SUMMARY AND DISCUSSION

CHAPTER 6

Several neuroinflammatory and neurodegenerative diseases are associated with blood-brain barrier (BBB) dysfunction. In particular during multiple sclerosis (MS), a chronic inflammatory disorder of the central nervous system (CNS), impaired function of the BBB is an early hallmark of lesion development due to a resulting influx of immune cells subsequently contributing to neurological damage and clinical symptoms. Therefore, preventing BBB dysfunction and/ or restoring BBB integrity in neuroinflammatory and neurodegenerative diseases associated with an impaired BBB may contribute to improved health outcomes. Better comprehension of molecular mechanisms and of involvement of additional cells of the CNS concerning BBB integrity is imperative to reveal new targets for treatment of MS. Studies described in this thesis were conducted to gain more insight into MS pathogenesis and more specific into mechanisms involved in BBB regulation. Below, the results are summarized and discussed.

TETRASPANINS REGULATE LEUKOCYTE TRANSMIGRATION INTO THE CNS

An essential function of the BBB is to regulate immune cell migration into the CNS during health and disease. The BBB is composed of highly specialized endothelial cells (ECs) that line the vessel wall forming a tight barrier by expressing tight junction proteins and membrane efflux pumps. ECs are enclosed together with pericytes within the basement membrane onto which astrocytes firmly project their endfeet. Together with neurons and microglia these cellular components make up the so-called neurovascular unit, which ensures optimal protection of the CNS from harmful compounds and cells thereby closely regulating its homeostasis. An early event in MS lesion formation is the influx of immune cells into the CNS. Once in the CNS, mainly monocyte-derived macrophages and T-cells can induce their damage resulting in demyelination and axonal loss. Migration of immune cells into the CNS is not a random process but a complex interplay between cells of the neurovascular unit and the immune cells. Essential steps in immune cell transmigration are tethering, rolling, adhesion, firm adhesion, and subsequent transmigration[1]. Each step in this process involves different key molecules with a.o. the vascular endothelial cell adhesion molecules (VCAM)-1 and Intercellular adhesion molecule (ICAM)-1 being essential in the tethering and rolling process. However, integrins, cytokines, chemokines and selectins are all essential in the transmigration process. Previous studies from our group and others have shown that tetraspanins are involved in the transendothelial migration process[2,3]. One mechanism by which tetraspanins contribute to leukocyte transmigration is through formation of tetraspanin microdomains (Tems)[4]. Tems are assembled signaling complexes of tetraspanins with cell adhesion molecules, integrins, and growth factor receptors and as such are regulators of processes such as migration, proliferation, cell adhesion and cell motility. However, expression of tetraspanins at the BBB and the potential role of other tetraspanins during neuroinflammation are lacking. In **chapter 2** we therefore studied the cellular distribution of tetraspanins in MS brain tissue and determined *in vitro* regulation of tetraspanin expression under inflammatory conditions. Subsequently, we determined the functional role of tetraspanins with respect to endothelial barrier function and transendothelial resistance. In our study, tetraspanins CD9 and CD81 and were expressed in the CNS. CD9 and CD81 were expressed by the vasculature while both molecules also showed extensive parenchymal expression. In vitro, expression of tetraspanins on the brain endothelial cell line hCMEC/D3 substantiate these findings as they express both CD9 and CD81 with concentrated expression at cell-cell contacts. Inflammatory conditions upregulate expression of endothelial CD9 but not CD81 and no regulation was found in human astrocytes. Functionally, endothelial CD9 and CD81 are involved in transendothelial migration of monocytes. Finally, blocking CD9 but not CD81 promotes endothelial barrier function, indicating that differential signaling process may underlie the tetraspanins.

By blocking endothelial CD9 and CD81 with monoclonal antibodies we observed reduced human monocyte migration through endothelial monolayers. In addition, a previous study from our group demonstrated that targeting the tetraspanin CD81 with monoclonal antibodies limits monocyte transmigration and

reduces EAE[5]. Tetraspanins localize to endothelial docking structures formed upon adhesion of leukocytes and the above findings are substantiated by a study that besides homophilic (CD9-CD9) and heterophilic (CD9-CD151) interactions demonstrates preferential interaction of CD9 with ICAM-1 and CD151 with VCAM-1 within endothelial adhesive platforms (EAPs)[6,7]. By using a CD9-blocking peptide this study demonstrates the importance of CD9 interactions within EAPs with respect to clustering of receptors and subsequent adhesiveness, a mechanism which may underlie our observed results concerning monocyte migration[8].

We show that by blocking CD9 on endothelial cells transendothelial resistance of endothelial cell monolayers increases. It has been shown that CD9 associates with β1-integrins and regulates their activity since CD9 silencing experiments showed inhibition of β1-integrin ligand binding[9]. Silencing of CD9 resulted in a reduced capacity of cell invasion of human cancer cells[10]. In concordance, in human microvascular endothelial cells it has been demonstrated that silencing CD9 expression inhibited both VEGF- and HGF-induced migration and invasion of these cells. Moreover, these outcomes were attributed to abnormal localization of integrins possibly resulting in prevention of angiogenesis[11]. An additional study in endothelial cells confirms a critical role of β1-integrins in capillary tube formation[12]. By blocking CD9 on endothelial cells angiogenic pathways may become suppressed which may translate into the observed increase in transendothelial resistance.

FTY720P EXERTS AN ANTI-INFLAMMATORY EFFECT IN THE CNS THROUGH ASTROCYTES

FTY720 (Gilenya, Novartis) is the first oral drug for treatment of RRMS which was approved by the Food and Drug Administration (FDA) in September 2010. The synthetic drug is derived from a natural occurring compound called myriocin produced by the fungus *Isaria sinclairii*. Upon oral administration FTY720 is readily phosphorylated after which it is capable to bind to all S1P receptors but $S1P_2$[13]. First, the drug was studied in clinical trials concerning organ transplantation due to the immunosuppressive property of the compound. Later on the drug was tested in clinical trials concerning RRMS and demonstrated efficacy in two large phase III trials (FREEDOMS, TRANSFORMS)[14,15]. Efficacy of FTY720 in RRMS is attributed to its effect on lymphocytes. Binding of FTY720P to its receptor $S1P_1$, expressed by lymphocytes, results in internalization of the receptor leaving the cells unresponsive towards a physiological S1P gradient and are therefore sequestered in secondary lymphoid tissues[16]. Resulting lymphopenia is thought to directly contribute to the observed beneficial effect of FTY720 in treatment of RRMS[17]. However, S1P receptor expression is not restricted to immune cells, they are differentially expressed by many tissues and cell types[18]. This dispersed expression of S1P receptors and the fact that FTY720 is a lipophillic molecule, and thus able to cross the BBB, led us to investigate S1P receptor expression in control brain tissue and in MS brain tissue in **chapter 3**. In our study we demonstrate that astrocytes express both $S1P_1$ and $S1P_3$ in control brain tissue. In MS brain tissue astrocytes increase $S1P_1$ and $S1P_3$ expression in both active and inactive MS lesions compared to control brain tissue and NAWM. *In vitro*, human astrocytes increase mRNA levels of both $S1P_1$ and $S1P_3$ under inflammatory conditions (TNF-α) confirming the previous finding concerning astrocytic S1P receptor expression in MS lesions. In the same experimental setup, astrocytes respond towards FTY720P in an anti-inflammatory manner suggesting a novel anti-inflammatory mechanism of FTY720P in the CNS, through inducing an anti-inflammatory effect on astrocytes through $S1P_1$ and $S1P_3$.

Recently, it was shown that in conditional null mouse mutants lacking $S1P_1$ on GFAP-expressing astrocytes in which EAE was induced, the beneficial effect of FTY720 was abated. All conditional null mutants displayed normal lymphocyte trafficking supporting the suggestion of a nonimmunological CNS mechanism for FTY720 in EAE[19]. Our results provide new data showing that human astrocytes under inflammatory conditions respond to FTY720 in an anti-inflammatory manner by reducing the secretion of MCP-1. Reactive astrocytes contribute to MS pathology in more than one way, they reduce BBB function, secrete pro-inflammatory cytokines and chemokines, inhibit remyelination and axonal sprouting, and regeneration by glial scar formation [20-22]. Astrocytes have increased MCP-1 mRNA levels during EAE and produce MCP-1 in MS[23,24]. Concerning BBB integrity, it is demonstrated that MCP-1 increases BBB permeability through disruption of TJ complexes between brain endothelial cells[25,26]. The clinical effect of reduced MCP-1 signalling is demonstrated in a study using CCR2 (-/)-mice which are resistant to EAE upon induction with MOG[27]. Therefore, dampening the inflammatory response of astrocytes by lowering the producton of MCP-1 through FTY720P administration is a promising new avenue to fight ongoing neuro-inflamation in MS.

ASTROCYTES DEMONSTRATE A DISTURBED SPHINGOMYELIN CYCLE IN ACTIVE MS LESIONS

The BBB confines both transcellular and paracellular passage of cells and molecules into the CNS leading to its foremost function of maintaining homeostasis in the CNS microenvironment. Astrocytes play an important role in BBB regulation since they are capable of regulating barrier function through several mechanisms. First, it is reported that astrocytes contribute to barrier formation by increasing tight junction formation[28]. Second, astrocytes mediate expression, functionality and polarized localization of transport proteins in endothelial cells[28-30]. And finally, astrocytes can increase functionality and expression of enzyme systems in endothelial cells[31,32]. In addition, astrocytes are equipped with an elaborate antioxidant enzyme system enabling them to scavenge reactive oxidant species (ROS) thereby preventing formation of lipid peroxidation products which are potent barrier disrupting molecules[33]. In recent years, evidence is emerging that the bioactive sphingolipid ceramide is involved in several pathologies including; diabetes, hereditary sensoric neuropathy type I, Batten's syndrome, Wilson's disease, and infectious diseases[34-41]. Ceramide is considered a pro-inflammatory sphingolipid and as such its role in MS and BBB regulation remains largely unknown. Therefore, in **chapter 4** we studied ceramide expression and cellular distribution of key enzymes involved in sphingolipid metabolism in MS lesions. In addition, we assessed whether FTY720P is a potent sphingomyelin cycle modulator under inflammatory conditions. Ceramide analysis on brain tissue comprising non-neurological control tissue, NAWM tissue, and active MS lesion tissue showed a strong reduction in total ceramide levels in active MS lesion homogenates compared to non-neurological controls. Interestingly, quantitative analysis of specific ceramide subspecies showed an increased ratio of C16/18 ceramide to C24 ceramide. Astrocytes in active MS lesions were determined to be the cellular source of these ceramide species. Observed increase in astrocytic ceramide was substantiated by the expression pattern of enzymes involved in sphingolipid metabolism, which was found to favor ceramide production in astrocytes in active MS lesions. FTY720P was able to downplay the inflammatory status of astrocytes by reducing ceramide levels in these astrocytes. Subsequently, in context of BBB functioning, this translated in reduced monocyte migration through endothelial cell monolayers exposed to supernatants of the treated astrocytes, demonstrating a novel anti-inflammatory mechanism of the compound through intervention in the sphingomyelin cycle in astrocytes.

Recently, it was shown in MS brain tissue and in two demyelinating animal models, the EAE model as a T-cell dependent disease model and the cuprizone model as a T-cell independent disease model, that reactive astrocytes around or in active demyelinated lesions indeed accumulate ceramide species[42]. Moreover, a study using neonatal rat hippocampal and differentiated oligodendrocytes demonstrated that exogenous S1P was rapidly metabolized into C16/18 ceramide. In this same study they also demonstrated reduced total ceramide and S1P levels in MS brain and in MS lesions compared to control tissue. However, they did find an increased C16/C18- to C24-ceramide ratio in these samples compared to control tissue[43]. Our ceramide data in MS lesions are precisely in concordance with these data and we

know that astrocytes rapidly metabolize exogenous S1P into ceramide as well (data not shown). However, we determined that in active MS lesions astrocytes are the cellular source for the observed increase in specific ceramide subspecies. Very interesting is the finding of reduced S1P levels in MS lesions by the same study. It is tempting to speculate that local S1P in MS brain is metabolized into specific ceramide subspecies and other sphingolipids by for example astrocytes. However, direct evidence to substantiate this speculation is lacking and therefore it remains unknown whether FTY720P in the CNS acts as compensator for diminished local S1P levels.

ENDOTHELIAL $S1P_5$ SIGNALING MAINTAINS BBB INTEGRITY AND IMMUNE QUIESCENCE

ECs express different S1P receptors and in studies, endothelial expression of $S1P_1$, $S1P_2$, and $S1P_3$ are described most often. In endothelial cells, many downstream effects of S1P receptor signaling are described comprising effects such as cytoskeletal changes, proliferation, migration, and survival. Permeability of EC monolayers is also studied extensively with regard to S1P signaling and generally studies demonstrate barrier enhancing effects upon S1P exposure[44,45]. Moreover, at least one study demonstrates a beneficial effect of S1P signaling on monocyte adhesion to endothelial cells under inflammatory conditions[46]. Since most effects of S1P signaling are attributed to all S1P receptors but $S1P_5$ and due to expression of $S1P_5$ on brain endothelial cells, we assessed the role of $S1P_5$ in BBB maintenance in **chapter 5**. Two $S1P_5$ agonists, FTY720P and a selective $S1P_5$ agonist, proved to positively regulate transendothelial resistance and permeability in a brain endothelial cell line. As a consequence monocyte migration across endothelial cell monolayers exposed to FTY720P and the selective $S1P_5$ agonist is reduced even under basal cell culture conditions. $S1P_5$ knockdown in this brain endothelial cell line resulted in negative regulation of both transendothelial resistance and endothelial permeability. This finding was substantiated by reduced expression levels of tight junction and adherent junction associated proteins like claudin-5 and VE-cadherin. In addition $S1P_5$ knockdown cells demonstrated reduced expression levels of BBB associated transporter proteins such as Pgp, GLUT-1, and BCRP-1. These findings suggest an essential role of $S1P_5$ signaling in brain endothelial cells regarding barrier integrity. Interestingly, $S1P_5$ knockdown in brain endothelial cells also resulted in an increased expression in mRNA levels of pro-inflammatory cytokines and chemokines such as; MCP-1, IL-8, IL-1β, and TNF-α. In addition these endothelial cells increased both mRNA expression and protein expression levels of ICAM-1 and VCAM-1. Functionally, these findings translate into increased monocyte adhesion and migration to and through endothelial cell monolayers. This inflammatory phenotype of $S1P_5$ knockdown brain endothelial cells is partly mediated by NF-κB activation. Together, these results imply an important role for $S1P_5$ signaling for proper brain endothelial barrier function and immune quiescence.

Literature about $S1P_5$ is scarce but most literature that is available is related to expression of $S1P_5$ in the CNS. Before going into more detail in $S1P_5$ studies concerning the CNS, an interesting study by Niedernberg et al. describes regulated and constitutive activation of $S1P_5$ induced pathways. In this study they demonstrate an reducing effect of $S1P_5$ expression on basal cAMP leves in $S1P_5$ stable transfected HEK239 cells that was independent of the presence S1P[47]. In addition it was demonstrated in CHO-K1 cells that $S1P_5$ inhibits basal ERK activity constitutive and is not sensitive to S1P. Functionally, they demonstrated that $S1P_5$ mediates cell rounding which is sensitive to S1P but does not require S1P[47]. Although these experiments are performed in different cell systems compared to our study and involve introduction of the $S1P_5$ receptor in these cell systems, they may provide clues about which intracellular signaling molecules are involved in ligand dependent and ligand independent $S1P_5$ signaling. In addition, in our study we most

likely interfere with both ligand dependent and ligand independent $S1P_5$ signaling pathways in our knockdown system. However, in the first part of our study we only trigger the ligand dependent signaling pathways with FTY720P and the selective $S1P_5$ agonist. This difference in involved pathways possibly explains the much broader range in consequent effects upon $S1P_5$ knockdown compared to exposure to $S1P_5$ ligands.

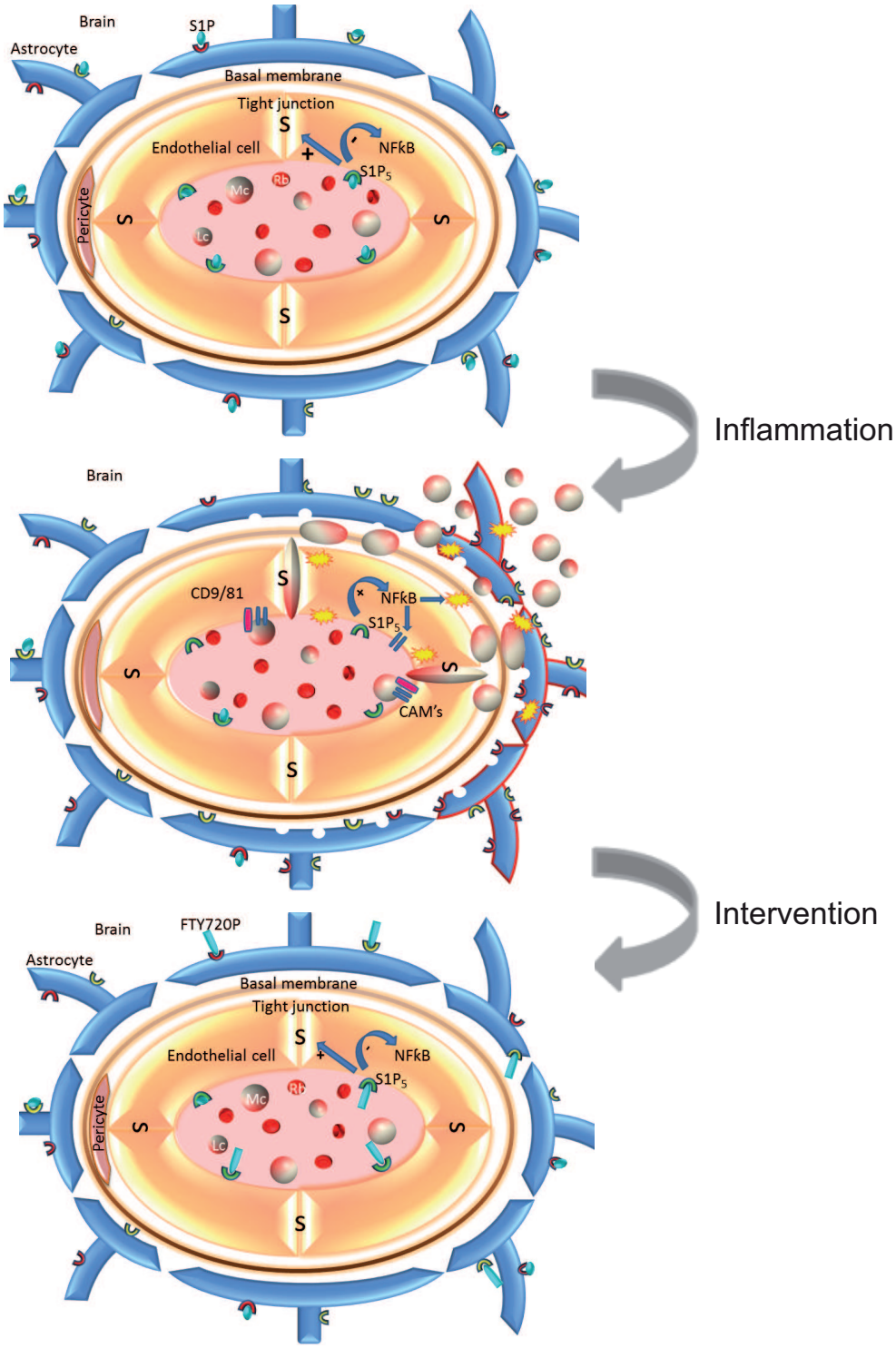
Most studies concerning $S1P_5$ describe $S1P_5$ expression and signaling in oligodendrocytes or oligodendrocyte precursor cells[48-51]. In pre-oligodendrocytes S1P signaling results in membrane retraction via $S1P_3$ or $S1P_5$ possibly through Rho GTPase-dependent signaling. In addition, S1P is a survival factor for mature oligodendrocytes acting through $S1P_5$ and downstream Akt dependent pathways[52]. In concordance with the S1P data, exposure of mature oligodendrocytes to FTY720P resulted in modulation of the cytoskeleton and promoted survival (Miron, Hall, 2008). Finally, it has been shown that FTY720P enhances remyelination in demyelinated slice cultures through $S1P_3$/ $S1P_5$ signalling[53]. All of the results mentioned here and our finding of $S1P_5$ signaling in endothelial cells, are very interesting since these results may describe a neuroprotective effect of Fingolimod in the CNS during pathology.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

An impaired BBB is an early hallmark of MS lesion formation. Therefore, comprehension of involved molecular mechanisms in BBB regulation during health and disease may contribute to identification of new or more specific targets for treatment of MS. In this thesis we demonstrate both novel endothelial and astrocytic molecular mechanisms involved in BBB regulation (Figure 1). Concerning endothelial cells we show involvement of CD9 and CD81 in transendothelial migration of monocytes. Additionally, CD9 but not CD81 signaling is directly involved in BBB integrity by increasing barrier function of the endothelial cells upon anti-CD9 antibody exposure. Currently, antibody therapies for pathologies such as Crohn's disease, Rheumatoid Arthritis, and Colitis Ulcerosa demonstrate great efficacy. Concerning treatment of MS, natalizumab (Tysabri) is an antibody therapy used in the clinic that shows a reduction in new Gd-enhanced lesions and fewer relapses in patients with relapsing MS[54]. However, natalizumab treatment is associated with progressive multifocal leukoencephalopathy (PML) which is an opportunistic viral infection of the CNS[55]. A recent study quantified the risk of PML among MS patients treated with natalizumab and demonstrated 2.1 cases of PML per 1000 treated patients[56]. Since mortality of PML within the first year lies between 80% and 90% other targets, such as molecules like CD9, for treatment of MS must be explored[57]. In addition, in this thesis we show that endothelial $S1P_5$ signaling is of great importance in maintaining proper endothelial cell barrier characteristics and maintaining immunoquiescence. Efficacy of the S1P receptor modulator FTY720P is demonstrated in treatment of RRMS[58]. However, FTY720P demonstrates affinity to 4 of the 5 S1P receptors and since these receptors demonstrate dispersed cellular expression, a broad range of cellular signaling events is elicited upon exposure to FTY720P. This may account for observed serious adverse events (SAEs) such as bradycardia and hypertension in MS patients treated with FTY720P. Moreover, exposure to FTY720P for a long period may induce additional SAE yet to be observed. Therefore, modulating individual S1P receptors is gaining interest and as demonstrated in this thesis selective targeting of $S1P_5$ may be of special interest in treatment of MS.

Concerning novel astrocytic molecular mechanisms involved in BBB regulation we first demonstrate that astrocytes are a target for FTY720P in active and inactive MS lesions. Exposure of these astrocytes to FTY720P results in a

Figure 1. The BBB during health, disease and upon intervention.
During health endothelial cells express tetraspanins such as CD9 and CD81 and GPCR's such as $S1P_5$. Signaling of S1P through $S1P_5$ during health remains endothelial cells quiescent resulting in appropriate BBB functioning. In addition, astrocytes express mainly two S1P receptors namely $S1P_1$ and $S1P_3$. During inflammation, signaling in endothelial cells through $S1P_5$ may decrease resulting in a pro-inflammatory phenotype of the endothelial cells, partly through activation of NFκB, and diminished expression of proteins necessary for proper BBB functioning. Moreover, endothelial CD9 and CD81 are involved in migration of monocytes through the BBB. Astrocytes strongly increase expression of $S1P_1$ and $S1P_3$ however, it remains to be elucidated whether there is increased or decreased signaling through these receptors during inflammation. Upon inflammation, astrocytes increase production of ceramide, a potent inflammatory agent, due to an altered sphingomyelin cycle. Targeting the S1P receptors of the endothelial cells and astrocytes with FTY720P elicits an anti-inflammatory response in both cell types. In addition targeting CD9 results in reduced monoctye migration.


Brain
S1P
Astrocyte
Basal membrane
Tight junction
Endothelial cell
NFkB
S1P5
Mc
Rb
Lc
Pericyte
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Inflammation
Brain
CD9/81
NFkB
S1P5
CAM's
Intervention
Brain
FTY720P
Astrocyte
Basal membrane
Tight junction
Endothelial cell
NFkB
S1P5
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Pericyte

reduced proinflammatory phenotype as measured by MCP-1 secretion suggesting that astrocytes present a novel target for FTY720P in treatment of MS. In addition, we demonstrate a shift in the sphingomyelin cycle in astrocytes present in active MS lesions favoring a proinflammatory phenotype through increased ceramide formation subsequently contributing to BBB dysfunction. FTY720P limits ceramide-induced BBB dysfunction by intervening in the sphingomyelin cycle favoring a less proinflammatory phenotype. Although we did not investigate a relationship between these two findings directly, it is very well conceivable that the proinflammatory status of reactive astrocytes is maintained through this altered sphingomyelin cycle generating pro-inflammatory cytokines such as MCP-1. Upon exposure of these reactive astrocytes to FTY720P signaling through $S1P_1$ and $S1P_3$ may be responsible for a shift in the sphingomyelin cycle favoring a less pro-inflammatory phenotype and thereby dampening MCP-1 secretion. Lymphocyte egress from lymph nodes is thought to be the main mode of action of FTY720P elucidating efficacy in treatment of RRMS. However, we and other groups demonstrate S1P receptor expression and direct FTY720P effects in the CNS. Astrocytes, oligodendrocytes and neurons all express several S1P receptor species and some of the downstream effects of FTY720P signaling in these cell types are migration, survival, and differentiation. FTY720P effects in the brain, by acting on these cell types, may comprise neuroprotective effects which besides an anti-inflammatory effect is a key component of a successful MS therapy. However, more research is needed to define which S1P receptor species is responsible for a certain downstream signaling effect within a specific cell type of the CNS and how this effect translates to neighboring cells within the CNS.