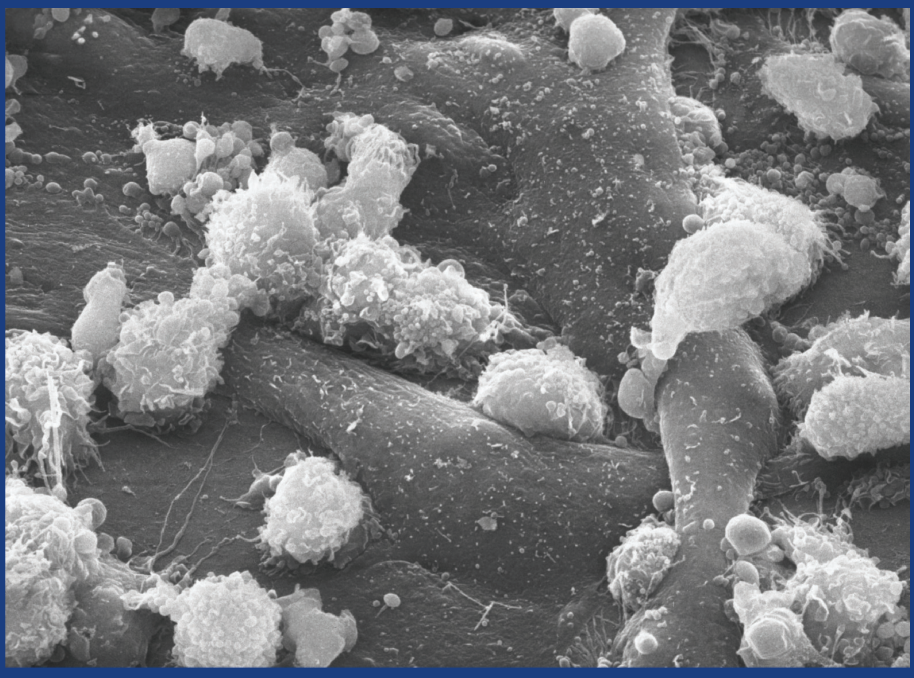
The Blood-Brain Barrier in Health and Disease

Volume 2: *Pathophysiology and Pathology*



Editor
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CRC Press
Taylor & Francis Group
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Editor

Katerina Dorovini-Zis

Professor of Pathology & Laboratory Medicine
The University of British Columbia
Vancouver General Hospital
Vancouver, BC
Canada

CRC Press
Taylor & Francis Group
Boca Raton London New York

CRC Press is an imprint of the
Taylor & Francis Group, an **informa** business

A SCIENCE PUBLISHERS BOOK

Cover illustration provided by the editor of the book, Prof. Katerina Dorovini-Zis

CRC Press
Taylor & Francis Group
6000 Broken Sound Parkway NW, Suite 300
Boca Raton, FL 33487-2742

Version Date: 20150914

International Standard Book Number-13: 978-1-4987-2709-9 (eBook - PDF)

Preface to Volume 2

Ο βίος βραχύς, η δε τέχνη μακρή
Life is short and art long

Hippocrates (c. 460-373 BC)
Aphorisms

The blood-brain barrier is a biological barrier that separates the brain from the blood and serves to facilitate the entry of essential nutrients into the brain while protecting it from unwanted and harmful substances and cells circulating in the blood. The endothelial cells that line the cerebral blood vessels have been recognized as the anatomical substrate of the blood-brain barrier. Their structural and functional integrity is the sine qua non of central nervous system homeostasis and neuronal function. For a long time since the first demonstration of a physical barrier at the blood-brain interface, the unassuming endothelial cells, so inconspicuous when brain sections stained with conventional dyes are viewed under the light microscope, were considered a little more than an unimpressive cell layer lining the vascular lumen. The past 50 years and in particular the last three decades have witnessed a great expansion of our knowledge of the complex structure, biology and function of the blood-brain barrier. As a result of fast-paced discoveries facilitated by the development of new *in vivo* and *in vitro* experimental tools, the cerebral endothelium has been propelled to a prominent status and is presently an attractive research subject in neurosciences. As a metabolically active cell it controls the traffic of substances into and out of the brain by means of a large number of enzymes, transporters and receptors and possesses a formidable system of tight junctions which, combined with a paucity of caveolae, keeps permeability tightly controlled. It can modify its shape and function in response to cues originating from the surrounding neural microenvironment and circulating substances, cells and organisms in the blood. It has attained a prominent stature as a master switch and mediator of immune responses, being capable of producing and responding to inflammatory mediators, regulating the entry of immune cells into the brain through the expression of adhesion receptors and chemoattractant cytokines and modifying its barrier function. An important concept that has emerged in recent years is that the barrier endothelium does not operate in isolation, rather its function influences and is influenced by neighboring cells. The concept of the neurovascular unit has thus evolved that provides a meaningful conceptual framework for the blood-

brain barrier by linking the function of the endothelium to that of other cells in the surrounding neural microenvironment.

This book presents, in an integrated fashion, generally accepted facts and new and exciting concepts on the structure, function and pathobiology of the blood-brain barrier. This first volume begins with a brief historical journey, which puts into perspective seminal past and recent work. This is followed by a detailed account of the development and composition of the human cerebral microvascular system, the structure, function and heterogeneity of the cerebral microvascular endothelium, the cellular components and function of the neurovascular unit, the expression and function of a steadily increasing number of ABC transporters at the blood-brain barrier and insights into the structure and function of the blood-cerebrospinal fluid barrier. The remaining chapters of this volume focus on the immune function of the blood-brain and blood-cerebrospinal fluid barriers and on the various inflammatory mediators and signaling molecules that modify the phenotype and permeability properties of the blood-brain barrier and contribute to the initiation of inflammatory responses in the central nervous system.

Dysfunction of the blood-brain barrier is increasingly recognized as contributing to the pathogenesis of a host of diverse central nervous system diseases. Accordingly, this second volume of the book addresses the active role of the endothelium as an initiator and regulator of biological responses and as a target in a broad spectrum of disorders including infections, inflammatory diseases, hypertension, ischemia, trauma, epilepsy, neurodegenerative diseases, metabolic disturbances, tumors, as well as radiation and drug-induced damage.

This book is aimed at graduate students who work towards a degree in neuroscience, postdoctoral fellows establishing a career and wishing to formulate new ideas, medical students with special interest in neurosciences and established clinicians and scientists wishing to update and expand their knowledge in this area. In spite of great accomplishments, our understanding of the blood-brain barrier remains incomplete. The ultimate goal of this book, therefore, is to provide information and serve as a stimulus for the next generation of researchers who will carry the torch of blood-brain barrier research to the next level.

In closing, I wish to express my thanks to my publisher, CRC Press. I remain grateful to the contributing authors for their generous contribution of precious time and effort. I would like to acknowledge my family for their patience, understanding and encouragement. I would also like to express my gratitude to Odysseas Zis for his kind and generous assistance with the complexities of organizing the reference libraries. This book is dedicated to the memory of my parents, Constantinos and Fanie Dorovinis.

Katerina Dorovini-Zis

Contents

List of Contributors

Fawaz M. Alotaibi
Microvascular/Molecular Neuro-oncology Laboratory, NYU Langone Medical Center MSB 521, 550 First Avenue, New York, NY 10016.
Email: Fawaz.Alotaibi@nyumc.org

Shilpa Buch
Department of Pharmacology and Experimental Neuroscience, 985880 Nebraska Medical Center (DRC 8011), University of Nebraska Medical Center, Omaha, NE 68198-5880.
Email: sbuch@unmc.edu

Roger F. Butterworth
Neuroscience Research Unit, Hôpital St-Luc (CHUM), University of Montreal, 45143 Cabot Trail, Englishtown, NS, Canada BOC1HO.
Email: rb@enceph.com

Adam Chodobski
Neurotrauma and Brain Barriers Research Laboratory, Department of Emergency Medicine, Alpert Medical School of Brown University, Coro Center West, Room 112, 1 Hoppin Street, Providence, RI 02903, USA.
Email: Adam_Chodobski@Brown.edu

Mahalia S. Desruisseaux
Albert Einstein College of Medicine, Jack and Pearl Resnick Campus, 1300 Morris Park Avenue, Bronx, New York, 10461, USA.
Email: m.desruis@einstein.yu.edu

Ruben van Doorn
Department of Molecular Cell Biology and Immunology (MCBI), VU University Medical Center, Neuroscience Campus Amsterdam, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands.
Email: r.vandoorn@vumc.nl

Katerina Dorovini-Zis
Department of Pathology and Laboratory Medicine, The University of British Columbia, Vancouver General Hospital, 855 West 12th Avenue, Vancouver, Canada, V5Z-1M9.
Email: dorovini@mail.ubc.ca

Scott G. Filler
Division of Infectious Diseases, Department of Medicine, Los Angeles Biomedical Research Institute at Harbor-UCLA Medical Center, Torrance, CA 90502, David Geffen School of Medicine at UCLA, Los Angeles, CA, 90095.
Email: sfiller@ucla.edu

Yun Gao
Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska, USA.
Email: terminator_Yun@hotmail.com

Kathy Guo
Department of Cellular and Molecular Medicine, Cleveland Clinic Lerner College of Medicine, NB-20 LRI, 9600 Euclid Ave Cleveland, OH 44195, USA.
Email: kguo2010@gmail.com

Henri van der Heyde
La Jolla Infectious Disease Institute, 3525 Del Mar Heights Road, San Diego, CA, 92130, USA.
Email: hvande@ljidi.org

Sheng T. Hou
Human Health Therapeutics, National Research Council Canada, 1200 Montreal Road, Ottawa, ON, Canada K1A 0R6.
Email: Sheng.Hou@nrc.ca

Rajan Jain
Department of Radiology, Clinical Co-Director, Center for Advanced Imaging and Research, NYU Langone Medical Center, 550 First Avenue, New York, NY 10016.
Email: Rajan.Jain@nyumc.org

Damir Janigro
Department of Cellular and Molecular Medicine, Cerebrovascular Research, Cleveland Clinic Lerner College of Medicine, NB-20 LRI, 9600 Euclid Ave Cleveland, OH 44195, USA.
Email: janigrd@ccf.org

Kwang Sik Kim
Division of Pediatric Infectious Diseases, Johns Hopkins University School of Medicine, 200 North Wolfe St., Room 3157, Baltimore, MD 21287.
Email: kwangkim@jhmi.edu

Lih-Fen Lue
Banner Sun Health Research Institute, 10515 West Santa Fe Drive, Sun City, Arizona, USA.
Email: lihfen.lue@bannerhealth.com

Yuri Chaves Martins
Albert Einstein College of Medicine, Department of Pathology, 1300 Morris Park Ave, Bronx, NY 10461, USA.
Email: yuri.chaves@einstein.yu.edu

Dorian B. McGavern
NINDS/NIH, 10 Center Drive, Bethesda, MD 20892.
Email: mcgavernd@mail.nih.gov

Steven F. Merkel
Department of Pathology and Laboratory Medicine, Temple University School of Medicine, 3500 North Broad Street, Medical Education and Research Building, 8th Floor, Room 880A, Philadelphia, Pennsylvania 19140 (USA).
Email: Tue39636@temple.edu

Mark Mizee
Department of Molecular Cell Biology and Immunology (MCBI), VU University Medical Center, Neuroscience Campus Amsterdam, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands.
Email: m.mizee@vumc.nl

Sukriti Nag
Department of Pathology, Rush University Medical Center, AAC, Suite1020 F, 600 South Paulina Street, Chicago, IL 60616 USA.
Email: Sukriti_Nag@ rush.edu

Souhel Najjar
Department of Neurology (Epilepsy Center Division), 223 E 34th St., New York, NY 10016.
Email: Souhel.Najjar@nyumc.org

Yuri Persidsky
Department of Pathology and Laboratory Medicine, Temple University School of Medicine, 3500 North Broad Street, Medical Education and Research Building, 8th Floor, Room 841, Philadelphia, Pennsylvania 19140 (USA).
Email: Yuri.Persidsky@tuhs.temple.edu

Nemani V. Prasadarao
Division of Infectious Diseases, Departments of Pediatrics and Surgery, Children's Hospital Los Angeles and University of Southern California School of Medicine, Los Angeles, CA 90027.
Email: pnemani@chla.usc.edu

Alexandre Prat
Neuroimmunology Research Laboratory, Hospital Complex of the Université de Montréal, Notre Dame Hospital, Faculty of Medicine, University of Montréal, 1560 Sherbrooke Street East, Montreal, Quebec, Canada H2L 4M1.
Email: a.prat@umontreal.ca

Vikram V. Puvenna
Department of Cellular and Molecular Medicine, Cleveland Clinic Lerner College of Medicine, NB-20 LRI, 9600 Euclid Ave Cleveland, OH 44195, USA.
Email: puvennv@ccf.org

Servio H. Ramirez
Department of Pathology and Laboratory Medicine, Temple University School of Medicine, 3500 North Broad Street, Medical Education and Research Building, 8th Floor, Room 844, Philadelphia, Pennsylvania 19140 (USA).
Email: Servio@temple.edu

Seema Shroff
Department of Pathology, NYU Langone Medical Center TH-444, 550 First Avenue, New York, NY 10016.
Email: Seema.Shroff@nyumc.org

Hany Soliman
Department of Radiation Oncology, Sunnybrook Health Sciences Centre, University of Toronto, 2075 Bayview Avenue, Toronto, ON Canada, M4N 3M5.
Email: hany.soliman@sunnybrook.ca

Danica Stanimirovic
Human Health Therapeutics, National Research Council Canada, 1200 Montreal Road, Ottawa, ON, Canada K1A 0R6.
Email: Danica.Stanimirovic@nrc.ca

Monique Stins
Johns Hopkins School of Medicine, Department of Neurology and Bloomberg School of Public Health, Malaria Research Institute, 600 N Wolfe Street, Mayer 6-113, Baltimore, MD 21287, USA.
Email: mstins@jhmi.edu

Phillip A. Swanson II
National Institute of Neurological Disorders & Stroke, National Institutes of Health, 10 Center Drive, Bethesda, MD 20892.
Email: swansonpa@mail.nih.gov

Joanna Szmydynger-Chodobska
Neurotrauma and Brain Barriers Research Laboratory, Department of Emergency Medicine, Alpert Medical School of Brown University, Coro Center West, Room 112, 1 Hoppin Street, Providence, RI 02903, USA.
Email: Joanna_Szmydynger-Chodobska@Brown.edu

Spencer Tung
Department of Pathology & Laboratory Medicine (Neuropathology), David Geffen School of Medicine at UCLA, Los Angeles, CALIFORNIA, USA.
Email: stung@mednet.ucla.edu

Harry V. Vinters
Departments of Pathology & Laboratory Medicine (Neuropathology) and Neurology, Brain Research Institute and Mental Retardation Research Center, David Geffen School of Medicine at UCLA, Los Angeles, CALIFORNIA, USA.
Email: hvinters@mednet.ucla.edu

Helga E. de Vries
Department of Molecular Cell Biology and Immunology (MCBI), VU University Medical Center, Neuroscience Campus Amsterdam, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands.
Email: he.devries@vumc.nl

C. Shun Wong
Department of Radiation Oncology, Sunnybrook Health Sciences Centre, University of Toronto, 2075 Bayview Avenue, Toronto, ON Canada, M4N 3M5.
Email: shun.wong@sunnybrook.ca

Douglas G. Walker
Banner Sun Health Research Institute, 10515 West Santa Fe Drive, Sun City, Arizona, USA.
Email: douglas.walker@bannerhealth.com

Honghong Yao
Department of Pharmacology, School of Medicine, Southeast University, Nanjing, Jiangsu, China.
Email: 101011704@seu.edu.cn

David Zagzag
Division of Neuropathology, Microvascular and Molecular Neuro-Oncology Laboratory, Department of Pathology, NYU Langone Medical Center, 550 First Avenue, TH-444, New York, NY 10016.
Email: David.Zagzag@nyumc.org

Brian J. Zink
Department of Emergency Medicine, Alpert Medical School of Brown University, Claverick 2, 593 Eddy Street, Providence, RI 02903, USA.
Email: BZink@Lifespan.org

Wandong Zhang
Human Health Therapeutics, National Research Council Canada, 1200 Montreal Road, Ottawa, ON, Canada K1A 0R6.
Email: Wandong.Zhang@nrc.ca

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Blood-Brain Barrier Disruption in Multiple Sclerosis

Mark Mizee,[1,a] *Ruben van Doorn,*[1,b] *Alexandre Prat*[2] and *Helga E. de Vries*[1,*]

Introduction

The blood-brain barrier is specialized to function as a barrier to protect the central nervous system by restricting entry of unwanted molecules and immune cells into the brain and inversely, to prevent central nervous system-born agents from reaching the systemic circulation. The blood-brain barrier endothelium, together with cells involved in its regulation forms the neurovascular unit. Blood-brain barrier dysfunction is an important hallmark of early multiple sclerosis pathophysiology, leading to a consequent loss of the imperative brain homeostasis and subsequent neuronal dysfunction and damage. The neuroinflammatory changes at the blood-brain barrier are numerous and include the loss of barrier function, altered communication with surrounding cells, and activation of both inflammation promoting and dampening mechanisms. A better understanding of blood-brain barrier alterations in neuroinflammation might lead to new ways to promote blood-brain barrier function in neurological diseases like multiple sclerosis.

[1] Department of Molecular Cell Biology and Immunology (MCBI), VU University Medical Center, Neuroscience Campus Amsterdam, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands.
[a] Email: m.mizee@vumc.nl
[b] Email: r.vandoorn@vumc.nl
[2] Neuroimmunology Research Laboratory, Hospital Complex of the Université de Montréal, Notre Dame Hospital, Faculty of Medicine, University of Montréal, 1560 Sherbrooke Street East, Montreal, Quebec, Canada H2L 4M1.
Email: a.prat@umontreal.ca
* Corresponding author: he.devries@vumc.nl

Multiple Sclerosis

Clinical Features and Diagnosis

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS). MS is characterized by the presence of focal inflammatory lesions scattered throughout the brain. Depending on their stage, lesions are hallmarked by inflammation, demyelination, gliosis, axonal injury and diffuse axonal degeneration (Frohman et al. 2006, Noseworthy et al. 2000). According to the World Health Organization, globally its median estimated prevalence is 30 per 100.000 resulting in over two million people affected with MS worldwide with a global women versus men ratio of 3:1. As the average age of onset is between 25 and 32 years, MS is one of the most common neurological disorders and causes of disability in young adults (World Health Organization 2008).

Presentation and symptoms of MS are characterized by great variability and diversity. In general, the initial symptoms and signs are sensory impairment, optic neuritis, motor deficits, limb ataxia and difficulty with balance (Weinshenker et al. 1989). The majority of MS patients are subject to a relapse with onset of MS, referred to as clinically isolated syndrome (CIS), which may eventually convert to MS (Miller et al. 2012). Over time, the clinical manifestation of MS varies and consensus was reached to describe three clinical course definitions. Relapsing–remitting (RR) MS, onset of disease in about 85% of MS patients, is described by clearly defined disease relapses with full recovery or with residual deficit upon recovery. Secondary-progressive (SP) MS is described by initial RR disease course followed by progression with or without occasional relapses, minor remissions, and plateaus (Lublin and Reingold 1996). Primary–progressive (PP) MS, onset of disease in about 10% of MS patients, is described by disease progression from onset with occasional plateaus and temporary minor improvements allowed.

The international Panel on the Diagnosis of Multiple Sclerosis announced new diagnostic criteria for MS in 2001. These criteria, the McDonald Criteria, were widely adopted by neurologists, providing them a diagnostic scheme for reliable diagnosis of MS (McDonald 2001). Diagnosis of MS is primarily based on clinical grounds comprising neurological examination and clinical history. If a diagnosis can not be made based on clinical presentation, radiological and laboratory assessments such as magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analysis may be essential for diagnosing MS. MRI analysis detects MS lesions in brain and in spinal cord and can therefore provide evidence of dissemination of MS lesions in both time and space, two potential criteria for the diagnosis of MS. CSF analysis may provide supportive evidence in the form of the presence of oligoclonal bands.

Aetiology

So far, the precise aetiology of MS remains unknown which is partly due to the complexity and heterogeneity of the disease. Epidemiological studies indicate that both environmental and genetic factors may contribute to development of MS (Dyment et al. 2004). It is suggested that development of MS must commence in genetically

susceptible individuals upon exposure to environmental factors (Ramagopalan et al. 2010).

Family studies have revealed that first degree relatives of MS patients are more likely to develop MS compared to non-related persons (Dyment et al. 2004, Sadovnick et al. 1988). Furthermore, supporting a genetic component in MS susceptibility, twin studies showed a higher concordance rate of MS in monozygotic twins compared to dizygotic twins (Ebers et al. 1986, Kinnunen et al. 1987, McFarland 1992). Concerning genetic associations, certain human leukocyte antigen (HLA) alleles are associated with susceptibility to MS. The allele with the strongest association with MS is HLA-DRB1*15 (HLA-DR2) showing consistency of effect across several western European and Scandinavian countries and the Unites States. In addition, various genetic mutations or polymorphisms in genes coding for cytokines (IL7, IL12A (p35), IL12B (p40)), cytokine receptors (CXCR5, IL2RA, IL7R, TNFRSF1A, IL12R), adhesion molecules (CD6, VCAM-1), and co-stimulatory molecules (CD37, CD40, CD80, CD86) are associated with MS pathogenesis (Sawcer et al. 2011).

Environmental risk factors described for MS are diverse in character. Several infectious agents such as varicella zoster virus, herpes viruses, and chlamydia are described as environmental risk factors however, current scientific interest is oriented towards the Epstein-Barr virus (EBV) (Ascherio et al. 2001, Enbom 2001, Friedman et al. 1999, Lassmann et al. 2011, Layh-Schmitt et al. 2000, Lunemann 2012, Moore and Wolfson 2002, Morre et al. 2001, Ohara 1999, Sriram et al. 1999). Involvement of EBV in MS pathology may be explained by its aptitude to elicit a persistent infection in the CNS inducing an immune response that contributes to pathology directly or through autoimmunity. Although literature about involvement of EBV in MS pathology is expanding, consensus about its complicity is not reached due to major controversies concerning sensitivity and specificity of detection methods of the virus in the CNS (Lassmann et al. 2011).

Two important non-infectious environmental risk factors for MS are latitude and vitamin D. Populations living at higher latitude show an increased prevalence of MS compared to populations living near the equator. This finding is most likely associated with adequate vitamin D serum levels in those populations living in closer proximity to the equator due to sun exposure. Interestingly, studies show that populations living at high latitude but with rich vitamin D food intake also show reduced MS prevalence (Kakalacheva and Lunemann 2011, Ramagopalan et al. 2010).

Pathogenesis

A distinct feature of MS pathology is the formation of demyelinated lesions, or plaques, in the CNS. Four patterns of demyelination were identified by systematic analysis of MS plaques: T-cell and macrophage-mediated demyelination, antibody and complement-mediated demyelination, oligodendrocyte dystrophy, and primary oligodendrocyte degeneration. To improve and standardize appropriate diagnosis and to support uniformity in research material, several different staging attempts have emerged in the last 20 years. These were named according to the pathologists

involved in these staging systems: The Bö/Trapp system, The De Groot/van der Valk modification, The Luchinetti/Lassmann/Brück system, and the Vienna consensus (van der Valk and De Groot 2000).

According to De Groot /van der Valk staging, MS lesions can be classified as preactive, active demyelinating, active but not demyelinating, chronic active, and chronic inactive lesions (van der Valk and De Groot 2000). Preactive lesions may be located near existing demyelinated plaques and in 'healthy' white matter areas. The lesions do not show demyelination but are characterized by modest white matter abnormalities including clusters of activated microglial cells and few perivascular leukocytes. In addition to the minimal leukocyte infiltration, there is a relative absence of demyelination and discrete basement membrane abnormalities. However, considerable redistribution of junctional proteins and increased expression of cell adhesion molecules (CAMs) were detected and suggest that barrier breach occurs at early stages of lesion formation, before significant immune cell infiltration and demyelination (meeting abstract FOCIS 2010, PACTRIMS 2012). In contrast to preactive lesions, active demyelinating lesions are characterized by loss of myelin and presence of abundant macrophages containing myelin degradation products. In addition, parenchymal and perivascular infiltrates of macrophages and lymphocytes are observed as well as randomly distributed reactive astrocytes. A chronic active MS lesion is a demyelinated lesion containing a hypocellular centre and a hypercellular rim of hypertrophic astrocytes, microglia, and macrophages (Zeinstra et al. 2003). Finally, chronic inactive lesions are demyelinated and hypocellular with only moderate expression of major histocompatability complex class II (MHCII) molecules and few $CD68^+$ oil-red-O^+ macrophages present (Serafini et al. 2006).

Complementary to demyelination, axonal damage is known to be of great importance in MS pathology. Early axonal damage is found at areas of acute demyelination and inflammation (Ferguson et al. 1997, Trapp et al. 1998). In paralyzed MS patients it is shown that axonal loss is a major cause of the irreversible neurological disability (Bjartmar et al. 2000). The irreversible nature of axonal damage and its association with inflammation suggest that anti-inflammatory treatment should be utilized early and that future therapies should include a neuroprotective component to prevent neurological deterioration.

Despite many advances in both molecular and clinical MS research, MS still remains incurable. Nevertheless, various therapies for treatment of MS are available and more therapies will most likely become available within the next one or two years since they currently reside in phase III clinical studies or are under review by regulatory agencies. Current MS therapies are limited to reduction of relapse rates, slowing down disease progression, accelerating recovery from relapses, and treatment of symptoms.

Blood-Brain Barrier

The vasculature of the brain is specialized to function as a barrier to protect the CNS by restricting entry of unwanted molecules and immune cells into the brain, by active removal of cytotoxic compounds from the brain, and by supplying the brain with

essential nutrients and oxygen through specific transport mechanisms. Therefore, the blood-brain barrier (BBB) is not static, but reacts dynamically to the local demands of neurons for their need of oxygen, glucose and other nutrients.

Several neuroinflammatory and neurodegenerative diseases like MS, HIV associated dementia, capillary cerebral amyloid angiopathy (capCAA) and Parkinson's disease are associated with impaired function of the BBB. Especially in MS, an altered BBB function leads to enhanced entry of immune cells and unwanted compounds into the CNS (Kooij et al. 2010), which will be reviewed below.

Barrier Properties of the Brain Endothelium

The BBB is composed of highly specialized brain endothelial cells (BECs) that line the vessel lumen. These BECs form a tight barrier by expression of tight junction (TJ) proteins and membrane efflux pumps. BECs 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 the close regulation of CNS homeostasis.

The BBB limits both transcellular and paracellular passage of cells and molecules into the CNS. Transcellular passage of hydrophilic molecules is limited due to a low rate of transcytotic vesicles, an extremely low pinocytotic activity, expression of active efflux membrane pumps of the ATP-binding cassette (ABC) family such as P-glycoprotein, and high metabolic activity (cytosolic enzymes and transporters). Paracellular diffusion of hydrophilic molecules and trafficking of immune cells is restricted by a network of TJ complexes which allow firm adhesion of BECs to each-another and sealing of the inter-endothelial space (Hamm et al. 2004, Loscher and Potschka 2005, Scherrmann 2002, Wolburg and Lippoldt 2002).

Adjacent BECs express continuous rows of transmembrane proteins that make homophilic contact in the intercellular space and form TJs (Van Itallie and Anderson 2004). Claudins and occludin are the most important membranous components of TJs, but the participation of junctional adhesion molecules (JAMs) and adherens junctions (Cadherins) are important as well (Wolburg and Lippoldt 2002). Occludin was the first TJ protein identified. Occludin is a phosphoprotein of about 65 kDa with two extracellular loops, four transmembrane domains and two cytoplasmic termini. The N-terminal cytoplasmic domain is involved in migration of neutrophils (Bazzoni 2006, Wolburg and Lippoldt 2002). The C-terminal cytoplasmic domain is associated with ZO-1 and ZO-2 which link occludin to the cytoskeleton. Claudins make up a family of proteins that consist of at least 23 closely related members. Claudins range in size from 20 to 25 kDa, contain four membrane-spanning domains, and have two extracellular loops (Morin 2005, Van Itallie and Anderson 2004). At the BBB, the presence of claudin-1,-3-,5 and recently -12 has been reported (Liebner et al. 2000, Nitta et al. 2003).

Astrocytes and the BBB

Astrocytes are strongly represented within the neurovascular unit, ensheathing over 95% of the abluminal microvascular surface. It was this observation that gave rise to the idea that astrocytic processes formed the BBB, until electron microscopic studies showed that BECs were responsible for barrier function in the brain microvasculature (Brightman and Reese 1969).

Astrocytes are able to influence a number of features of the BECs, leading to increased integrity of the BBB, TJ expression and TJ complex formation and maturation, expression and localization of BEC transporters, and specialized enzyme systems have been shown to be upregulated under astrocyte influence (Abbott et al. 2006). The notion that astrocytes can induce and maintain BBB properties in BECs through physical interaction and secreted agents has been widely accepted (Haseloff et al. 2005). Astrocyte processes extending towards CNS microvessels terminate in specialized (perivascular) endfeet structures onto the basal lamina surrounding the BECs. Astrocyte endfeet associated with BECs show a high density of orthogonal arrays of particles (OAPs), organized arrays of ion- and volume-regulating membrane particles identified by freeze fracture (Dermietzel 1974), containing channels like the water channel aquaporin-4 (AQP4) and the potassium ion channel Kir4.1 (Nagelhus et al. 2004). Membrane proteins in OAPs represent a strong polarization of perivascular astrocyte function and correlate with the expression of the basement membrane molecule agrin, an important proteoglycan for BBB integrity (Noell et al. 2007), which is responsible for the correct localization of AQP4. The distribution of these channels in OAPs is most likely important in the regulation of BBB homeostasis, as disruption of their distribution is associated with microvascular damage in, among other pathologies, Alzheimer's disease (Berzin et al. 2000).

The observation of astrocyte conditioned medium inducing junction formation in BECs *in vitro* (Arthur et al. 1987) gave rise to the idea that astrocyte-derived secreted factors were able to influence the BBB properties of BECs. Numerous astrocyte-derived agents have since then been described, mainly by *in vitro* studies, as modulators of BEC barrier function. Transforming growth factor-β (TGFβ) secreted by astrocytes has been shown to mediate the regulation of tissue plasminogen activator and the anticoagulant thrombomodulin (Tran et al. 1999). Glial-derived neurotrophic factor (GDNF) has been found to enhance barrier function in BECs trough the induction of TJ expression (Igarashi et al. 1999). Fibroblast growth factor (FGF) was found to decrease BBB permeability, consistent with the observation that FGF knockout mice show decreased levels of TJ proteins and BBB integrity loss (Reuss et al. 2003) and Angiopoietin-1 (ANG1) was shown to be an astrocyte secreted factor that increases TJ expression of BECs with an important effect on BBB permeability (Lee et al. 2003). Recently, we have also demonstrated that sonic hedgehog (sHh), a member of the Hh pathway, was produced and secreted by perivascular astrocytes in the human and mouse adult brain and that microvascular BECs expressed the receptors and the intracellular machinery to respond to Hh ligands (Alvarez et al. 2011). We also showed that pharmacological neutralization of Hh receptors or genetic deletion of Hh receptors lead to enhanced permeability of the BBB, loosening of the TJs and worsening of CNS inflammatory events, both *in vivo* in mouse and *in vitro* in human. These observations confirm the

important role of perivascular astrocytes in the regulation of the BBB in the adult CNS, and identify numerous and somewhat redundant astrocyte-dependant molecular pathways which converge onto BECs to favor and confer optimal BBB functioning.

The BBB in MS

In MS pathology, numerous changes in BBB structure and function have been described. These observations, derived from *in vitro*, *in vivo* animal models, and patient tissue studies, show significant involvement of the disruption of BBB integrity and function in MS pathology. The combined outcome of these studies has led to the notion that BBB disruption represents an early event in MS lesion formation, preceding both the massive infiltration of leukocytes (mainly T lymphocytes and monocyte-derived macrophages) and nervous tissue destruction (Minagar and Alexander 2003). Even before clinical symptoms arise, MRI scans of animals with experimental allergic encephalomyelitis (EAE), a well-established and validated animal model for the inflammatory phase of MS, show leakage of the BBB before monocytes infiltrate (Floris et al. 2004). However, before leukocytes adhere and transmigrate through the BBB, the cerebral endothelium must be activated by inflammatory mediators which induce expression of CAMs on BECs, with which leukocytes interact.

Inflammation at the BBB in MS

Tumour necrosis factor α (TNF-α) and chemokine (C-C motif) ligand 2 (CCL2) are two examples of numerous proinflammatory molecules which can cause upregulation of endothelial CAMs such as E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) (Librizzi et al. 2006), activated leukocyte cell adhesion molecule (ALCAM) (Cayrol et al. 2008) and melanoma cell adhesion molecule (MCAM) (Larochelle et al. 2012). While it remains unclear what triggers initial vascular activation in MS, reactive astrocytes and perivascular microglia are potent contributors to endothelial inflammation since they secrete proinflammatory cytokines and chemokines such as TNF-α, interleukin (IL)1β, IL6, IL12 and CCL2 during the disease process (Abbott 2002, Hayashi et al. 1995, Stalder et al. 1997). Through secretion of pro-inflammatory molecules, astrocytes and microglia not only contribute to direct disruption of the BBB, but also facilitate upregulation of CAMs thereby promoting recruitment and adhesion of leukocytes to BECs.

Inflammation induced tissue damage in the CNS of MS patients is driven by both autoreactive, antigenic $CD4^+$ T cells and $CD8^+$ T cells (Bajramovic et al. 2000, Berthelot et al. 2008, Bielekova et al. 2000, Bielekova et al. 2004, de Rosbo et al. 2004, Greer et al. 1997, Pette et al. 1990, Zhang et al. 1994). In addition, IL17 producing memory $CD4^+$ T cells (so called T_H17 lymphocytes) are found within active MS lesions (Kebir et al. 2007, Kebir et al. 2009) where IL17 gene expression is upregulated (Lock et al. 2002, Tzartos et al. 2008). Of the antigen presenting cells (APCs), infiltrating monocyte-derived macrophages are thought to possess a crucial role in orchestrating processes such as demyelination and axonal damage (Adams et al. 1989, Bruck et al. 1996, Cuzner et al. 1988, Esiri and Reading 1987, Hauser et al. 1986).

Before entering the CNS, leukocytes have to transmigrate through the specialized ECs of the BBB. Monocytes, the effector cells within MS lesions, are attracted to the perivascular space in high numbers. Within the process of monocyte trafficking across the BBB, it has been demonstrated that reactive oxygen species (ROS) play a dominant role. ROS are produced by monocytes upon firm adhesion to ECs and subsequently enhance migration and adhesion of monocytes (van der Goes et al. 2001). Treatment of EAE animals with antioxidants such as flavonoids and lipoic acid suppressed the development of EAE by lowering the entry of inflammatory cells into the CNS. Histological examination demonstrated a reduced number of infiltrated T-cells and macrophages, suggesting a role for ROS in BBB permeability (Hendriks et al. 2004, Schreibelt et al. 2006). Moreover, it was shown that super oxide is the predominant ROS which induces BBB disruption by inducing TJ rearrangements and cytoskeletal changes, allowing cell migration (van der Goes et al. 2001).

Immune Cell Trafficking Across the Brain Endothelium

The transmigration of leukocytes across the vascular wall requires the sequential activation and interaction of numerous molecular effectors expressed by BECs and immune cells, including selectins, chemokines, adhesion molecules of the immunoglobulin superfamily and their integrin counter ligands. The importance of leukocyte migration in MS is highlighted by the fact that the healthy CNS is devoid of immune cells and has been further demonstrated by the clinical efficacy of pharmacological blockers of migration in human MS patients. Interfering with leukocyte extravasation and diapedesis by blocking the adhesion cascade has indeed proven to be beneficial in reducing clinical disease activity and pathological indices in MS. Natalizumab, which blocks VLA-4, the ligand of VCAM-1, is reported to reduce migration of most leukocyte subtypes into the brain. Therefore, validation of the biological importance and of the clinical relevance of immune cell trafficking in MS is provided by the important clinical benefit of anti-VLA-4 blocking therapies. These VLA-4 blocking strategies prevent immune cell recruitment to the CNS, reduce myelin and axonal damage and alleviate clinical symptoms and disease progression in both animal models of MS (Vajkoczy et al. 2001) and in MS patients (Miller et al. 2003).

Although the presence of leukocytes within demyelinating lesions is indisputable in MS and EAE, the route and adhesion molecules by which these cells access the CNS are still not fully understood. As immune cell transmigration across BECs represents a critical step for the initiation of CNS-directed immune reactions, a better understanding of the molecular mechanisms involved in leukocyte diapedesis could identify novel therapeutic targets to modulate CNS immune responses. In this sense, VCAM-1, ICAM-1, ALCAM, JAM-L, CD99 and CD137 have all previously been shown to influence leukocyte transmigration in a non-restrictive manner, affecting the recruitment of antigen-presenting cells, but also of T and B lymphocytes. Furthermore, since ICAM-1 and VCAM-1 blockade only partially restrict migration of immune cells across BECs, it was suggested that additional CAMs are involved in the leukocyte transmigration process. These new CAMs need still to be identified.

MCAM, also known as CD146, is a new molecule of particular interest. MCAM is a member of the immunoglobulin superfamily, such as ALCAM, ICAM and VCAM. The only ligand reported to bind MCAM is MCAM itself (homotypic interaction), although we recently reported on the binding of MCAM to the matrix protein laminin 411 (Flanagan et al. 2012). MCAM is expressed by endothelial and smooth muscle cells. MCAM associates with the actin cytoskeleton and could contribute to the stabilization of inter-endothelial junctions. MCAM is also reported to mediate rolling of immortalized immune cells on BECs, although such data have not been confirmed using primary cells. Recently, MCAM was shown to be expressed by subsets of human peripheral blood memory $CD4^+$ and $CD8^+$ T lymphocytes. Interestingly, MCAM-expressing T lymphocytes are $CCR7^{neg}$ and thus bear the phenotypic properties of immune cells that have the capacity to migrate to inflamed organs. Blocking MCAM *in vivo* delayed disease and reduced the severity of EAE, using MOG-injected C57/BL6 animals (Larochelle et al. 2012). Taken together, these observations suggest that MCAM is an adhesion molecule expressed by activated T_H17 lymphocytes and used to enter the CNS by binding to either MCAM expressed by the BBB, or by binding to the matrix protein laminin 411.

Recent evidences also suggest that encephalitogenic T_H17 lymphocytes can migrate to the CNS via capillary structures of the choroid plexus, and not through the BBB. This seems to be uniquely dependent on the chemokine CCL20 and the chemokine receptor CCR6 (Reboldi et al. 2009). However, entry of encephalitogenic lymphocytes via choroid plexi remains a matter of debate, as other groups have not been able to confirm these data or have provided some contradictory findings (Elhofy et al. 2009, Villares et al. 2009).

In addition to the family of CAMs, members of another class of cell surface molecules are involved in the transendothelial migration process. The transmembrane 4 superfamily (TM4SF), or tetraspanins, are small membrane proteins differentially expressed by all mammalian cells. The size of tetraspanins ranges from 204 to 355 amino acids and they contain four transmembrane domains, intracellular termini and two (one large and one small) extracellular loops (Seigneuret et al. 2001). The long extracellular loop in combination with the four transmembrane domains are important in promoting associations of the tetraspanin with additional proteins such as other tetraspanins, integrins, CAMs, and intracellular signalling molecules (Levy and Shoham 2005). Resulting structures are referred to as tetraspanin-enriched microdomains (TEMs) and they operate as molecular organizers for other transmembrane proteins (Hemler 2008). The biological function of tetraspanins depends on their ability to organize TEMs. Biological functions associated with tetraspanins include adhesion, proliferation, differentiation, and motility of many different cell types (Hemler 2001, Hemler 2003, Hemler 2005). Of the more than 30 mammalian tetraspanins, three are associated with intercellular junctions in endothelial cells (Yanez-Mo et al. 1998). Moreover, the tetraspanins CD9, CD81, and CD151, also localize to docking structures on endothelial cells which are formed at sites of leukocyte adhesion (Barreiro et al. 2005). More specifically, microdomains containing tetraspanins and adhesion receptors were present on activated endothelial cells even before leukocytes adhered and studies demonstrated that CD81 and CD9 play a role in the transendothelial migration of immune cells (Barreiro et al. 2008, Rohlena et al. 2009).

Astrocyte-Endothelial Interactions in MS

During MS pathogenesis, reactive astrocytes participate in various mechanisms that contribute to neuroinflammation. Reactive astrocytes aggravate inflammation by increasing vascular activation and leukocyte accumulation in the CNS, and are involved in loss of BBB integrity, possibly mediated by the local release of pro-inflammatory molecules like IL-1β, IL6, and CCL2 (Didier et al. 2003, Quintana et al. 2009, Stamatovic et al. 2006). In addition, once inflammation has abated, astrocytes are the major cell type involved in glial scar formation and are thereby directly associated with inhibition of axonal regeneration (Davies et al. 1997). In contrast, during pathology astrocytes may also exert protective properties and promote cellular regeneration. Astrocytes are able to produce antioxidant enzymes and glutamate metabolizing enzymes and transporters suggesting an important role in scavenging reactive oxygen species (ROS) and extracellular glutamate (van Horssen et al. 2008, Newcombe et al. 2008). Furthermore, reactive astrocytes maintain the capacity to secrete T-cell suppressive factors (Kort et al. 2006), anti-inflammatory cytokines, and neurotrophic factors (Bsibsi et al. 2006). Finally, astrocytes in active MS lesions produce semaphorins, which are known to form chemotactic gradients for developing oligodendroglial cells, thereby possibly promoting remyelination (Williams et al. 2007). The data mentioned above accentuates the important and dual role of astrocytes in the CNS, with specific features discussed below.

The Hedgehog Pathway

Neuroinflammatory conditions such as MS are associated with breakdown of the BBB. We have shown that primary cultures of human astrocytes treated with TNF-α and IFN-γ increased SHh expression and that BECs grown in astrocyte media and treated with TNF-α and IFN-γ increased their expression of Hh receptors Ptch-1 and Smo (Alvarez et al. 2011). We further demonstrated that addition of SHh to BEC cultures induced a reduction in both CAM expression and chemokine secretion. Within control brain tissue and normal-appearing white matter (NAWM) obtained from MS brains, astrocyte processes and endfeet surrounding parenchymal vessels displayed SHh immunoreactivity. However, SHh immunoreactivity was strikingly enhanced in hypertrophic astrocytes and processes throughout active demyelinating MS lesions, and the Hh transcription factor Gli-1 was increased in BBB-ECs (Alvarez et al. 2011, Wang et al. 2008). Upon inflammatory stimulation, astrocyte-secreted SHh therefore induces expression of Hh receptors in BECs, which leads to the translocation of the Hh transcription factor Gli-1 into the nucleus of BECs. The hedgehog pathway, where Hh ligands are secreted by astrocytes and Hh receptors are expressed by BECs, therefore acts as a molecular repressor of CNS inflammation and promotes BBB repair.

Aquaporin-4 and Kir4.1 in Astrocyte Endfeet

Astrocytes with endfeet terminating in the neurovascular unit perform specific functions in the maintenance of perivascular ion and water homeostasis (Simard and Nedergaard 2004). Extracellular potassium ions released by neurons require spatial buffering by

astrocytes to maintain homeostasis. The inwardly rectifying Kir4.1 potassium channels which are highly expressed in the polarized astrocyte endfeet meet this need for potassium buffering. Potassium ion buffering by astrocytes is accompanied by osmotic changes and slight cell swelling. The AQP4 water channels present at high densities in the OAPs of astrocytic endfeet regulate these osmotic changes by redistribution of excess water. The tight regulation of expression and distribution of the ion and water channels on astrocytic endfeet is necessary for homeostasis and disruption of this compensatory system has been shown for BBB disruption in Alzheimer's disease (Berzin et al. 2000) and glioblastomas (Warth et al. 2004), both involving aberrant agrin expression. The increase of AQP4 expression observed in brain edema, probably serving as an adaptive mechanism, tends to aggravate the BBB disruption (Zador et al. 2007). AQP4 upregulation has also been shown in reactive (hypertrophic) astrocytes in response to injury, correlating with BBB disruption (Vizuete et al. 1999). Recently, active MS lesions were shown to have increased levels of AQP4 expression (Sinclair et al. 2007), which could possibly contribute to further edema-induced BBB damage after initial disruption.

The observation that astrocytes with the highest AQP4 expression are located at the outer rim of active MS lesions, resembling ischemic foci (Oki-Yoshino et al. 2005), suggests that altered AQP4 expression, localization, or regulation by agrin could be contributing to aggravation of MS pathology.

Connexin43

Astrocytes in the neurovascular unit are coupled together via gap junctions (GJ), mainly formed by connexin43 (Cx43) (Nagy and Rash 2000). The coupling through GJ provides the network of astrocytes with a cytoplasmic continuity which allows the free and fast passage of (signaling) ions and metabolites between astrocytes. This syncytium of cells provides the BBB with a network of continuously communicating astrocytes, where fast responsiveness can be crucial in maintaining homeostasis.

In EAE a decrease in astrocytic Cx43 expression was observed in the inflammatory lesions, suggesting a decreased astrocytic connectivity in these areas (Brand-Schieber et al. 2005). Whether reduced astrocyte-astrocyte communication during inflammation is detrimental or beneficial remains to be determined, although the possible involvement of Cx43 in maintaining BBB integrity through co-localization with TJ-proteins in porcine BEC has recently been reported (Nagasawa et al. 2006). The effects of the loss of GJ-contact between astrocytes on astrocyte activation, BBB integrity, and inflammatory response should be investigated further to address the questions about effects on MS pathology.

P-glycoprotein

The drug-efflux transporter P-gp is an ATP-dependent efflux pump highly expressed on the luminal side of BEC, responsible for the active removal of a broad range of hydrophobic molecules from the BEC cytoplasm (Bellamy 1996). P-gp function leads to the prevention of potentially neurotoxic molecules entering the CNS tissue, also leading to the low penetration of CNS-therapeutic drugs (Fromm 2004). The

expression of P-gp is not confined to BECs, but expression was also shown to localize in astrocytic endfeet (Pardridge et al. 1997). In a recent study, P-gp expression in the inferior colliculus was shown to be markedly reduced in BECs following a chemically induced focal loss of astrocyte contact. Interestingly, P-gp expression returned to normal when astrocytes were seen to repopulate the affected area (Willis et al. 2007). This observation indicates a role for astrocytes in the induction and maintenance of P-gp expression by BECs.

Recent data by our group showed a significant reduction of microvessel P-gp expression in various MS lesions in patients, compared to normal appearing white matter (Kooij et al. 2010). These results suggest that a loss of P-gp expression might be involved in lesion formation or aggravation. A follow-up study showed that P-gp expression increased in astrocytes in MS lesions, suggesting a possible role for astrocytes as a complementary drug resistance barrier in areas of BBB disruption. However, P-gp was found to mediate the release of CCL2 and the proinflammatory lipid platelet activating factor (Kooij et al. 2011) which may actively contribute to the neuroinflammatory process by attracting more immune cells into the lesion.

Sphingolipid Metabolites

In recent years, it has become increasingly clear that sphingomyelin metabolism plays a key role in biological processes in the CNS. Sphingomyelin is the major sphingolipid present in cell membranes, where it serves as a building block for biological membranes and in addition it plays an important role in proper membrane function (Gensure et al. 2006, Lande et al. 1995, Simons and van 1988). Moreover, sphingomyelin is the predominant source for bioactive sphingomyelin metabolites, such as ceramide and sphingosine 1-phosphate (S1P). Evidence is now emerging that alterations in sphingolipid metabolism, leading to enhanced proinflammatory ceramide production, occur in several neurological disorders (Dawkins et al. 2001, Esen et al. 2001, Grassme et al. 2003, Hauck et al. 2000, Lang et al. 2007, Puranam et al. 1997, Puranam et al. 1999). Importantly, inflammatory mediators, including TNF-α, ROS, and IL-1β induce the production of ceramide through activation of acid sphingomyelinase (ASM), which in turn amplifies the inflammatory cascade by either direct activation of downstream targets or by affecting membrane organization (Hofmeister et al. 1997, Sanvicens and Cotter 2006, Schutze et al. 1992).

Recently, we demonstrated an increase in the production of ceramide in reactive astrocytes in active MS lesions. Interestingly, astrocytes isolated from active MS lesions maintain increased ASM mRNA expression in culture which may be the result of continuous ceramide-induced autocrine activation through proinflammatory cytokines. During MS pathogenesis, stress signals such as ROS, TNF-α, and IFN-γ are present in the inflamed brain parenchyma and may be responsible for the observed increase in astrocytic ceramide. In addition, ceramide induces IL-6 mRNA and protein levels in a human astrocytoma cell line and ASM is able to induce release of microparticles containing IL-1β in astrocytes most likely mediated through ceramide formation (Bianco et al. 2009, Fiebich et al. 1995). In turn, ceramide was found to impair the function of the BBB *in vitro* (van Doorn et al. 2012), illustrating the impact of the reactive astrocyte phenotype on the barrier properties in MS.

Strikingly, reactive astrocytes were found to have an induced expression of the S1P receptors which after triggering with the S1P analogue Fingolimod (FTY-720P) resulted in a diminished production of pro-inflammatory mediators (van Doorn et al. 2010, van Doorn et al. 2012). Together, these data indicate that the dampening of the reactive astrocyte phenotype is an attractive new therapeutic strategy (Lassmann 2012).

A schematic overview of the healthy BBB and the inflammatory changes in MS is depicted in Fig. 1.

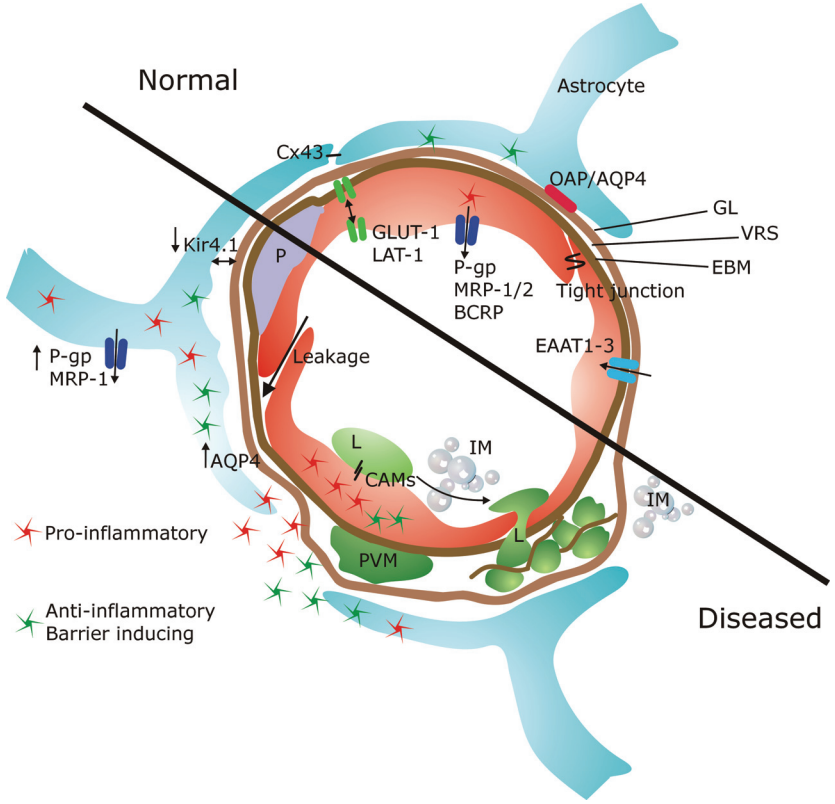

Figure 1. Schematic overview of the BBB in health and neuroinflammation.

Under normal conditions, the endothelial cells of the BBB are tightly interconnected via TJs, have polarized expression of ABC-transporters (P-gp, MRP-1/2, and BCRP), nutrient transporters (GLUT-1 and LAT-1), and excitatory amino acid transporters (EEAT1-3). The endothelial basement membrane (EBM) lies adjacent to the glia limitans (GL), with a very narrow Virchow-Robin space (VRS). Astrocyte endfeet, communicating with other astrocytes via Cx43 gap junctions, cover the abluminal side of the BBB and support endothelial barrier function. Water homeostasis is ensured by expression of AQP4 and Kir4.1 in OAPs. During neuroinflammation in MS, endothelial barrier function is disrupted, leading to leakage of serum-components into the perivascular space. Leukocyte (L) migration is facilitated by endothelial CAM expression and the presence of chemokines. Leukocytes themselves also release immune modulators (IM) affecting the endothelial cells of the BBB. BEC expression of ABC transporters is reduced, resulting in decreased detoxification potential. Astrocytes release both pro-inflammatory (e.g., IL-6 and CCL2) and anti-inflammatory mediators (e.g., sHh and RA) and show deregulation of Kir4.1 and AQP4 expression in OAPs. ABC-transporter expression (P-gp and MRP-1) is increased in astrocytes, resulting in enhanced secretion of pro-inflammatory chemokines.

Future Perspectives

The BBB is specialized to function as a barrier to protect the CNS by restricting entry of unwanted molecules and immune cells into the brain. An important hallmark of MS pathology is a dysfunctional BBB and consequent loss of the imperative CNS homeostasis. The unrestrained access of immune cells and harmful compounds into the CNS play a central role in demyelination and axonal damage, two hallmarks of MS pathology strongly contributing to the clinical symptoms of MS.

Strategies aimed at restoring the impaired function of the BBB in MS are therefore a promising new tool to combat disease progression, together with the dampening of the inflammatory phenotype of reactive astrocytes.

As discussed in this chapter, 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 neuronal cell types. Interestingly, developmental pathways involved in BBB development are now emerging as possible protective mechanisms to reduce BBB damage in neuroinflammation, as illustrated by the increased expression of sHh. Recently, retinoic acid (RA), an important astrocyte-derived morphogen in CNS development, has been shown to play a role in the induction of the BBB (Mizee et al. 2013). Recent data from our group indicates that, similar to the expression of sHh, RA production re-emerges during neuroinflammation in MS pathology (Mizee et al. 2014). We furthermore show protective effects of RA on the disrupted BBB, in line with reports that show anti-inflammatory (Xu and Drew 2006) and neuroprotective effects (Katsuki et al. 2009) of RA in the CNS. The association of other pathways that have been associated with BBB development, the Wnt/β-catenin pathway (Daneman et al. 2009, Liebner et al. 2008) and the early association of CNS pericytes with the developing BBB (Daneman et al. 2010), 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. Unravelling ways of boosting this self-regenerative capacity of the CNS to repair BBB disruption shows significant promise as a possible therapeutic avenue in MS, working side by side with the current immune-dampening therapeutic strategies.

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2

Portals of Viral Entry into the Central Nervous System

Phillip A. Swanson II[1] and *Dorian B. McGavern*[2]

Introduction

Humans and other vertebrates have evolved highly sophisticated barrier systems to prevent the entry of potentially harmful substances into the central nervous system (CNS). These structures include the blood-brain barrier (BBB), formed in part by non-fenestrated vascular endothelial cells within the CNS parenchyma, and the blood-cerebrospinal fluid barrier (BCSFB), comprised of tightly linked mesenchymal or epithelial cells in structures that border CSF-filled spaces (Fig. 1). Despite these complex barriers, many viruses have evolved strategies to infect the CNS, which can give rise to acute and chronic diseases such as meningitis, encephalitis, myelitis, paralysis, etc. Some viruses are able to directly manipulate the BBB or BCSFB to enter the CNS, whereas others hijack host immune cells or travel within peripheral nerves. This chapter will begin with a general description of the structure and function of the BBB/BCSFB and follow with a detailed synopsis of the mechanisms used by viruses to bypass these structures in order to infect the CNS.

Anatomy of CNS Barrier Structures

Gross Anatomy

The parenchyma of the CNS is enveloped by a series of membranes called meninges (Figs. 1, 2). The outermost layer, the dura mater, directly connects to the skull or

[1] National Institute of Neurological Disorders & Stroke, National Institutes of Health, 10 Center Drive, Bethesda, MD 20892.
Email: swansonpa@mail.nih.gov
[2] NINDS/NIH, 10 Center Drive, Bethesda, MD 20892.
* Corresponding author: mcgavernd@mail.nih.gov

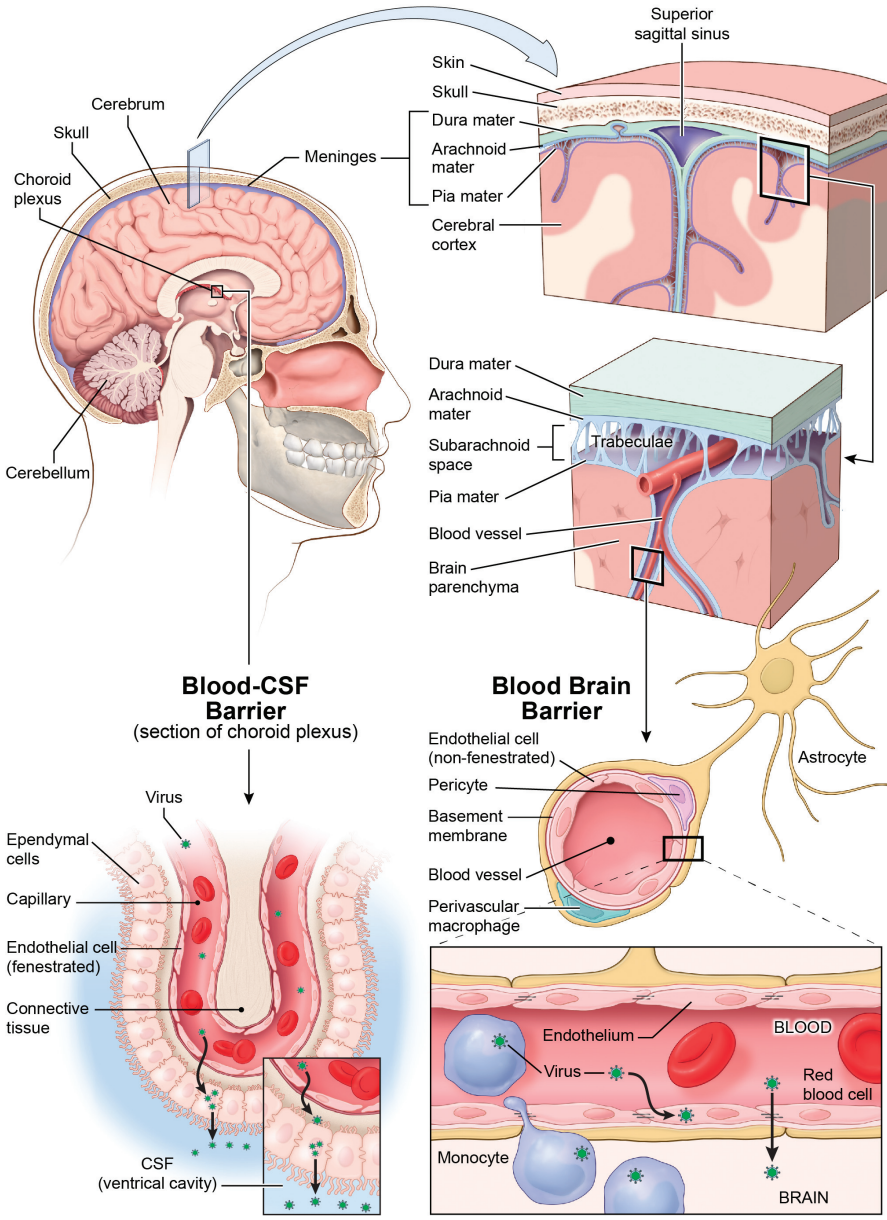

Figure 1. Anatomy of the blood-brain and blood-cerebrospinal fluid barriers.

bones of the vertebral canal and lies superficial to the second layer, the arachnoid mater. The arachnoid mater is attached to the dura mater, but is separated from the final meningeal layer (pia mater) by a CSF-filled cavity called the subarachnoid space (SAS). Vascular endothelial cells within the dura mater are fenestrated, allowing free