is required to assess the causal role of BBB damage in cortical neuroinflammation in MS. Dedicated *in vitro* studies might further shed light on differences in the astrocytic response to either serum components or inflammatory cytokines.
In recent years it has become increasingly clear that the involvement of pericytes in BBB regulation is of significant importance. Not only are pericytes crucial in the development of the BBB but their involvement in neurodegenerative disorders recently became apparent trough the investigation of pericytes-deficient animal models. Considering the embedded location of pericytes within the endothelial basement membrane, and their extensive coverage of the CNS microvasculature, pericytes seem to be ideal candidates to monitor endothelial cell function and to communicate with perivascular astrocytes. This “tripartate” regulation of the BBB warrants further investigation in both animal models of neuroinflammation, as well as in *in vitro* models of the BBB.
In conclusion, this thesis reflects that our current knowledge of BBB dysfunction in MS, and the close relationship with activated astrocytes in neuroinflammation is increasing at a fast pace. The findings in this thesis have contributed to the knowledge in this field and contribute to the notion that modulation of astrocyte behavior at the BBB might serve as novel therapeutic avenue in the treatment of MS. It furthermore illustrates that the continued combination of fundamental and applied scientific research is needed to elucidate the role of BBB alteration in cortical MS pathology, and that lessons from BBB development could continue to uncover novel protective mechanisms for BBB damage in neuroinflammation.

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