Springer Series in Translational Stroke Research

Jun Chen
John H. Zhang
Xiaoming Hu *Editors*

Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke

Springer

Springer Series in Translational Stroke Research

Series Editor
John Zhang

More information about this series at http://www.springer.com/series/10064

Jun Chen • John H. Zhang • Xiaoming Hu
Editors

Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke

Springer

Editors
Jun Chen
Department of Neurology
University of Pittsburgh
Pittsburgh, PA, USA

Pittsburgh Institute of Brain Disorders and Recovery
University of Pittsburgh
Pittsburgh, PA, USA

State Key laboratory of Medical Neurobiology
Fudan University
Shanghai, China

Xiaoming Hu
Department of Neurology
University of Pittsburgh
Pittsburgh, PA, USA

Pittsburgh Institute of Brain Disorders and Recovery
University of Pittsburgh
Pittsburgh, PA, USA

State Key laboratory of Medical Neurobiology
Fudan University
Shanghai, China

John H. Zhang
Department of Anesthesiology
Loma Linda University School of Medicine
Loma Linda, CA, USA

Department of Pharmacology
Loma Linda University School of Medicine
Loma Linda, CA, USA

Department of Physiology
Loma Linda University School of Medicine
Loma Linda, CA, USA

Center for Neuroscience Research
Loma Linda University School of Medicine
Loma Linda, CA, USA

ISSN 2363-958X ISSN 2363-9598 (electronic)
Springer Series in Translational Stroke Research
ISBN 978-3-319-32335-0 ISBN 978-3-319-32337-4 (eBook)
DOI 10.1007/978-3-319-32337-4

Library of Congress Control Number: 2016944470

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG Switzerland

Introduction: Nonneuronal Mechanisms and Targets for Stroke

For several decades now, clinically effective neuroprotection has been an elusive goal. Although much progress has been made in terms of dissecting molecular pathways and cellular mechanisms, true translation has not succeeded for patients suffering from stroke, brain trauma, and neurodegeneration. Excitotoxicity, oxidative stress, and programmed cell death all represent logical targets for preventing neuronal demise. But it is now increasingly apparent that saving neurons alone may not be enough.

Based on these challenges, the neurovascular unit was proposed as a conceptual framework for reassessing neuroprotection, the fundamental premise being that central nervous system (CNS) function is not solely based on neuronal activity. The brain is more than just action potentials! For neurotransmission to work, release–reuptake kinetics must be coordinated between neurons and astrocytes. For myelinated signals to connect different brain networks, axons need to be in constant homeostatic communication with oligodendrocytes. For the blood–brain barrier to be manifested, crosstalk is required between glial endfeet and cerebral endothelium. Altogether, CNS function is based on cell–cell signaling between multiple cells. Therefore, neuroprotection requires one to do much more than just prevent neuronal death. Rescuing function and cell–cell crosstalk between all cell types in neuronal, glial, and vascular compartments should be required. It is in this context that this monograph *Nonneuronal Mechanisms of Brain Damage and Repair After Stroke* represents a significant addition to the literature and field.

This monograph is divided into five well-integrated sections. The first section focuses on microvascular integrity. Chapters here include analyses of the structural biology of tight junctions, the role of pericytes, glial regulation of barrier function, blood–brain barrier damage in neonatal stroke, and a reconsideration of angiogenesis after stroke. The second section covers the complex actions of glial cells and includes chapters on astrocyte protection, biphasic effects of microglia, and crosstalk between cerebral endothelium and oligodendrocyte precursor cells. The third section then goes on to examine the multifactorial pathways in stroke neuroinflammation, with analyses of peripheral immune activators, monocyte/macrophage responses, T cells, B cells, mast cells and neutrophils, and the web of cytokines that

all contribute to stroke pathophysiology. A critical part of stroke that is relatively less investigated comprises white matter response, and this is the focus of the fourth section of the monograph. In this section, chapters are devoted to assessing the age dependence of white matter injury and subsequently investigating the role of oligodendrogenesis for white matter plasticity. Finally, the last collection of chapters builds on the mechanistic themes explored thus far to develop potential therapeutic approaches. In this final section, chapters span a comprehensive range, including the targeting of leukocyte–endothelial interactions, methods to repair the entire neurovascular unit, immune-based treatments, and cell-based therapies that all seek to achieve neuroprotection by restoring crosstalk amongst the nonneuronal population of CNS cells.

Taken together, the chapters here represent the very best in cutting-edge hypotheses and translational ideas. The mechanisms dissected herein may eventually lead us to testable targets for stroke patients. Curated by editors and authors who are experts in their field, this is an impressive collection of stroke science.

Eng H. Lo, Ph.D.
Lo@helix.mgh.harvard.edu
Neuroprotection Research Laboratory
Department of Radiology
Massachusetts General Hospital and
Harvard Medical School
149 13th Street, Charlestown, MA, 02129, USA

Contents

Contributors

Luis Alarcon-Martinez, B.Sc., M.Sc., Ph.D. Institute of Neurological Sciences and Psychiatry, Hacettepe University, Ankara, Turkey

Anuska V. Andjelkovic, M.D., Ph.D. Department of Pathology, University of Michigan, Ann Arbor, MI, USA

Department of Neurosurgery, University of Michigan Health System, Ann Arbor, MI, USA

Josef Anrather Feil Family Brain and Mind Research Institute, Weill Cornell Medical College, New York, NY, USA

Ken Arai Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA

Selva Baltan, M.D., Ph.D. Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, OH, USA

Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, USA

Chinthasagar Bastian, M.B.B.S., Ph.D. Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, USA

Cesar V. Borlongan Department of Neurosurgery and Brain Repair, University of South Florida Morsani College of Medicine, Tampa, FL, USA

Sylvain Brunet, B.Sc., Ph.D. Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University,, Cleveland, OH, USA

Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH, USA

Sherrefa R. Burchell, B.Sc. Department of Physiology, Loma Linda University School of Medicine, Loma Linda, CA, USA

Center for Neuroscience Research, Loma Linda University School of Medicine, Loma Linda, CA, USA

Jun Chen Department of Neurology, University of Pittsburgh, Pittsburgh, PA, USA

Pittsburgh Institute of Brain Disorders and Recovery, University of Pittsburgh, Pittsburgh, PA, USA

State Key laboratory of Medical Neurobiology, Fudan University, Shanghai, China

Turgay Dalkara, M.D., Ph.D. Faculty of Medicine, Department of Neurology, Hacettepe University, Ankara, Turkey

Institute of Neurological Sciences and Psychiatry, Hacettepe University, Ankara, Turkey

Department of Radiology, Massachusetts General Hospital, Harvard University, Boston, MA, USA

Thomas P. Davis, Ph.D. Department of Pharmacology, University of Arizona College of Medicine, Tucson, AZ, USA

Department of Medical Pharmacology, College of Medicine, University of Arizona, Tucson, AZ, USA

Shinghua Ding, Ph.D. Department of Bioengineering, Dalton Cardiovascular Research Center, University of Missouri, Columbia, MO, USA

Naohiro Egawa Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA

Yanqin Gao State Key Laboratory of Medical Neurobiology and Institute of Brain Sciences, Fudan University, Shanghai, China

Center of Cerebrovascular Disease Research, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Wing-Mann Ho, M.D. Department of Physiology, Loma Linda University School of Medicine, Loma Linda, CA, USA

Center for Neuroscience Research, Loma Linda University School of Medicine, Loma Linda, CA, USA

Department of Neurosurgery, Medical University Innsbruck, Innsbruck, Tyrol, Austria

Xiaoming Hu Department of Neurology, University of Pittsburgh, Pittsburgh, PA, USA

Pittsburgh Institute of Brain Disorders and Recovery, University of Pittsburgh, Pittsburgh, PA, USA

State Key laboratory of Medical Neurobiology, Fudan University, Shanghai, China

Kanako Itoh Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA

Glen C. Jickling Department of Neurology, MIND Institute, University of California at Davis Medical Center, Sacramento, CA, USA

Richard F. Keep, Ph.D. Department of Neurosurgery, University of Michigan Health System, Ann Arbor, MI, USA

Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, USA

Christopher C. Leonardo, B.S., Ph.D. Molecular Pharmacology and Physiology, University of South Florida, Tampa, FL, USA

Anna C. Liang Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA

Arthur Liesz, M.D. Institute for Stroke and Dementia Research, Klinikum der Universität München, Munich, Germany

Perttu J. Lindsberg, M.D., Ph.D. Neurology, Clinical Neurosciences, Helsinki University Hospital, Helsinki, Finland

Molecular Neurology, Research Programs Unit, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland

Eng H. Lo Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA

Josephine Lok Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA

Department of Pediatrics, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA

Takakuni Maki Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, MA, USA

Olli S. Mattila, M.D. Neurology, Clinical Neurosciences, Helsinki University Hospital, Helsinki, Finland

Molecular Neurology, Research Programs Unit, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland

Nobukazu Miyamoto Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA

Hung Nguyen Department of Neurosurgery and Brain Repair, University of South Florida Morsani College of Medicine, Tampa, FL, USA

Takayuki Noro Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA, USA

Keith R. Pennypacker, B.A., M.S., Ph.D. Molecular Pharmacology and Physiology, College of Medicine Neurosurgery, University of South Florida, Tampa, FL, USA

Katie Poinsatte Department of Neurology and Neurotherapeutics, UT Southwestern Medical Center, Dallas, TX, USA

Patrick T. Ronaldson, Ph.D. Department of Pharmacology, University of Arizona College of Medicine, Tucson, AZ, USA

Department of Medical Pharmacology, College of Medicine, University of Arizona, Tucson, AZ, USA

Uma Maheswari Selvaraj Department of Neurology and Neurotherapeutics, UT Southwestern Medical Center, Dallas, TX, USA

Frank R Sharp Department of Neurology, MIND Institute, University of California at Davis Medical Center, Sacramento, CA, USA

Hong Shi State Key Laboratory of Medical Neurobiology and Institute of Brain Sciences, Fudan University, Shanghai, China

Department of Anesthesiology of Shanghai Pulmonary Hospital, Tongji University, Shanghai, China

Yejie Shi Center of Cerebrovascular Disease Research, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Akihiro Shindo Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical Schoo, Charlestown, MA, USA

Ann M. Stowe Department of Neurology and Neurotherapeutics, UT Southwestern Medical Center, Dallas, TX, USA

Daniel Strbian, M.D., Ph.D. Neurology, Clinical Neurosciences, Helsinki University Hospital, Helsinki, Finland

Molecular Neurology, Research Programs Unit, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland

Yang Sun, M.D. Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Naoki Tajiri Department of Neurosurgery and Brain Repair, University of South Florida Morsani College of Medicine, Tampa, FL, USA

Jiping Tang, M.D. Department of Physiology, Loma Linda University School of Medicine, Loma Linda, CA, USA

Center for Neuroscience Research, Loma Linda University School of Medicine, Loma Linda, CA, USA

Zinaida S. Vexler, Ph.D. Department of Neurology, University of California, San Francisco, San Francisco, CA, USA

Mingyue Xu State Key Laboratory of Medical Neurobiology and Institute of Brain Sciences, Fudan University, Shanghai, China

Center of Cerebrovascular Disease Research, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Tuo Yang, M.D. Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Xinxin Yang Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Muge Yemisci, M.D., Ph.D. Faculty of Medicine, Department of Neurology, Hacettepe University, Ankara, Turkey

Institute of Neurological Sciences and Psychiatry, Hacettepe University, Ankara, Turkey

Ke-Jie Yin, M.D., Ph.D. Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

Feng Zhang, M.D., Ph.D. Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

John H. Zhang, M.D., Ph.D. Department of Anesthesiology, Loma Linda University School of Medicine, Loma Linda, CA, USA

Department of Pharmacology, Loma Linda University School of Medicine, Loma Linda, CA, USA

Department of Physiology, Loma Linda University School of Medicine, Loma Linda, CA, USA

Center for Neuroscience Research, Loma Linda University School of Medicine, Loma Linda, CA, USA

Heng Zhao, Ph.D. Department of Neurosurgery, Stanford University School of Medicine, Stanford, CA, USA

Part I
Microvascular Integrity in Stroke

Structural Alterations to the Endothelial Tight Junction Complex During Stroke

Anuska V. Andjelkovic and Richard F. Keep

1 Introduction

Cerebral endothelial cells and their linking tight junctions (TJs) form the blood–brain barrier (BBB) [1]. That blood/brain interface regulates the movement of compounds and cells into and out of brain. The BBB controls nutrient supply, aids in the removal of potential neurotoxic compounds from brain, and is an essential component regulating the composition of brain extracellular environment which is vital for normal neuronal function [1].

Stroke, including ischemic and hemorrhagic forms, causes BBB dysfunction [2–4]. Thus, stroke causes increased BBB permeability to blood-borne molecules, cerebral edema formation, and leukocyte infiltration. Such changes may enhance stroke-induced brain injury and worsen stroke outcome. This chapter describes the effects of stroke on the cerebral endothelium and the impact of those changes on brain injury. It examines the underlying mechanisms and potential therapeutic

A.V. Andjelkovic, M.D., Ph.D.
Department of Pathology, University of Michigan,
1150 West Medical Center Dr, Ann Arbor, MI 48109-5602, USA

Department of Neurosurgery, University of Michigan Health System,
109 Zina Pitcher Place, Ann Arbor, MI 48109-2200, USA
e-mail: anuskaa@med.umich.edu; anuskaa@umich.edu

R.F. Keep, Ph.D. (✉)
Department of Neurosurgery, University of Michigan Health System,
109 Zina Pitcher Place, Ann Arbor, MI 48109-2200, USA

Department of Molecular and Integrative Physiology, University of Michigan,
1150 West Medical Center Dr, Ann Arbor, MI 48109-5602, USA
e-mail: rkeep@umich.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_1

approaches to reduce stroke-induced BBB dysfunction. It particularly focuses on alterations in endothelial TJs, but it also addresses the potential role of enhanced endothelial transcytosis after stroke.

2 Normal BBB Structure and Function.

In contrast to systemic capillaries, brain capillary endothelial cells are linked by TJs and have a low basal rate of transcytosis (Fig. 1; [1, 5]). These characteristics limit the para- and transcellular pathways across the endothelium resulting in a very low permeability to many compounds. Thus, for example, the transendothelial electrical resistance, a measure of ionic impermeability, is orders of magnitude greater in cerebral compared to systemic capillaries [6, 7]. Exceptions to such low permeability are compounds which can diffuse across the endothelial cell membrane (e.g., O_2, CO_2, H_2O and molecules with high lipophilicity) or those with specific BBB influx transporters, such as D-glucose [1]. There are also an array of efflux transporters, such as *p*-glycoprotein, that enhance brain to blood transport [1]. Such transporters are involved in preventing potential neurotoxins from entering the brain and clearing metabolites from brain. They are a significant obstacle to the delivery of therapeutic agents for neurological disorders [1]. The BBB also has an array of enzymes that can degrade neuroactive compounds preventing their entry from blood into brain (i.e., it is also a metabolic barrier [1, 5]).

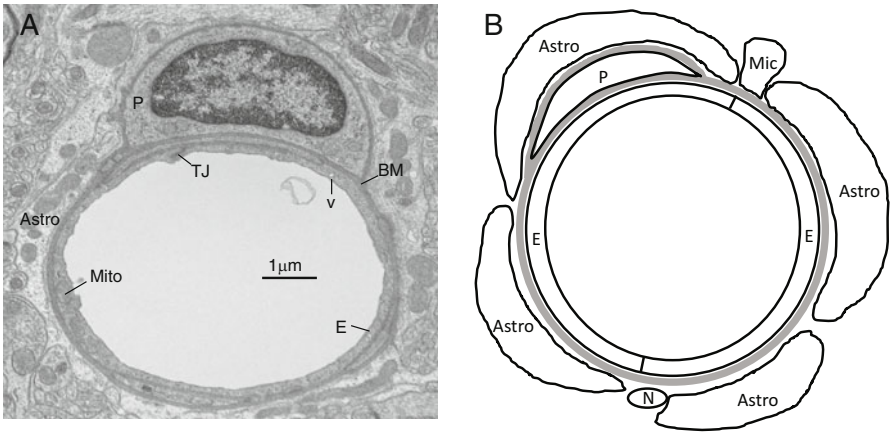

Fig. 1 (**a**) An electron micrograph showing the structure of a normal mouse cerebral capillary. (**b**) A schematic showing the relationship between the endothelium and other elements of the neurovascular unit. Brain capillary endothelial cells (E) are linked by tight junctions (TJ), show few vesicles (v) and have more mitochondria (mito) than systemic capillaries. Sharing the same basement membrane (bm) as the endothelium are pericytes (P). Capillaries are surrounded by astrocyte endfeet (astro), with occasional neuronal (N) and microglial processes (Mic). The endothelial cells and the ensheathing cells/basement membrane are called the neurovascular unit

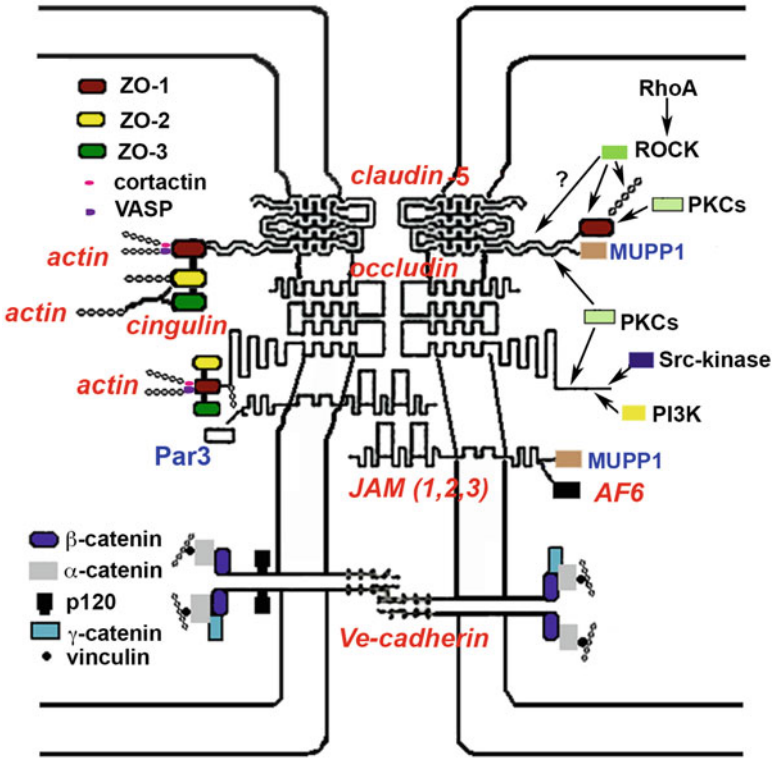

Fig. 2 Schematic representation of the basic components of the brain endothelial junctional complex. The *left* of the panel shows potential structural components of the TJ complex. Claudin-5, occludin, and the JAMs are transmembrane proteins that link adjacent cells. Claudin-5 is the major occlusive protein at the BBB while the cytoplasmic plaque protein, ZO-1, is important for clustering of claudin-5 and occludin and establishing connection with actin filaments. The roles of ZO-2, ZO-3, MUPP1, Par3, Af6 are less clear in brain endothelial cells. Brain endothelial cells also possess adherens junctions, containing the transmembrane protein, Ve-cadherin, and cytosolic accessory proteins (e.g., catenins). The *right* of the panel shows signaling molecules involved in modulating TJ complex assembly and disassembly

Endothelial TJs are integral for BBB function. They are comprised of transmembrane proteins, which form the links between adjacent endothelial cells, and cytoplasmic plaque proteins that form a physical scaffold for the TJs and regulate TJ function [1, 8–10]. In addition, there are links between TJs and the actin cytoskeleton and the adherens junctions that are important for TJ stability and formation (Fig. 2) [9, 10]. TJs are dynamic structures with, for example, claudin-5 and occludin having half-lives of 70–90 min and 6 h, respectively [8].

Transmembrane proteins: These include the claudins, occludin, and the junctional associated molecules (JAMs). In addition, tricellulin, a molecule with homology to occludin, is present at points of three cell contact. The transmembrane

proteins can form *trans*-interactions with proteins on other cells or *cis*-interactions within the same plasma membrane [8].

The claudins are a 27 gene family which are involved in closing the paracellular space between cells (e.g., claudin-5) and in forming ion pores (e.g., claudin-2) [8]. At the BBB, claudin-5 is by far the most predominant claudin although there is evidence of some other claudins (-3, -12, and possibly -1) [8]. Claudin-5 structure and function is regulated by phosphorylation (see below; [8]). It can undergo proteasomal degradation after ubiquitination as well as lysosomal degradation [11]. The claudin-5 knockout results in increased BBB permeability to small molecular weight compounds [12].

Although occludin is not directly involved limiting paracellular permeability, it is thought to be a central regulatory component of the TJ being involved in TJ formation and maintenance [8, 13]. It is a major link to cytoplasmic plaque proteins via binding to ZO-1. Like claudin-5, occludin structure and function is regulated by phosphorylation and it also undergoes ubiquitination and proteasomal degradation [8, 13].

Junctional adhesion molecules (JAM-A, -B, -C) are members of the immunoglobulin superfamily. Their role at the BBB has received much less attention than claudin-5 and occludin, but evidence indicates they are involved in regulating not only paracellular permeability but also leukocyte/endothelial interactions [14–16].

Cytoplasmic plaque proteins: As well as the transmembrane proteins, there are an array of cytosolic proteins associated with the TJs that form the cytoplasmic plaque (Fig. 2). These proteins can be divided on the basis of whether they contain PDZ binding domains. Those which do include members of the membrane-associated guanylate-kinase (MAGUK) superfamily, ZO-1, -2 and -3, as well as Par3, Par6, and AF6 [9]. As many of these proteins contain multiple PDZ domains (e.g., the ZO family members contain three [17]), they can act as scaffolding proteins linking different elements of the TJ (e.g., transmembrane proteins, cytoplasmic plaque proteins, and the actin cytoskeleton).

Cytoplasmic plaque proteins that do not contain PDZ domains include cingulin, 7H6, Rab13, ZONAB, AP-1, PKCζ, and PKCλ, as well as several G proteins (Gαi, Gαs, Gα12, Gαo). These proteins have multiple functions. Thus, for example, it is thought that cingulin acts as a cross link between TJ transmembrane proteins and the actin-myosin cytoskeleton and that the PKC isoforms and the G proteins regulate TJ assembly and maintenance [9].

Adherens junctions: Cerebral endothelial cells possess adherens junctions (AdJs) as well TJs. AdJs are also a complex of transmembrane (Ve-cadherin) and cytosolic accessory proteins (α, β catenin, p120) closely associated with actin filaments [9, 10]. Crosstalk between AdJs and TJs has been proposed to regulate TJ function [9, 10]. For example, Ve-cadherin upregulates claudin-5 expression in brain endothelial cells [18].

Cell cytoskeleton: The cytoskeleton comprises of actin microfilaments, intermediate filaments, and microtubules. The actin cytoskeleton is linked to the TJs via cytoplasmic plaque proteins such as ZO-1 and to AdJs via catenins (Fig. 2 [9]). Increasing evidence indicates that the actin cytoskeleton is a major regulator of TJ

function [19]. Changes in that cytoskeleton may impact TJ function by altering the physical support (scaffold) for the junction and by transmitting physical forces to the junction proteins.

Neurovascular unit (NVU): Although the cerebral endothelial cells and their linking TJs are the ultimate determinants of BBB permeability, perivascular cells (pericytes, astrocytes, neurons, microglia) have a large role in regulating that permeability. In particular, pericytes, which share the same basement membrane as the endothelium, and astrocytes, whose endfeet almost completely surrounds cerebral capillaries (Fig. 1), both regulate barrier permeability [20–22]. These cells, together with smooth muscle cells in larger vessels, act in concert to regulate cerebrovascular function (blood flow and barrier function), forming the NVU.

3 Alterations in BBB Function After Stroke

The hallmarks of ischemic and hemorrhagic stroke include an increased influx of compounds, such as plasma proteins (Fig. 3), into brain from blood, brain swelling due to a net movement of fluid from blood to brain (brain edema) and brain leukocyte infiltration [4, 23–25]. BBB dysfunction participates in all three of these processes.

Ischemic and hemorrhagic stroke cause increased BBB permeability to both small and large molecules [4, 23–25]. The degree and time course BBB disruption varies depending on the severity of the injury, the location of the tissue being sampled relative to the injury and whether or not there is reperfusion. The BBB disruption can be biphasic [24]. In animal models, BBB barrier disruption reaches a peak

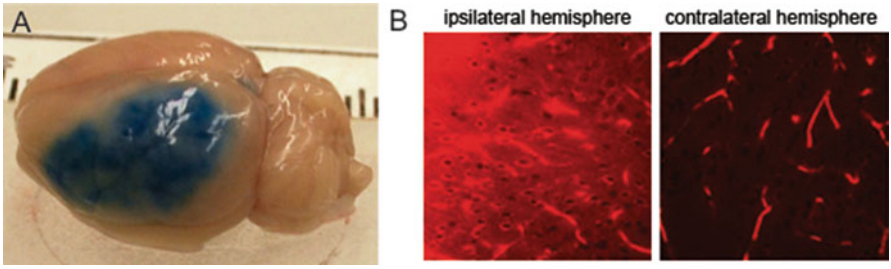

Fig. 3 (**a**) An example of BBB disruption after rat focal cerebral ischemia at the macroscopic level. A hyperglycemic rat underwent 2 h of middle cerebral artery (MCA) occlusion followed by 2 h of reperfusion. The rat was injected intravenously with Evans blue, which binds to albumin in the bloodstream and is excluded from normal brain by the BBB. In the ischemic MCA territory there was marked extravasation of the Evans blue. (**b**) An example of BBB disruption after mouse focal cerebral ischemia at the microscopic level. A mouse underwent 30 min of MCA occlusion followed by 24 h of reperfusion. Enhanced dextran-Texas red (40 kDa) leakage through the disrupted BBB was detected by confocal laser scanning micrographs of the brain ipsi- and contralateral to the occlusion. Note that the dextran was confined to the blood vessels in the contralateral tissue but had spread into the parenchyma in the ischemic tissue

~3–7 days after stroke and then gradually resolves [26]. However, there is evidence that there can be a chronic low level of BBB dysfunction after stroke [27–29]. BBB disruption may result in vasogenic edema and contribute to neuroinflammation. Thus, for example, an influx of prothrombin across the damaged BBB results in the production of thrombin in the brain which is pro-inflammatory and can cause brain edema [30]. Similarly, the entry of fibrinogen from blood to brain is a pro-inflammatory signal [31]. One potential beneficial effect of BBB dysfunction is that it may allow greater entry of therapeutics.

Brain edema is a major consequence of stroke which can result in increased intracranial pressure (ICP) and brain herniation. It is a major cause of morbidity and mortality in ischemic and hemorrhagic stroke [32, 33]. Brain edema is classically classified as cytotoxic or vasogenic dependent on whether the underlying cause is injury to parenchymal cells or the cerebrovasculature [33, 34]. It should be noted, however, that in stroke there is injury to both (mixed edema) and that in both cytotoxic and vasogenic there is a net influx of water from blood to brain across the BBB.

Stroke results in leukocyte (neutrophils, macrophages, and lymphocytes) infiltration into brain [35, 36]. That involves a stepwise process with leukocyte:endothelium interactions causing rolling, adhesion and then diapedesis of the leukocyte across the endothelium [35]. The expression of adhesion molecules on the endothelial luminal membrane plays a prominent role in that process. Leukocytes have been implicated in having a detrimental role in stroke, but they are also involved in tissue repair [35, 36].

The effects of stroke on other barrier functions, such as transport and enzyme activity, have received much less attention, although such changes might have important consequences (e.g., for edema formation and drug delivery) [37, 38]. In addition, although the cerebral and systemic vasculatures possess different properties, they share hemostatic properties that are very important in the occurrence and response to ischemia and hemorrhage [39].

4 Alterations to TJ Structure After Stroke

Alterations in TJ function may result from many different processes, including TJ protein modification (e.g., phosphorylation), altered TJ protein/protein interaction, TJ protein relocation, TJ protein degradation and alterations in transcription/translation (Figs. 4 and 5). There is evidence for each of these processes occurring in stroke and they are intimately linked (e.g., phosphorylation may lead to relocation and degradation). The relative importance of each of the processes likely depends on stroke severity and time point.

Protein modifications: Alterations in the phosphorylation state of TJs proteins are crucial factors influencing BBB permeability. Nevertheless, there is controversy due to the actions of different kinases on distinct residues on the same TJ proteins [40–43]. Most evidence on the effects of phosphorylation/dephosphorylation is on three TJ proteins, the transmembrane proteins occludin and claudin-5, and the scaffolding protein ZO-1.

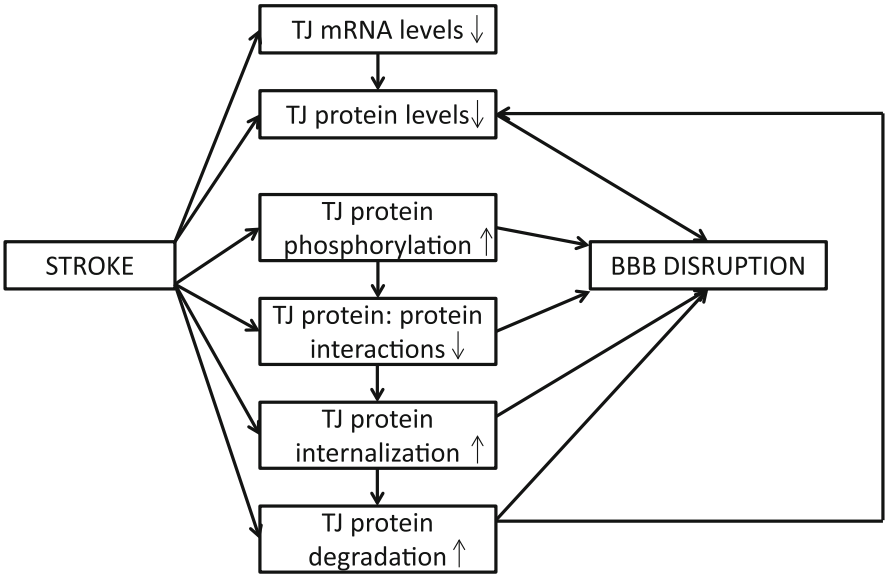

Fig. 4 Schematic showing changes occurring in TJ proteins after stroke that participate in BBB hyperpermeability. Although a potential link between stroke-induced TJ protein phosphorylation, loss of TJ protein interactions, TJ protein internalization and degradation is shown, stroke may affect those processes by several mechanisms, e.g., it may also affect TJ protein degradation by MMP activation

Occludin has several Ser, Thr, and Tyr phosphorylation sites on the C-terminus [40, 44, 45]. Elevated Ser/Thr phosphorylation of occludin is associated with BBB dysfunction during inflammation in the encephalitic human brain [46]. Particularly important findings on the role of Ser/Thr phosphorylation status and barrier function have come from the analysis of vascular endothelial growth factor (VEGF)-induced barrier disruption in retinal endothelial cells, a mechanism underlying diabetic retinopathy [47]. VEGF was shown to induce occludin phosphorylation at sites Thr-168, Thr-404, Sr-408, Ser-471, and Ser-490 via PKCβ, with Ser-490 impacting occludin/ZO-1 interaction and leading to occludin ubiquitination and degradation [47, 48]. In stroke, direct evidence regarding occludin phosphorylation on these Ser/Thr residues is still lacking. There is though evidence that protein kinase C (PKC) isozymes (nPKC-θ and aPKC-ζ) are activated during hypoxia/reoxygenation injury and that cPKCα and Rho-kinase activation is induced by the chemokine CCL2, a major driver of leukocyte entry during stroke-induced brain injury [49, 50].

In epithelial cells, occludin Tyr residues are normally minimally phosphorylated and their phosphorylation is associated with barrier disruption [51]. Tyr-398 and Tyr-402 on occludin are involved in the interaction with ZO-1 and their phosphorylation destabilizes barrier function [44]. At the cerebral endothelium, focal cerebral ischemia, and glutamate treatment in vitro induces occludin Tyr phosphorylation. A Src-kinase inhibitor, PP2, reduces the occludin Tyr phosphorylation and BBB dysfunction found during ischemia/reperfusion (I/R) injury [52–54].

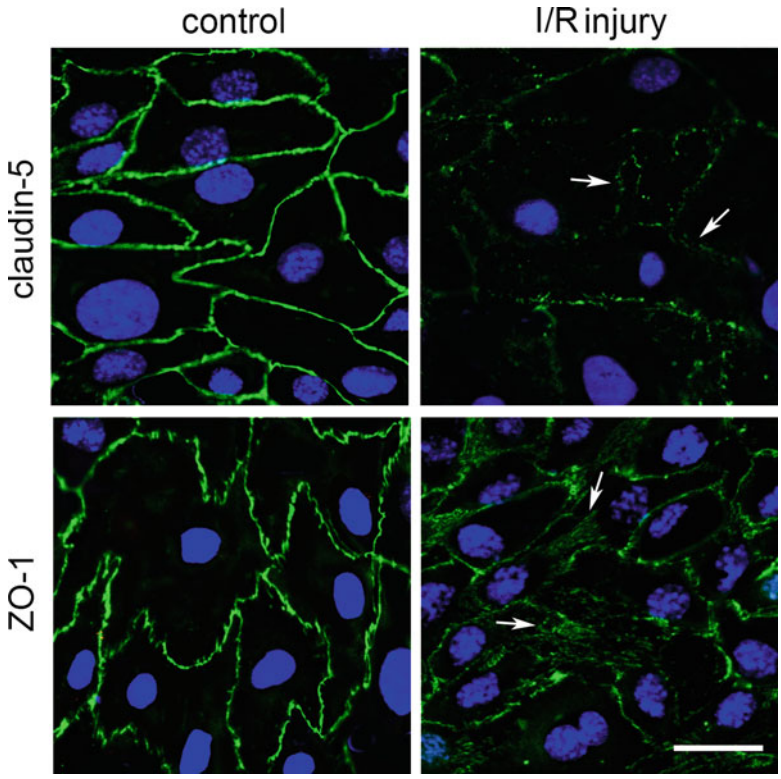

Fig. 5 Mouse brain endothelial cells were exposed to oxygen–glucose deprivation (OGD/ischemia) for 5 h followed by normal oxygen and glucose condition (reperfusion) for 1 h as a model of ischemia/reperfusion (I/R) injury. Control represents cells exposed to normal oxygen and glucose. Immunofluorescent staining was used to visualize the localization of claudin-5 and ZO-1 under the control and I/R conditions. Notice the alteration in claudin-5 and ZO-1 localization/expression on the lateral border of the brain endothelial cells after I/R injury (*arrows*). Scale bar = 20 μm

Phosphorylation of claudin-5 on the Thr-207 residue in the C-terminal domain by PKA or Rho kinase generally affects TJ integrity in brain endothelial cells and increases barrier permeability [46, 55]. However, direct evidence on the pattern of claudin-5 phosphorylation in stroke is still lacking. The activation of nPKC-θ, aPKC-ζ, cPKC-α cPKC-β, PI3Kγ, p38MAPK in different phases of stroke injury and correlation with alterations in claudin-5 localization and expression indirectly point to an essential role of Ser/Thr phosphorylation of claudin-5 in TJ complex disassembly [49, 50, 56, 57].

Evidence indicates that ZO-1 phosphorylation regulates barrier permeability in different systems [58–60]. VEGF increase pTyr-ZO-1 levels in retinal endothelial cells and causes barrier dysfunction [61]. In inflammation, ZO-1 phosphorylation by PKC-ε on threonine 770/772 causes endothelial barrier disruption [62]. Several recent studies also pinpoint that in brain endothelial cell models, cytokine-induced

injury (TNF-α, IL-6 or CCL2) significantly induces Tyr, Thr, and Ser phosphorylation of ZO-1 [43, 49].

The phosphorylation of TJ proteins is tightly associated with the "status" of protein/protein interactions in the junctional complex. Claudin-5/ZO-1, occludin/ZO-1, ZO-1/JAM-A, and ZO-1/actin cytoskeleton interactions are considered essential for the localization and stability of the TJ complex as well as for establishing the trans-interactions and adhesion properties of claudin-5 between adjacent cells [8, 9]. Thus, occludin phosphorylation on Thr 424/Thr438 by PKCζ is required for TJ assembly in epithelial cells, while phosphorylation on Tyr (Tyr398 and Tyr402) and Ser residues (Ser490) attenuates interaction with ZO-1 and promotes dislocation from the lateral membrane in oxidative stress-induced barrier alterations [45, 46, 48, 63, 64]. A similar effect is also described for occludin phosphorylation on Ser408, with dissociation from ZO-1 and increased paracellular permeability [45]. In transient cerebral ischemia, occludin interaction with ZO-1 is diminished due to increased Tyr phosphorylation of occludin and inhibiting Src-kinase (inhibitor PP2) reduces BBB dysfunction [54].

The C-terminus of claudin-5 contains a PDZ binding motif for direct binding to ZO-1, ZO-2, and ZO-3 [65, 66]. Direct evidence regarding diminished interaction between claudin-5 and ZO-1 in the TJ complex during pathological conditions such as ischemia/reoxygenation is lacking although the disappearance of claudin-5 from the cell boundary and increased Ser and Thr phosphorylation suggest diminished interaction with ZO-1 in TJ complex disassembly [50, 56, 57, 67].

Relocation: Besides phosphorylation and diminished protein/protein interactions, disassembly of TJ complex is associated with redistribution of transmembrane TJ proteins. Both occludin and claudin-5 are internalized in inflammatory as well as ischemic conditions [68, 69]. Redistribution of claudin-5 in early brain ischemic conditions is mediated by caveolae [68]. CCL2, a chemokine increased in brain after I/R injury, also causes claudin-5 and occludin by caveolae-mediated internalization [69]. On the other hand, occludin may also be redistributed by clathrin-dependent pathways described in retinal endothelial cells [47]. In contrast, another transmembrane TJ protein, JAM-A, shows macropinocytotic-dependent redistribution under inflammatory conditions [16]. It is relocated from the lateral to the apical side of brain endothelial cells during I/R injury and BBB disruption, as well as during inflammation [15, 16]. JAM-A contains LFA binding sites in the C2 domain and has a role in leukocyte adhesion and diapedesis. Thus, the pattern of JAM-A relocation during TJ disassembly is closely associated with its role in leukocyte infiltration [15, 16].

Degradation: Many studies have described a loss of TJ immunostaining after stroke (e.g., [70–75]). This may result from in situ degradation or internalization of the proteins followed by degradation by the proteasome or lysosomes. The most extensively studied in situ degradation is via matrix metalloproteinases (MMPs). MMP-2 and -9 have been shown to degrade occludin and claudin-5 after cerebral ischemia [68, 75]. Intracellular proteasomal and lysosomal degradation of TJ proteins can occur after ubiquitination, with polyubiquitination generally favoring proteasomal degradation and monoubiquitination lysosomal [11]. With cerebral

ischemia there is evidence of ubiquitination of occludin at the BBB by an E3 ubiquitin ligase, Itch, followed by degradation [76]. Similarly, with retinal ischemia, occludin undergoes polyubiquitination after phosphorylation at the blood retinal barrier [77].

TJ mRNA expression: As well as degradation, loss of TJ proteins after stroke may result from decreased transcription or translation. Reductions in claudin-5, occludin, and ZO-1 mRNA levels have been described after ischemic stroke [67, 75, 78].

Oligomerization and the redox response of TJ proteins: Occludin and claudin-5 have cysteine residues in their cytosolic C-termini that are involved in oligomerization through disulfide bridge formation [40–42]. By building disulfide bridge formation occludin, for example, may become increasingly oligomerized [79]. Such bridges are redox-dependent with normoxic conditions supporting occludin oligomerization and TJ assembly, while hypoxia-reoxygenation results in TJ disruption [79].

5 Signaling Mechanisms Underlying Alterations in TJ Structure in Stroke

Direct and indirect effects of stroke on the endothelium. The effects of ischemic stroke on the endothelial TJs, and thus BBB disruption, may be directly on the endothelium or indirectly via effects on other cell types within the NVU or infiltrating leukocytes which then signal to the endothelium. It has been difficult to assess the relative importance of direct endothelial cell effects in vivo, but the generation of endothelial-specific knockout and transgenic mice will help to examine this question. That the endothelium can be directly affected by ischemia is demonstrated by in vitro experiments on brain endothelial monocultures using oxygen glucose deprivation (OGD) to partially mimic ischemia (Fig. 5). OGD with and without reoxygenation causes disruption of the barrier formed by brain endothelial cell monolayers [68, 80, 81] and this is associated with marked changes in endothelial cell TJs (e.g., relocation and/or loss of TJ proteins [68, 81]).

One of the major components of ischemic and hemorrhagic injury is inflammation with an infiltration of leukocytes into brain, the activation of resident microglia and the production of inflammatory mediators. Inflammation and inflammatory mediators have a major impact on the brain endothelial TJ complex. Thus, different cytokines (IL-1β, TNF-α), chemokines (IL-8, CCL2), MMPs (MMP-2, MMP-9), adhesion molecules (ICAM-1), transcription factors (NfκB), Poly(ADP-ribose) polymerase-1 (PARP) affect TJ structure and function either directly or indirectly (by attracting leukocytes which bind to the endothelium and also produce inflammatory mediators) (e.g., [81–84]). Inflammation and inflammatory mediators are strong modulators of TJ complex disassembly and BBB disruption after cerebral ischemia (e.g., [81, 83, 85, 86]). Long-term changes in the TJ complex and BBB permeability after stroke may fuel alterations in the NVU towards development of chronic inflammatory foci which may lead to further disruption of BBB and progression of inflammation and injury[27–29].

Apart from direct effects of stroke on the cerebral endothelium and indirect effects due to inflammation, ischemic and hemorrhagic stroke may impact the endothelium by other effects on the NVU (e.g., altered trophic support). The reader is referred to other chapters in this book concerning the relationships between cells of the NVU.

Protein kinase C: Hypoxic, ischemic, and I/R injury trigger a variety of signaling pathways involved in regulating TJs and barrier permeability. Among the most extensively studied TJ regulators is the PKC family of serine/threonine kinases. They regulate a variety of cell functions including proliferation, gene expression, cell cycle, differentiation, cytoskeletal organization cell migration, and apoptosis [87–89]. The PKC family includes many different isozymes that are involved in signal transduction from membrane receptors to the nucleus. The activation of cPKC-βII, nPKC-θ, PKC-ζ, PKC-α, and PKC-μ during hypoxia and post-hypoxic reoxygenation or under influence of endothelin-1 or CCL2 regulate TJ disassembly [50, 90–95].

Protein tyrosine kinases: The protein tyrosine kinases (PTKs) are another set of signaling molecules essential for regulating BBB integrity. There are two main classes of PTKs: receptor PTKs and cellular, or non-receptor, PTKs [96–98]. The receptor PTKs have extracellular domains comprised of one or more identifiable structural motifs. They are involved in the BBB effects of growth factors such as VEGF and PDGF [99, 100]. For example, Flt-1 activation and activation of signaling molecules associated with intracellular domain of receptor tyrosine kinases (phosphatidylinositol 3-kinase/Akt (PI3-K/Akt) nitric oxide syntheses (NOS) and protein kinase G (PKG)) mediate hypoxia/VEGF-induced brain endothelial hyperpermeability and brain edema formation [101, 102].

The cellular PTKs (SRC, JAK, ABL, FAK, FPS, CSK, SYK, and BTK) are involved in some of critical events in TJ assembly and disassembly. For example, the Src-family kinases can modulate tyrosine phosphorylation on occludin attenuating its interaction with ZO-1, ZO-2, and ZO-3. They also regulate MMP activity and occludin degradation as well as caveolin-1 phosphorylation and activation and, thus, relocalization of TJ proteins, particularly during I/R-induced BBB disruption [52–54, 68, 103]. Some cellular PTKs, the JAKs, phosphorylate members of the signal transducer and activator of transcription family (the JAK/STAT pathway). That pathway is important in regulating claudin-5, ZO-1 and inflammation in the brain endothelial cells exposed to HIV [104]. PTKs are a major target of reactive oxygen species (ROS) [105]. Thus, for example, ROS generated during ischemia, brain injury, monocyte/neutrophil activation or alcohol exposure, can activate MMPs (-1, -2, and -9) and decrease tissue inhibitors of MMPs (TIMP-1 and -2) in a PTK-dependent manner [68, 106, 107].

MAP kinases. Besides tyrosine kinases, mediators of oxidative stress can also activate a complex system of MAP kinases (p38, Erk1/2) and regulate brain endothelial permeability [57, 108–110]. MAP kinases may be a nodal point in, for example, PTK signaling but they may also directly phosphorylate TJ proteins as occurs during exposure to 4-hydroxy-2-nonenal (4-HNE), one of the major biologically active aldehydes formed during inflammation and oxidative stress [110].

Rho GTPases: Another important regulator of TJs and paracellular permeability is the Rho family of GTPases. That family regulate TJs both indirectly, via effects on the actin cytoskeleton, and directly. Rho, GEF-H1 a guanine nucleotide exchange factor for Rho, Rac, and ROCK regulate cytoskeletal contractile responses via myosin ATPase activity [111]. ROCK, for example, promotes phosphorylation of the regulatory light-chain of myosin (MLC) on Ser19 and Thr18 through phosphorylation of the myosin light-chain phosphatase (MLCP) and, thus, blocks MLC dephosphorylation [112, 113]. This site-specific phosphorylation of MLC in turn elevates myosin ATPase activity, leading to actin-myosin contraction [111, 113]. Besides the specific actin filament polymerization (stress fiber formation), which generate in turn contractile intraendothelial forces impacting the TJs, all of these factors may also induce phosphorylation of TJ and AdJ proteins and their redistribution [112]. These data indicate that Rho/ROCK not only causes remodeling of actin cytoskeleton but also directly or indirectly via activation other signal pathways induces phosphorylation and remodeling of the TJ and AdJ complexes [70, 114]. In addition, ROCK is involved in regulating the expression of the proteolytic enzymes, such as MMP-9 and urokinase-type plasminogen activator (uPA), that impact BBB permeability [115]. Recent evidence has highlighted the beneficial effect of ROCK inhibition, with fasudil or Y2763, in treating BBB disruption in stroke and post-stroke conditions [70, 116–118].

Cerebral hemorrhage. In contrast to the signaling events occurring in the cerebral endothelium during cerebral ischemia, little is known about the events after cerebral hemorrhage [2]. A component of ICH-induced brain injury is related to clot-derived factors such as thrombin, hemoglobin, and iron [23]. The direct impact of such factors on brain endothelial cell signaling and TJ function in relation to ICH is generally understudied. However, there is evidence that thrombin can disassemble claudin-5 from endothelial TJs [119], that hemoglobin exposure can reduce claudin-5 and ZO-1 levels at the BBB [120] and that iron participates in the loss of BBB occludin and ZO-1 after cerebral ischemia [121]. Thus, it is very likely that clot-derived factors impact BBB TJs after hemorrhagic stroke.

6 Non-TJ Pathways Involved in BBB Hyperpermeability After Stroke

Although there is evidence for a role of TJ alterations in BBB disruption after stroke, two other pathways have also been implicated, increased transcytosis at the cerebral endothelium and endothelial cell death [122, 123] (Fig. 6). While cerebral endothelial cells have very few vesicles compared to systemic capillaries, there is some transcytosis under basal conditions. Such receptor-mediated transcytosis, via clathrin- and caveolin-1-coated vesicles, is important for the uptake of a number of macromolecules into brain (e.g., transferrin and leptin, [1, 124, 125]). In addition, some heavily cationized proteins undergo adsorptive-mediated transcytosis [1]. After ischemic stroke there is a marked increase in the number of vesicles in the

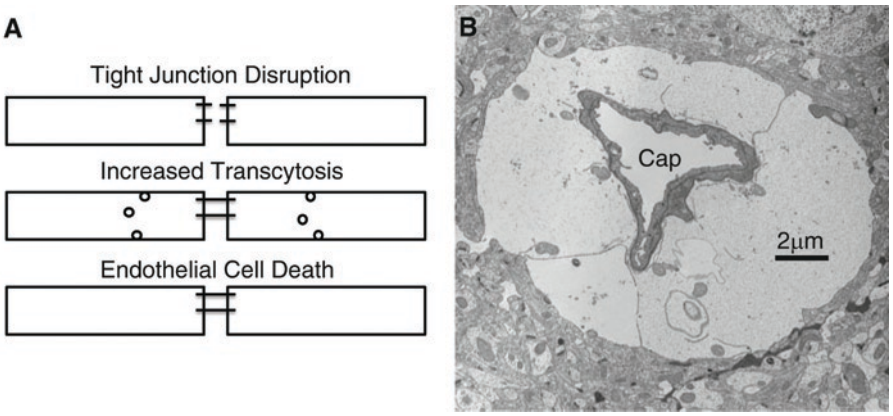

Fig. 6 (**a**) Routes proposed to participate in BBB disruption after stroke. Note that these routes are not mutually exclusive. (**b**) Electron micrograph of a cerebral capillary (cap) from the ischemic penumbra in a mouse that underwent 3 h of middle cerebral artery occlusion. Note the marked perivascular edema in the absence of marked changes in the rest of the parenchyma (compare to Fig. 1a)

cerebral endothelium [122, 126, 127]. If the increase in vesicles reflects increased transcytosis, this may contribute to increased BBB permeability after stroke. This may be particularly important for large molecular weight compounds as this pathway would be relatively size independent. Knowland et al. [122] recently proposed that such an increase in transcytosis is responsible for BBB disruption to albumin (but not a smaller tracer, Biocytin-TMR) observed 6 h after ischemic stroke, whereas profound TJ changes are responsible for later disruption at 2 days. Mice without caveolin-1, an essential component of caveolae, had reduced levels of endothelial- and parenchymal-associated albumin after stroke. It should be noted that caveolae are also involved in internalization of claudin-5 and occludin [69] and, therefore, the caveolin-1 knockout may have also affected TJ function after stroke although Knowland et al. did not identify such an effect [122].

One method to elucidate the relative importance of the transcellular (vesicular) and paracellular routes involved in BBB hyperpermeability is examining size selectivity. Preston and Webster [128] examined the relative permeability of two tracers (sucrose and inulin) of different molecular weights after cerebral ischemia and found that the absolute increase in permeability was greater for the smaller compound. They concluded that the results fitted with diffusion through a pore (with some steric hindrance) rather than vesicular transport. However, it should be noted that those experiments were 24–72 h after the ischemic event.

It has also recently been suggested that endothelial cell death is the primary cause of BBB disruption after cerebral ischemia [123]. Such endothelial cell death has been noted by other investigators after cerebral ischemia [129, 130]. A question that arises with a loss of endothelial cells being the route of BBB disruption is the extent to which such vessels continue to remain patent. Endothelial damage results in the formation of a platelet plug and activation of the coagulation cascade.

7 Therapeutic Interventions

Despite decades of work on developing neuroprotective agents for stroke [131] only reperfusion-based therapy has so far shown clinical efficacy. Tissue plasminogen activator [132] and now mechanical thrombectomy [133] improve outcomes in ischemic stroke. Those approaches do not directly target BBB changes after stroke. Indeed, tPA is associated with increased risk of cerebral hemorrhage after ischemic stroke [132]. There have been preclinical [134, 135] and now clinical trials (NCT02222714 and the European trial, Imatinib Treatment in Acute Ischemic Stroke) of agents that might protect the cerebral vasculature if given in combination with tPA. Reducing the occurrence of ICH would alleviate one impediment to its use in stroke patients. It might also increase the time window over which tPA can be given.

In preclinical models, many agents have been shown to decrease BBB hyperpermeability and alterations in TJ structure after ischemic stroke (e.g., [70–75]). A question arises as to whether those BBB effects are direct or indirect (e.g., via reducing parenchymal damage)? Endothelial-specific genetic manipulations should address this question and also whether BBB damage may contribute to parenchymal damage. It should be noted that if the BBB is a therapeutic target, drug delivery to the BBB avoids many of the problems with delivery to the brain parenchyma (e.g., brain penetration).

8 Conclusions

BBB disruption occurs in both ischemic and hemorrhagic stroke. It may contribute to brain injury by enhancing edema formation, leukocyte influx, and entry of neurotoxic compounds from blood to brain. While endothelial transcytosis and cell death have been proposed as being important in BBB dysfunction after stroke, considerable evidence indicates a major role for TJ modification. The extent to which the BBB is a primary therapeutic target for limiting brain injury, with and without reperfusion therapy, still needs to be fully clarified, but the tools necessary for such studies are becoming available.

Acknowledgments This work was supported by grants NS-034709 (R.F.K.), NS-093399 (R.F.K.), NS062853 (A.V.A.), and NS075757 (A.V.A.) from the National Institutes of Health. The content is solely the responsibility of the authors and does not necessarily represent the official view of the NIH.

References

1. Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. Neurobiol Dis. 2010;37(1):13–25.
2. Keep RF, Zhou N, Xiang J, Andjelkovic AV, Hua Y, Xi G. Vascular disruption and blood-brain barrier dysfunction in intracerebral hemorrhage. Fluids Barriers CNS. 2014;11:18.

3. Rosenberg GA. Neurological diseases in relation to the blood-brain barrier. J Cereb Blood Flow Metab. 2012;32(7):1139–51.
4. Yang Y, Rosenberg GA. Blood-brain barrier breakdown in acute and chronic cerebrovascular disease. Stroke. 2011;42(11):3323–8.
5. Strazielle N, Ghersi-Egea JF. Physiology of blood-brain interfaces in relation to brain disposition of small compounds and macromolecules. Mol Pharm. 2013;10(5):1473–91.
6. Butt AM, Jones HC, Abbott NJ. Electrical resistance across the blood-brain barrier in anaesthetized rats: a developmental study. J Physiol. 1990;429:47–62.
7. Crone C, Christensen O. Electrical resistance of a capillary endothelium. J Gen Physiol. 1981;77(4):349–71.
8. Haseloff RF, Dithmer S, Winkler L, Wolburg H, Blasig IE. Transmembrane proteins of the tight junctions at the blood-brain barrier: structural and functional aspects. Semin Cell Dev Biol. 2015;38:16–25.
9. Stamatovic SM, Keep RF, Andjelkovic AV. Brain endothelial cell-cell junctions: how to "open" the blood brain barrier. Curr Neuropharmacol. 2008;6(3):179–92.
10. Weiss N, Miller F, Cazaubon S, Couraud PO. The blood-brain barrier in brain homeostasis and neurological diseases. Biochim Biophys Acta. 2009;1788(4):842–57.
11. Mandel I, Paperna T, Volkowich A, Merhav M, Glass-Marmor L, Miller A. The ubiquitin-proteasome pathway regulates claudin 5 degradation. J Cell Biochem. 2012;113(7): 2415–23.
12. Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. J Cell Biol. 2003;161(3):653–60.
13. Blasig IE, Bellmann C, Cording J, Del Vecchio G, Zwanziger D, Huber O, et al. Occludin protein family: oxidative stress and reducing conditions. Antioxid Redox Signal. 2011;15(5):1195–219.
14. Lamagna C, Meda P, Mandicourt G, Brown J, Gilbert RJ, Jones EY, et al. Dual interaction of JAM-C with JAM-B and alpha(M)beta2 integrin: function in junctional complexes and leukocyte adhesion. Mol Biol Cell. 2005;16(10):4992–5003.
15. Sladojevic N, Stamatovic SM, Keep RF, Grailer JJ, Sarma JV, Ward PA, et al. Inhibition of junctional adhesion molecule-A/LFA interaction attenuates leukocyte trafficking and inflammation in brain ischemia/reperfusion injury. Neurobiol Dis. 2014;67:57–70.
16. Stamatovic SM, Sladojevic N, Keep RF, Andjelkovic AV. Relocalization of junctional adhesion molecule A during inflammatory stimulation of brain endothelial cells. Mol Cell Biol. 2012;32(17):3414–27.
17. Itoh M, Furuse M, Morita K, Kubota K, Saitou M, Tsukita S. Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. J Cell Biol. 1999;147(6):1351–63.
18. Taddei A, Giampietro C, Conti A, Orsenigo F, Breviario F, Pirazzoli V, et al. Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. Nat Cell Biol. 2008;10(8):923–34.
19. Lai CH, Kuo KH, Leo JM. Critical role of actin in modulating BBB permeability. Brain Res Rev. 2005;50(1):7–13.
20. Abbott NJ, Ronnback L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci. 2006;7(1):41–53.
21. Armulik A, Genove G, Mae M, Nisancioglu MH, Wallgard E, Niaudet C, et al. Pericytes regulate the blood-brain barrier. Nature. 2010;468(7323):557–61.
22. Daneman R, Zhou L, Kebede AA, Barres BA. Pericytes are required for blood-brain barrier integrity during embryogenesis. Nature. 2010;468(7323):562–6.
23. Keep RF, Hua Y, Xi G. Intracerebral haemorrhage: mechanisms of injury and therapeutic targets. Lancet Neurol. 2012;11(8):720–31.
24. Sandoval KE, Witt KA. Blood-brain barrier tight junction permeability and ischemic stroke. Neurobiol Dis. 2008;32(2):200–19.
25. Xi G, Keep RF, Hoff JT. Mechanisms of brain injury after intracerebral haemorrhage. Lancet Neurol. 2006;5(1):53–63.

26. Menzies SA, Betz AL, Hoff JT. Contributions of ions and albumin to the formation and resolution of ischemic brain edema. J Neurosurg. 1993;78(2):257–66.
27. Doyle KP, Quach LN, Sole M, Axtell RC, Nguyen TV, Soler-Llavina GJ, et al. B-lymphocyte-mediated delayed cognitive impairment following stroke. J Neurosci. 2015;35(5):2133–45.
28. Garbuzova-Davis S, Haller E, Williams SN, Haim ED, Tajiri N, Hernandez-Ontiveros DG, et al. Compromised blood-brain barrier competence in remote brain areas in ischemic stroke rats at the chronic stage. J Comp Neurol. 2014;522(13):3120–37.
29. Liu Y, Tang G, Li Y, Wang Y, Chen X, Gu X, et al. Metformin attenuates blood-brain barrier disruption in mice following middle cerebral artery occlusion. J Neuroinflammation. 2014;11:177.
30. Xi G, Reiser G, Keep RF. The role of thrombin and thrombin receptors in ischemic, hemorrhagic and traumatic brain injury: deleterious or protective? J Neurochem. 2003;84(1):3–9.
31. Davalos D, Akassoglou K. Fibrinogen as a key regulator of inflammation in disease. Semin Immunopathol. 2012;34(1):43–62.
32. Balami JS, Buchan AM. Complications of intracerebral haemorrhage. Lancet Neurol. 2012;11(1):101–18.
33. Khanna A, Kahle KT, Walcott BP, Gerzanich V, Simard JM. Disruption of ion homeostasis in the neurogliovascular unit underlies the pathogenesis of ischemic cerebral edema. Transl Stroke Res. 2014;5(1):3–16.
34. Marmarou A. A review of progress in understanding the pathophysiology and treatment of brain edema. Neurosurg Focus. 2007;22(5):E1.
35. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. Nat Med. 2011;17(7):796–808.
36. Liesz A, Hu X, Kleinschnitz C, Offner H. Functional role of regulatory lymphocytes in stroke: facts and controversies. Stroke. 2015;46(5):1422–30.
37. O'Donnell ME. Blood-brain barrier Na transporters in ischemic stroke. Adv Pharmacol. 2014;71:113–46.
38. Shah K, Abbruscato T. The role of blood-brain barrier transporters in pathophysiology and pharmacotherapy of stroke. Curr Pharm Des. 2014;20(10):1510–22.
39. del Zoppo GJ, Izawa Y, Hawkins BT. Hemostasis and alterations of the central nervous system. Semin Thromb Hemost. 2013;39(8):856–75.
40. Cummins PM. Occludin: one protein, many forms. Mol Cell Biol. 2012;32(2):242–50.
41. Dorfel MJ, Huber O. Modulation of tight junction structure and function by kinases and phosphatases targeting occludin. J Biomed Biotechnol. 2012;2012:807356.
42. Gonzalez-Mariscal L, Tapia R, Chamorro D. Crosstalk of tight junction components with signaling pathways. Biochim Biophys Acta. 2008;1778(3):729–56.
43. Rochfort KD, Cummins PM. Cytokine-mediated dysregulation of zonula occludens-1 properties in human brain microvascular endothelium. Microvasc Res. 2015;100:48–53.
44. Elias BC, Suzuki T, Seth A, Giorgianni F, Kale G, Shen L, et al. Phosphorylation of Tyr-398 and Tyr-402 in occludin prevents its interaction with ZO-1 and destabilizes its assembly at the tight junctions. J Biol Chem. 2009;284(3):1559–69.
45. Raleigh DR, Boe DM, Yu D, Weber CR, Marchiando AM, Bradford EM, et al. Occludin S408 phosphorylation regulates tight junction protein interactions and barrier function. J Cell Biol. 2011;193(3):565–82.
46. Yamamoto M, Ramirez SH, Sato S, Kiyota T, Cerny RL, Kaibuchi K, et al. Phosphorylation of claudin-5 and occludin by rho kinase in brain endothelial cells. Am J Pathol. 2008;172(2):521–33.
47. Murakami T, Felinski EA, Antonetti DA. Occludin phosphorylation and ubiquitination regulate tight junction trafficking and vascular endothelial growth factor-induced permeability. J Biol Chem. 2009;284(31):21036–46.
48. Murakami T, Frey T, Lin C, Antonetti DA. Protein kinase cbeta phosphorylates occludin regulating tight junction trafficking in vascular endothelial growth factor-induced permeability in vivo. Diabetes. 2012;61(6):1573–83.

49. Stamatovic SM, Dimitrijevic OB, Keep RF, Andjelkovic AV. Protein kinase Calpha-RhoA cross-talk in CCL2-induced alterations in brain endothelial permeability. J Biol Chem. 2006;281(13):8379–88.
50. Willis CL, Meske DS, Davis TP. Protein kinase C activation modulates reversible increase in cortical blood-brain barrier permeability and tight junction protein expression during hypoxia and posthypoxic reoxygenation. J Cereb Blood Flow Metab. 2010;30(11):1847–59.
51. Rao R. Occludin phosphorylation in regulation of epithelial tight junctions. Ann N Y Acad Sci. 2009;1165:62–8.
52. Kago T, Takagi N, Date I, Takenaga Y, Takagi K, Takeo S. Cerebral ischemia enhances tyrosine phosphorylation of occludin in brain capillaries. Biochem Biophys Res Commun. 2006;339(4):1197–203.
53. Mao XW, Pan CS, Huang P, Liu YY, Wang CS, Yan L, et al. Levo-tetrahydropalmatine attenuates mouse blood-brain barrier injury induced by focal cerebral ischemia and reperfusion: involvement of Src kinase. Sci Rep. 2015;5:11155.
54. Takenaga Y, Takagi N, Murotomi K, Tanonaka K, Takeo S. Inhibition of Src activity decreases tyrosine phosphorylation of occludin in brain capillaries and attenuates increase in permeability of the blood-brain barrier after transient focal cerebral ischemia. J Cereb Blood Flow Metab. 2009;29(6):1099–108.
55. Soma T, Chiba H, Kato-Mori Y, Wada T, Yamashita T, Kojima T, et al. Thr(207) of claudin-5 is involved in size-selective loosening of the endothelial barrier by cyclic AMP. Exp Cell Res. 2004;300(1):202–12.
56. Camire RB, Beaulac HJ, Brule SA, McGregor AI, Lauria EE, Willis CL. Biphasic modulation of paracellular claudin-5 expression in mouse brain endothelial cells is mediated through the phosphoinositide-3-kinase/AKT pathway. J Pharmacol Exp Ther. 2014;351(3):654–62.
57. Dong L, Qiao H, Zhang X, Zhang X, Wang C, Wang L, et al. Parthenolide is neuroprotective in rat experimental stroke model: downregulating NF-kappaB, phospho-p38MAPK, and caspase-1 and ameliorating BBB permeability. Mediators Inflamm. 2013;2013:370804.
58. Howarth AG, Singer KL, Stevenson BR. Analysis of the distribution and phosphorylation state of ZO-1 in MDCK and nonepithelial cells. J Membr Biol. 1994;137(3):261–70.
59. Kurihara H, Anderson JM, Farquhar MG. Increased Tyr phosphorylation of ZO-1 during modification of tight junctions between glomerular foot processes. Am J Physiol. 1995;268(3 Pt 2):F514–24.
60. Van Itallie CM, Balda MS, Anderson JM. Epidermal growth factor induces tyrosine phosphorylation and reorganization of the tight junction protein ZO-1 in A431 cells. J Cell Sci. 1995;108(Pt 4):1735–42.
61. Antonetti DA, Barber AJ, Hollinger LA, Wolpert EB, Gardner TW. Vascular endothelial growth factor induces rapid phosphorylation of tight junction proteins occludin and zonula occluden 1. A potential mechanism for vascular permeability in diabetic retinopathy and tumors. J Biol Chem. 1999;274(33):23463–7.
62. Ivey NS, Renner NA, Moroney-Rasmussen T, Mohan M, Redmann RK, Didier PJ, et al. Association of FAK activation with lentivirus-induced disruption of blood-brain barrier tight junction-associated ZO-1 protein organization. J Neurovirol. 2009;15(4):312–23.
63. Dorfel MJ, Westphal JK, Bellmann C, Krug SM, Cording J, Mittag S, et al. CK2-dependent phosphorylation of occludin regulates the interaction with ZO-proteins and tight junction integrity. Cell Commun Signal. 2013;11(1):40.
64. Jain S, Suzuki T, Seth A, Samak G, Rao R. Protein kinase Czeta phosphorylates occludin and promotes assembly of epithelial tight junctions. Biochem J. 2011;437(2):289–99.
65. Poliak S, Matlis S, Ullmer C, Scherer SS, Peles E. Distinct claudins and associated PDZ proteins form different autotypic tight junctions in myelinating Schwann cells. J Cell Biol. 2002;159(2):361–72.
66. Wu J, Peng D, Zhang Y, Lu Z, Voehler M, Sanders CR, et al. Biophysical characterization of interactions between the C-termini of peripheral nerve claudins and the PDZ(1) domain of zonula occludens. Biochem Biophys Res Commun. 2015;459(1):87–93.

67. Jiao H, Wang Z, Liu Y, Wang P, Xue Y. Specific role of tight junction proteins claudin-5, occludin, and ZO-1 of the blood-brain barrier in a focal cerebral ischemic insult. J Mol Neurosci. 2011;44(2):130–9.
68. Liu J, Jin X, Liu KJ, Liu W. Matrix metalloproteinase-2-mediated occludin degradation and caveolin-1-mediated claudin-5 redistribution contribute to blood-brain barrier damage in early ischemic stroke stage. J Neurosci. 2012;32(9):3044–57.
69. Stamatovic SM, Keep RF, Wang MM, Jankovic I, Andjelkovic AV. Caveolae-mediated internalization of occludin and claudin-5 during CCL2-induced tight junction remodeling in brain endothelial cells. J Biol Chem. 2009;284(28):19053–66.
70. Gibson CL, Srivastava K, Sprigg N, Bath PM, Bayraktutan U. Inhibition of Rho-kinase protects cerebral barrier from ischaemia-evoked injury through modulations of endothelial cell oxidative stress and tight junctions. J Neurochem. 2014;129(5):816–26.
71. Jin G, Arai K, Murata Y, Wang S, Stins MF, Lo EH, et al. Protecting against cerebrovascular injury: contributions of 12/15-lipoxygenase to edema formation after transient focal ischemia. Stroke. 2008;39(9):2538–43.
72. Kraft P, Schwarz T, Gob E, Heydenreich N, Brede M, Meuth SG, et al. The phosphodiesterase-4 inhibitor rolipram protects from ischemic stroke in mice by reducing blood-brain-barrier damage, inflammation and thrombosis. Exp Neurol. 2013;247:80–90.
73. Reischl S, Li L, Walkinshaw G, Flippin LA, Marti HH, Kunze R. Inhibition of HIF prolyl-4-hydroxylases by FG-4497 reduces brain tissue injury and edema formation during ischemic stroke. PLoS One. 2014;9(1):e84767.
74. Wang Y, Jia J, Ao G, Hu L, Liu H, Xiao Y, et al. Hydrogen sulfide protects blood-brain barrier integrity following cerebral ischemia. J Neurochem. 2014;129(5):827–38.
75. Yang Y, Estrada EY, Thompson JF, Liu W, Rosenberg GA. Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. J Cereb Blood Flow Metab. 2007;27(4):697–709.
76. Zhang GS, Tian Y, Huang JY, Tao RR, Liao MH, Lu YM, et al. The gamma-secretase blocker DAPT reduces the permeability of the blood-brain barrier by decreasing the ubiquitination and degradation of occludin during permanent brain ischemia. CNS Neurosci Ther. 2013;19(1):53–60.
77. Muthusamy A, Lin CM, Shanmugam S, Lindner HM, Abcouwer SF, Antonetti DA. Ischemia-reperfusion injury induces occludin phosphorylation/ubiquitination and retinal vascular permeability in a VEGFR-2-dependent manner. J Cereb Blood Flow Metab. 2014;34(3):522–31.
78. Wang Z, Xue Y, Jiao H, Liu Y, Wang P. Doxycycline-mediated protective effect against focal cerebral ischemia-reperfusion injury through the modulation of tight junctions and PKCdelta signaling in rats. J Mol Neurosci. 2012;47(1):89–100.
79. McCaffrey G, Willis CL, Staatz WD, Nametz N, Quigley CA, Hom S, et al. Occludin oligomeric assemblies at tight junctions of the blood-brain barrier are altered by hypoxia and reoxygenation stress. J Neurochem. 2009;110(1):58–71.
80. Culot M, Mysiorek C, Renftel M, Roussel BD, Hommet Y, Vivien D, et al. Cerebrovascular protection as a possible mechanism for the protective effects of NXY-059 in preclinical models: an in vitro study. Brain Res. 2009;1294:144–52.
81. Dimitrijevic OB, Stamatovic SM, Keep RF, Andjelkovic AV. Effects of the chemokine CCL2 on blood-brain barrier permeability during ischemia-reperfusion injury. J Cereb Blood Flow Metab. 2006;26(6):797–810.
82. Argaw AT, Zhang Y, Snyder BJ, Zhao ML, Kopp N, Lee SC, et al. IL-1beta regulates blood-brain barrier permeability via reactivation of the hypoxia-angiogenesis program. J Immunol. 2006;177(8):5574–84.
83. Orion D, Schwammenthal Y, Reshef T, Schwartz R, Tsabari R, Merzeliak O, et al. Interleukin-6 and soluble intercellular adhesion molecule-1 in acute brain ischaemia. Eur J Neurol. 2008;15(4):323–8.

84. Rom S, Zuluaga-Ramirez V, Dykstra H, Reichenbach NL, Ramirez SH, Persidsky Y. Poly(ADP-ribose) polymerase-1 inhibition in brain endothelium protects the blood-brain barrier under physiologic and neuroinflammatory conditions. J Cereb Blood Flow Metab. 2015;35(1):28–36.
85. Matsumoto T, Ikeda K, Mukaida N, Harada A, Matsumoto Y, Yamashita J, et al. Prevention of cerebral edema and infarct in cerebral reperfusion injury by an antibody to interleukin-8. Lab Invest. 1997;77(2):119–25.
86. Strecker JK, Minnerup J, Schutte-Nutgen K, Gess B, Schabitz WR, Schilling M. Monocyte chemoattractant protein-1-deficiency results in altered blood-brain barrier breakdown after experimental stroke. Stroke. 2013;44(9):2536–44.
87. Andreeva AY, Krause E, Muller EC, Blasig IE, Utepbergenov DI. Protein kinase C regulates the phosphorylation and cellular localization of occludin. J Biol Chem. 2001;276(42): 38480–6.
88. Rosse C, Linch M, Kermorgant S, Cameron AJ, Boeckeler K, Parker PJ. PKC and the control of localized signal dynamics. Nat Rev Mol Cell Biol. 2010;11(2):103–12.
89. Xiao H, Liu M. Atypical protein kinase C in cell motility. Cell Mol Life Sci. 2013;70(17):3057–66.
90. Bright R, Raval AP, Dembner JM, Perez-Pinzon MA, Steinberg GK, Yenari MA, et al. Protein kinase C delta mediates cerebral reperfusion injury in vivo. J Neurosci. 2004; 24(31):6880–8.
91. Miettinen S, Roivainen R, Keinanen R, Hokfelt T, Koistinaho J. Specific induction of protein kinase C delta subspecies after transient middle cerebral artery occlusion in the rat brain: inhibition by MK-801. J Neurosci. 1996;16(19):6236–45.
92. Selvatici R, Marino S, Piubello C, Rodi D, Beani L, Gandini E, et al. Protein kinase C activity, translocation, and selective isoform subcellular redistribution in the rat cerebral cortex after in vitro ischemia. J Neurosci Res. 2003;71(1):64–71.
93. Srivastava K, Shao B, Bayraktutan U. PKC-beta exacerbates in vitro brain barrier damage in hyperglycemic settings via regulation of RhoA/Rho-kinase/MLC2 pathway. J Cereb Blood Flow Metab. 2013;33(12):1928–36.
94. Sun X, Budas GR, Xu L, Barreto GE, Mochly-Rosen D, Giffard RG. Selective activation of protein kinase C in mitochondria is neuroprotective in vitro and reduces focal ischemic brain injury in mice. J Neurosci Res. 2013;91(6):799–807.
95. Wang LF, Li X, Gao YB, Wang SM, Zhao L, Dong J, et al. Activation of VEGF/Flk-1-ERK pathway induced blood-brain barrier injury after microwave exposure. Mol Neurobiol. 2015;52(1):478–91.
96. al-Obeidi FA, Wu JJ, Lam KS. Protein tyrosine kinases: structure, substrate specificity, and drug discovery. Biopolymers. 1998;47(3):197–223.
97. Bannerman DD, Goldblum SE. Direct effects of endotoxin on the endothelium: barrier function and injury. Lab Invest. 1999;79(10):1181–99.
98. Carbajal JM, Schaeffer Jr RC. H2O2 and genistein differentially modulate protein tyrosine phosphorylation, endothelial morphology, and monolayer barrier function. Biochem Biophys Res Commun. 1998;249(2):461–6.
99. Ma Q, Huang B, Khatibi N, Rolland 2nd W, Suzuki H, Zhang JH, et al. PDGFR-alpha inhibition preserves blood-brain barrier after intracerebral hemorrhage. Ann Neurol. 2011;70(6):920–31.
100. Ma Y, Zechariah A, Qu Y, Hermann DM. Effects of vascular endothelial growth factor in ischemic stroke. J Neurosci Res. 2012;90(10):1873–82.
101. Leung JW, Chung SS, Chung SK. Endothelial endothelin-1 over-expression using receptor tyrosine kinase tie-1 promoter leads to more severe vascular permeability and blood brain barrier breakdown after transient middle cerebral artery occlusion. Brain Res. 2009; 1266:121–9.
102. Vogel C, Bauer A, Wiesnet M, Preissner KT, Schaper W, Marti HH, et al. Flt-1, but not Flk-1 mediates hyperpermeability through activation of the PI3-K/Akt pathway. J Cell Physiol. 2007;212(1):236–43.

103. Bai Y, Xu G, Xu M, Li Q, Qin X. Inhibition of Src phosphorylation reduces damage to the blood-brain barrier following transient focal cerebral ischemia in rats. Int J Mol Med. 2014;34(6):1473–82.
104. Chaudhuri A, Yang B, Gendelman HE, Persidsky Y, Kanmogne GD. STAT1 signaling modulates HIV-1-induced inflammatory responses and leukocyte transmigration across the blood-brain barrier. Blood. 2008;111(4):2062–72.
105. Knock GA, Ward JP. Redox regulation of protein kinases as a modulator of vascular function. Antioxid Redox Signal. 2011;15(6):1531–47.
106. Haorah J, Knipe B, Leibhart J, Ghorpade A, Persidsky Y. Alcohol-induced oxidative stress in brain endothelial cells causes blood-brain barrier dysfunction. J Leukoc Biol. 2005;78(6):1223–32.
107. Haorah J, Ramirez SH, Schall K, Smith D, Pandya R, Persidsky Y. Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. J Neurochem. 2007;101(2):566–76.
108. Fischer S, Wiesnet M, Renz D, Schaper W. H2O2 induces paracellular permeability of porcine brain-derived microvascular endothelial cells by activation of the p44/42 MAP kinase pathway. Eur J Cell Biol. 2005;84(7):687–97.
109. Qin LH, Huang W, Mo XA, Chen YL, Wu XH. LPS induces occludin dysregulation in cerebral microvascular endothelial cells via MAPK signaling and augmenting MMP-2 levels. Oxid Med Cell Longev. 2015;2015:120641.
110. Usatyuk PV, Parinandi NL, Natarajan V. Redox regulation of 4-hydroxy-2-nonenal-mediated endothelial barrier dysfunction by focal adhesion, adherens, and tight junction proteins. J Biol Chem. 2006;281(46):35554–66.
111. Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. Annu Rev Cell Dev Biol. 2005;21:247–69.
112. Nelson CM, Pirone DM, Tan JL, Chen CS. Vascular endothelial-cadherin regulates cytoskeletal tension, cell spreading, and focal adhesions by stimulating RhoA. Mol Biol Cell. 2004;15(6):2943–53.
113. Noma K, Oyama N, Liao JK. Physiological role of ROCKs in the cardiovascular system. Am J Physiol. 2006;290(3):C661–8.
114. Persidsky Y, Heilman D, Haorah J, Zelivyanskaya M, Persidsky R, Weber GA, et al. Rho-mediated regulation of tight junctions during monocyte migration across the blood-brain barrier in HIV-1 encephalitis (HIVE). Blood. 2006;107(12):4770–80.
115. Yu Y, Qin J, Liu M, Ruan Q, Li Y, Zhang Z. Role of Rho kinase in lysophosphatidic acid-induced altering of blood-brain barrier permeability. Int J Mol Med. 2014;33(3):661–9.
116. Liu K, Li Z, Wu T, Ding S. Role of rho kinase in microvascular damage following cerebral ischemia reperfusion in rats. Int J Mol Sci. 2011;12(2):1222–31.
117. Posada-Duque RA, Barreto GE, Cardona-Gomez GP. Protection after stroke: cellular effectors of neurovascular unit integrity. Front Cell Neurosci. 2014;8:231.
118. Rikitake Y, Kim HH, Huang Z, Seto M, Yano K, Asano T, et al. Inhibition of Rho kinase (ROCK) leads to increased cerebral blood flow and stroke protection. Stroke. 2005;36(10):2251–7.
119. Kondo N, Ogawa M, Wada H, Nishikawa S. Thrombin induces rapid disassembly of claudin-5 from the tight junction of endothelial cells. Exp Cell Res. 2009;315(17):2879–87.
120. Yang S, Chen Y, Deng X, Jiang W, Li B, Fu Z, et al. Hemoglobin-induced nitric oxide synthase overexpression and nitric oxide production contribute to blood-brain barrier disruption in the rat. J Mol Neurosci. 2013;51(2):352–63.
121. Won SM, Lee JH, Park UJ, Gwag J, Gwag BJ, Lee YB. Iron mediates endothelial cell damage and blood-brain barrier opening in the hippocampus after transient forebrain ischemia in rats. Exp Mol Med. 2011;43(2):121–8.
122. Knowland D, Arac A, Sekiguchi KJ, Hsu M, Lutz SE, Perrino J, et al. Stepwise recruitment of transcellular and paracellular pathways underlies blood-brain barrier breakdown in stroke. Neuron. 2014;82(3):603–17.

123. Krueger M, Bechmann I, Immig K, Reichenbach A, Hartig W, Michalski D. Blood-brain barrier breakdown involves four distinct stages of vascular damage in various models of experimental focal cerebral ischemia. J Cereb Blood Flow Metab. 2015;35(2):292–303.
124. de Lange EC. The physiological characteristics and transcytosis mechanisms of the blood-brain barrier (BBB). Curr Pharm Biotechnol. 2012;13(12):2319–27.
125. Smith MW, Gumbleton M. Endocytosis at the blood-brain barrier: from basic understanding to drug delivery strategies. J Drug Target. 2006;14(4):191–214.
126. Cole DJ, Matsumura JS, Drummond JC, Schultz RL, Wong MH. Time- and pressure-dependent changes in blood-brain barrier permeability after temporary middle cerebral artery occlusion in rats. Acta Neuropathol. 1991;82(4):266–73.
127. Dietrich WD, Nakayama H, Watson BD, Kanemitsu H. Morphological consequences of early reperfusion following thrombotic or mechanical occlusion of the rat middle cerebral artery. Acta Neuropathol. 1989;78(6):605–14.
128. Preston E, Webster J. Differential passage of [14C]sucrose and [3H]inulin across rat blood-brain barrier after cerebral ischemia. Acta Neuropathol. 2002;103(3):237–42.
129. Dietrich WD, Busto R, Watson BD, Scheinberg P, Ginsberg MD. Photochemically induced cerebral infarction. II. Edema and blood-brain barrier disruption. Acta Neuropathol. 1987;72(4):326–34.
130. Ito U, Ohno K, Yamaguchi T, Takei H, Tomita H, Inaba Y. Effect of hypertension on blood-brain barrier. Change after restoration of blood flow in post-ischemic gerbil brains. An electronmicroscopic study. Stroke. 1980;11(6):606–11.
131. Ginsberg MD. Neuroprotection for ischemic stroke: past, present and future. Neuropharmacology. 2008;55(3):363–89.
132. Emberson J, Lees KR, Lyden P, Blackwell L, Albers G, Bluhmki E, et al. Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischaemic stroke: a meta-analysis of individual patient data from randomised trials. Lancet. 2014;384(9958):1929–35.
133. Campbell BC, Donnan GA, Lees KR, Hacke W, Khatri P, Hill MD, et al. Endovascular stent thrombectomy: the new standard of care for large vessel ischaemic stroke. Lancet Neurol. 2015;14(8):846–54.
134. Su EJ, Fredriksson L, Geyer M, Folestad E, Cale J, Andrae J, et al. Activation of PDGF-CC by tissue plasminogen activator impairs blood-brain barrier integrity during ischemic stroke. Nat Med. 2008;14(7):731–7.
135. Wang Y, Zhao Z, Chow N, Rajput PS, Griffin JH, Lyden PD, et al. Activated protein C analog protects from ischemic stroke and extends the therapeutic window of tissue-type plasminogen activator in aged female mice and hypertensive rats. Stroke. 2013;44(12):3529–36.

Role of Pericytes in Neurovascular Unit and Stroke

Turgay Dalkara, Luis Alarcon-Martinez, and Muge Yemisci

Abbreviations

ACA	Anterior cerebral artery
BBB	Blood–brain barrier
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CNS	Central nervous system
MCA	Middle cerebral artery
NVU	Neurovascular unit
PA	Penetrating arteries

T. Dalkara, M.D., Ph.D. (✉)
Faculty of Medicine, Department of Neurology, Hacettepe University, Ankara, Turkey

Institute of Neurological Sciences and Psychiatry, Hacettepe University, Ankara 06100, Turkey

Department of Radiology, Massachusetts General Hospital, Harvard University, Boston, MA, USA
e-mail: tdalkara@hacettepe.edu.tr

L. Alarcon-Martinez, B.Sc., M.Sc., Ph.D.
Institute of Neurological Sciences and Psychiatry, Hacettepe University, Ankara 06100, Turkey
e-mail: lalarcon@um.es

M. Yemisci, M.D., Ph.D.
Faculty of Medicine, Department of Neurology, Hacettepe University, Ankara, Turkey

Institute of Neurological Sciences and Psychiatry, Hacettepe University, Ankara 06100, Turkey
e-mail: myemisciozkan@gmail.com

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_2

PDGFRβ	Platelet-derived growth factor receptor-β
PDGFβ	Platelet-derived growth factor-β
RBCs	Erythrocytes = red blood cells
SMCs	Smooth muscle cells
tPA	Tissue plasminogen activator
αSMA	Smooth muscle α-actin

1 Neurovascular Unit and Stroke

The neurovascular unit (NVU) is composed of the endothelia, pericytes, basal membrane ensheathing them, astrocyte end-feet around the vessels and, neurons innervating the intraparenchymal vasculature (Fig. 1a). It plays an integrating role in matching the metabolic demand with the blood flow in addition to regulating the development and maintenance of the blood–brain barrier (BBB) [1–5], leukocyte trafficking across the BBB [6–9], and angiogenesis [10–12]. Ischemic injury to the NVU unfavorably impacts the stroke-induced damage and brain edema by

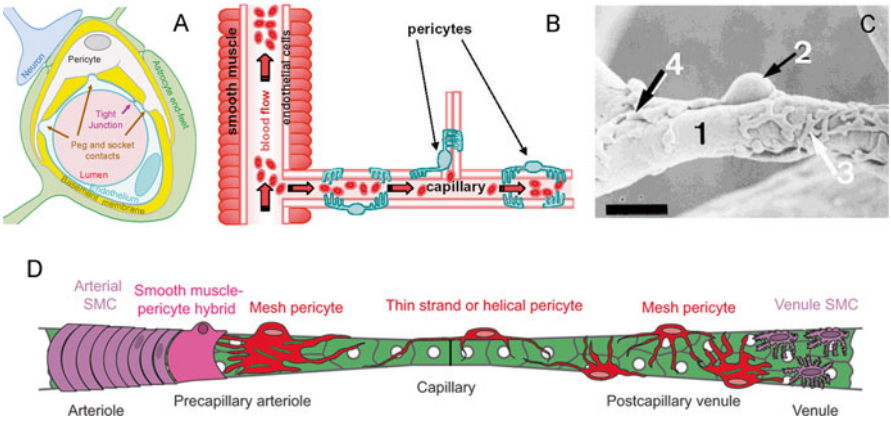

Fig. 1 Neurovascular unit and pericytes. (**a**) The neurovascular unit is composed of the endothelia and tight junctions between them, pericytes, the basal lamina ensheating endothelia and pericytes, and astrocyte end-feet surrounding the microvessel. Note the peg and socket type contacts between endothelia and pericytes. (**b**) Potential blood flow control sites in cerebral vasculature: smooth muscles on arterioles and pericytes on capillaries (Reproduced from Peppiatt et al., 2006 with permission). (**c**) Scanning micrograph of a vascular cast of a cortical capillary (*1*) with a pericyte-like structure (*2*) having primary and secondary processes (*3*) distributed around the vascular cast and the capillary branching points (*4*). Scale bar = 11.5 μm. (Reproduced from Rodriguez-Baeza et al., 1998 with permission). (**d**) Pericyte processes are highly varied with shapes ranging from thin singular strands that run parallel to the microvasculature to more complex mesh processes that enwrap the entire vessel lumen. Pericytes located closer to the arteriolar end of the microcirculation exhibit more circular processes that may be essential to their contractile function (Reproduced from Hartmann et al., 2015 with permission)

disrupting microvascular blood flow and BBB integrity, whereas ischemia-triggered signaling in the NVU of vasculature within the peri-infarct area positively impacts stroke outcome by promoting post-stroke angiogenesis and neurogenesis [13, 14].

2 Pericytes

First described by Rouget in 1873 [15], pericytes were later named by Zimmerman in 1923 based on their prominent location at the abluminal wall of microvessels [16]. However, their importance within the NVU has not been recognized until recently. Pericytes communicate with other cells of the NVU and contribute to the control of several microcirculatory functions such as neurovascular coupling, maintenance of the BBB and basal lamina, regulation of the angiogenesis, immune cell entry to the central nervous system (CNS) and scar formation [3, 11, 17–21]. They also function as pluripotent stem cells after injury to the NVU [22]. Although pericytes are also present on peripheral microvessels, the density of pericytes on microvessels is highest in the CNS and retina in accordance with their role in focal regulation of the microcirculatory blood flow and maintenance of the blood–brain/retina barrier [2, 21, 23, 24].

Pericytes are located on pre-capillary arterioles, capillaries, and post-capillary venules [2, 25, 26] (Fig. 1b–d). Pericytes are present on straight parts as well as branching points of the capillaries; 56 % of the pericyte somas are located at a capillary junction [27]. Unlike smooth muscle cells (SMCs), pericytes are embedded within two layers of basement membrane [24]. They form peg and socket type contacts and gap junctions with the endothelia through the basement membrane, facilitating the communication between pericytes and endothelia [28, 29] (Fig. 1a). Adjoining membranes of the neighboring pericytes are interconnected with gap junctions, which probably serve as a conduit for transmitting messages along the microvascular wall [19, 30]. Pericytes extend processes along and around the microvessels, which cover 30–90 % of the microvessel wall in the CNS [4, 23, 31, 32]. Processes are more circumferential at the arteriole side of the microvascular bed and at branching points, more longitudinal in the middle of the capillary bed, and have a stellate morphology at the venule end of the microcirculation (Fig. 1d). Junctional pericytes extend processes at least a cell width away from the junction [27]. It has long been recognized that pericyte morphology varies along the course of microvasculature, presumably to accommodate differing functions [25, 27, 33]. Not only their morphology, but also their protein expression varies [2, 34]. Several transitional forms are observed along the vascular bed at various developmental stages or after pathological stimuli [2, 25, 26, 35]. The transition from smooth muscle α-actin (αSMA) expressing SMCs to pericytes is also not sharp. Smooth muscle-pericyte "hybrid" cells precede the prearteriolar pericytes having mesh-like circular processes [26, 27]. Pericytes that give out more circumferential processes express more αSMA, when assessed either with immunohistochemistry of brain sections ex vivo [27, 34] or in mice cortex expressing reporter dyes under control of the

αSMA promoter in vivo [33]. It should be noted that immunohistochemistry directly detects the αSMA protein (mainly in the cytoplasm of the soma and processes), whereas reporter dyes expressed under the control of αSMA promoter are membrane-bound, therefore, basically label the pericyte membrane. Mid-capillary pericytes do also express αSMA [36]. However, the detection of the small pool of αSMA in their relatively short processes by immunohistochemistry requires rapid fixation before αSMA depolymerization, whereas low level of αSMA expression may be difficult to visualize due to dispersion of the limited amount of reporter fluorescent protein expressed over the large surface area of the pericyte membrane.

Pericytes can be identified by a number of proteins that they express. Unfortunately, the antibodies against to majority of them either detect only a subpopulation of pericytes or do not have the desired specificity to unambiguously distinguish pericytes from other cell types [2, 25]. Recent studies with transgenic mice expressing fluorescent reporter proteins under the control of the growth factor platelet-derived growth factor receptor-β (PDGFRβ) and of the proteoglycan NG2, suggest that PDGFRβ detects a larger population of pericytes compared to NG2 and, interestingly, CD13 immunohistochemistry is reportedly to match the performance of fluorescent reporters expressed under the control of PDGFRβ promoter [27, 33, 37].

2.1 Regulation of Microcirculatory Blood Flow and BBB by Pericytes

Pericytes are considered contractile cells since their discovery [15, 16]. However, this view was challenged in the past few decades based on the premise that the blood flow increase evoked by neuronal activity was mediated by relaxation of SMCs around arterioles as well as by failure of detection of αSMA in capillary pericytes in some studies. As reviewed in detail by Díaz-Flores et al. [12], the pericyte contractility is supported by several lines of evidence including their characteristic morphology with processes that envelop the microvessels as well as ultrastructural and immunohistochemical demonstration of contractile proteins [24, 34, 36, 38–45] in addition to the presence of receptors for vasoactive mediators on their surface [19, 30, 46]. In vitro studies on cerebellar, cerebral, and retinal slices or on isolated microvessels and in vivo studies have clearly disclosed that pericytes contract or dilate in response to vasoactive mediators applied [30, 46, 47]. A recent in vivo study showed that cortical capillaries dilated before arterioles during sensory stimulation, supporting the view that microvascular blood flow in the CNS is regulated by pericytes in response to the very focal demand originating from a small group of nearby cells as a final step of flow regulation after the arterioles, which serve a larger cohort of cells [18]. This flow regulation with fine spatial resolution may be essential for tissues with high functional specialization such as the brain and retina. However, it should be noted that all microvascular pericytes are not contractile and proportion of the contractile ones may vary with the tissue, species, and developmental stage as well as along the arteriovenous axis as noted above [20, 33, 48].

It has also been shown that a close interaction between the endothelia and pericytes as well as astrocytes is required for development and functioning of the NVU and BBB [4, 49]. Pericytes promote the formation of tight junctions and inhibit transendothelial vesicular transport [4, 49, 50]. The number of pericytes per endothelial cell and the surface area of the vascular wall covered by pericytes determine the relative permeability of capillaries. Accordingly, pericyte dysfunction or deficiency causes increased BBB permeability [2, 4, 21, 49].

2.2 Pericytes Are Vulnerable to Ischemic Injury

The pericyte contractility is regulated by intracellular Ca^{2+} concentration as in vascular SMCs surrounding the upstream vessels [19, 46, 51]. Accordingly, these highly dynamic cells bear the risk of Ca^{2+} overload when they are unable to maintain low intracellular Ca^{2+} concentrations. The energy insufficiency is a well-characterized cause of loss of the intracellular Ca^{2+} equilibrium, which relies on several energy demanding processes. In addition, factors such as reactive oxygen species (ROS) may also contribute to an uncontrolled rise in intracellular Ca^{2+} [52, 53]. Pericytes have large elongated mitochondria, which follow the central core of the pericyte longitudinally, and may be a significant source of ROS under pathological conditions [31]. NADPH oxidase, a major superoxide-producing enzyme is highly expressed in brain pericytes [54, 55]. Indeed, ROS has been shown to cause a sustained increase in Ca^{2+} in cultured human brain microvascular pericytes [52, 53]. Reactive oxygen and nitrogen species, especially peroxynitrite, have been reported to induce pericyte contraction during focal ischemia/reperfusion in the intact mouse brain [56]. In addition to unregulated Ca^{2+} rise, several other processes such as ATP and thromboxane A2 released from the ischemic brain or platelets, which are potent constrictors of pericytes, may also contribute to pericyte contraction [48].

3 Changes in Pial and Penetrating Arteries Shortly After Stroke

On occlusion of the middle cerebral artery (MCA), collaterals between the anterior cerebral artery (ACA) and MCA are opened, large arterial branches but especially surface pial network and penetrating arteries (PA) dilate while flow directions are reorganized to sustain the flux rates in PAs as high as possible [57–59]. While these compensatory changes can preserve cell viability at the periphery of the MCA area, creating an opportunity for recovery if MCA recanalization can be attained within a couple of hours, the severe decrease in blood flow velocity, volume, and distal capillary perfusion in the core ischemic area makes infarction unavoidable [58, 60–62] (Fig. 2). In vivo imaging of cerebral circulation in intact mice under anesthesia has unequivocally illustrated that the PAs, especially those with smaller luminal

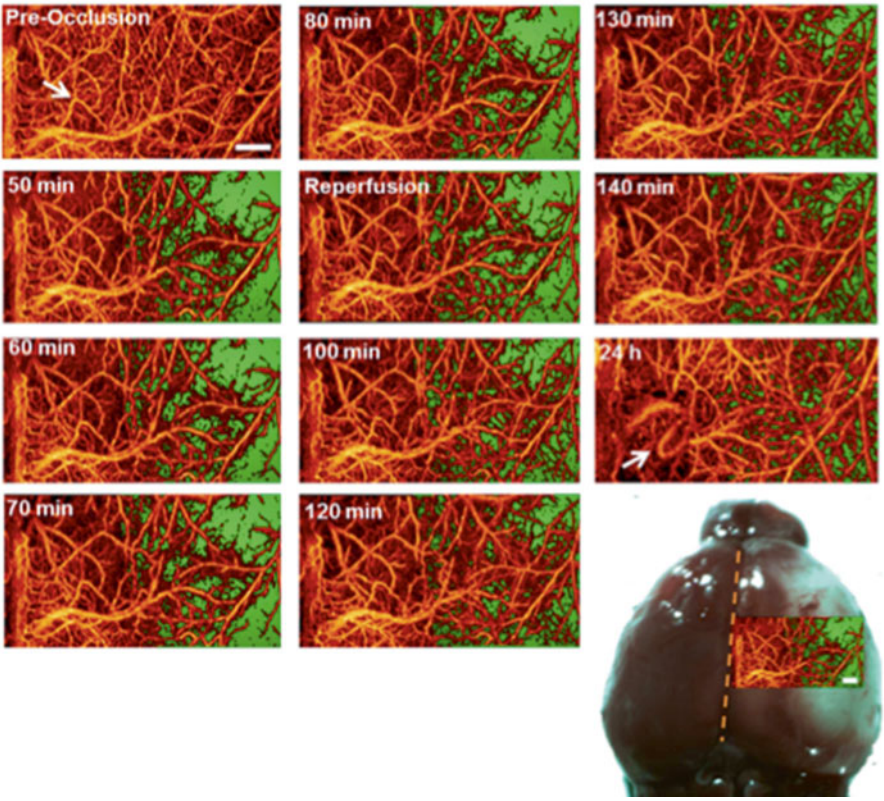

Fig. 2 Incomplete microcirculatory reflow after recanalization. Dynamic imaging of cortical blood flow using optical microangiography during 90-min proximal MCA occlusion followed by recanalization illustrates the lack of microcirculatory blood flow in the MCA territory (the *green area*) during occlusion and its partial recovery after recanalization (incomplete microcirculatory reperfusion) in the mouse. Consecutive images are shown at 10-min intervals. Image size is 2.2×4.4 mm^2. The image in the *lower right* is the optical microangiography image taken at 50 min overlaid on the 24 h infarct analysis by histological staining as the area of pallor. Scale bar = 500 μm (Reproduced from Dziennis et al., 2015 with permission)

diameter dilated to compensate for the low perfusion pressure [58, 61]. The magnitude of dilation decreased with the distance from the pial arteriolo-arteriolar anastomoses with sufficient collateral flow and was replaced by constriction in areas further away [58]. Majority of these changes are reversible if recanalization is achieved within a short time. However, when perfusion deficit is prolonged some of these changes are not reversible and may negatively impact the recovery after stroke. For example, in the mouse brain, part of the microcirculatory flow cannot be reinstituted after MCA occlusion lasting more than an hour despite complete reopening of the MCA [33, 56].

4 Incomplete Microcirculatory Reflow After Recanalization

An impaired tissue reperfusion due to microvascular constrictions (no-reflow phenomenon) was first noted after global and focal cerebral ischemia more than half a century ago [63, 64]. The emergence of "no-reflow" depends on the duration and severity of ischemia as well as the brain region studied although these variables have not been systematically compared in the setting of focal cerebral ischemia. In the mouse, MCA occlusion induces nodal microvascular constrictions that generally do not recover after recanalization starting 1 h after ischemia and affecting more than half of the microvessels within 2 h [33, 56] (Fig. 2). Narrowed microvessel lumina are filled with entrapped erythrocytes (RBCs), leukocytes, and fibrin-platelet deposits [65–69]. RBCs are the predominant cell types in aggregates as they are the most prevalent cells in circulation. In addition to the constricted segments observed at the arteriolar end of microcirculation and capillaries, leukocytes adhered to post-capillary venules for entering to the parenchyma also induce luminal aggregates together with fibrin and platelets [65, 69–71].

Experimental data strongly suggest that incomplete restoration of the microcirculatory blood flow negatively impacts tissue recovery even if reopening of the occluded artery is achieved when there is still salvageable penumbral tissue. Pharmacological agents and genetic manipulations reducing microvascular clogging by inhibiting leukocyte adherence, platelet activation or fibrin–platelet interactions have been shown to restore microcirculation and improve stroke outcome in animal models [65, 72–75]. Importantly, neuroprotection obtained with some BBB-impermeable agents strongly support the idea that restoring microvascular patency can improve stroke outcome independently of parenchymal mechanisms [56, 76]. Consequently, restitution of the microcirculatory reperfusion emerges as an exciting target to improve the success rate of recanalization therapies.

In the past, microvessel constrictions were thought to be caused by swollen astrocyte end-feet encircling microvessels [66, 67]. Recently, pericytes on microvessels were proposed to play an important role in incomplete microcirculatory reperfusion because they contracted during ischemia and remained contracted despite reopening of the occluded artery [18, 56, 77] (Fig. 3). Although it has been claimed that αSMA expressing microvascular cells with contractile capability should be defined as SMCs [33], the mural cells with a bump-on-a-log morphology located on the abluminal wall of microvessels downstream to arterioles, including their transitory forms to SMCs, are named as pericytes since their original description by Zimmerman [16, 78]. The fact that pericytes are a heterogeneous group of cells sharing some transitional features with SMCs and that some but not all express αSMA have always been a matter of confusion and a source of debate. Nomenclature disagreements notwithstanding the important point for the stroke pathophysiology is that contractile cells on brain microvessels impede reperfusion after ischemia and unfavorably impact the outcome of recanalization. It should be noted that even small decreases in capillary radius caused by subtle pericyte contractions can lead to erythrocyte entrapments because capillary luminal size hardly allows passage of

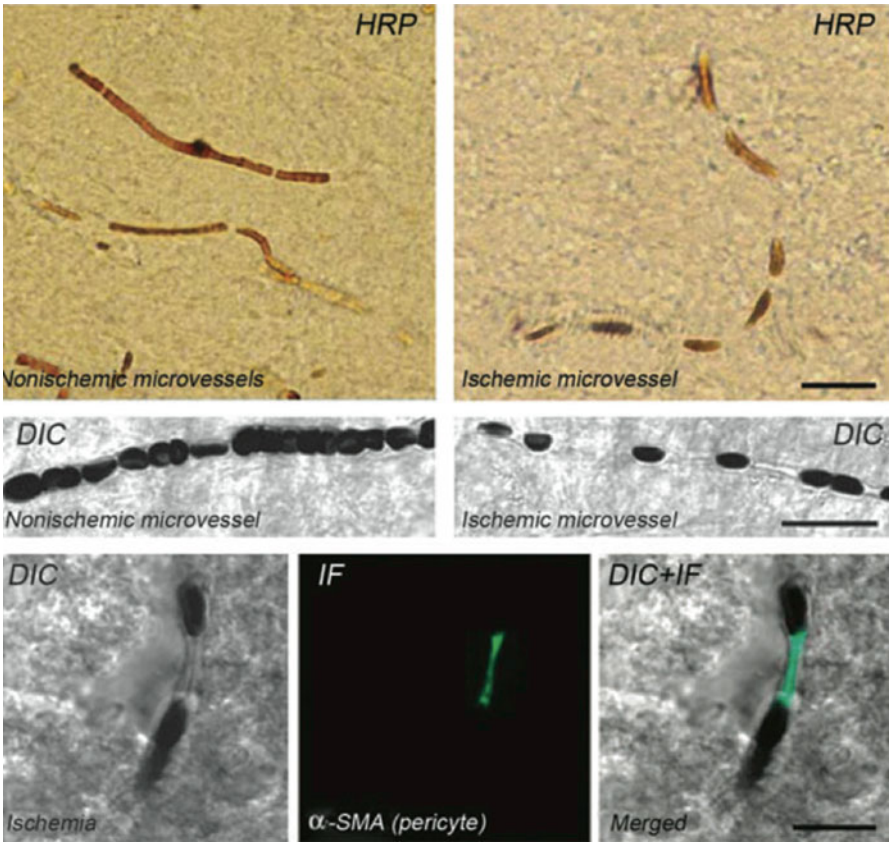

Fig. 3 Ischemia causes persistent pericyte contraction, which is not restored after complete recanalization of the occluded artery. Mice were subjected to 2 h of proximal MCA occlusion and intravenously injected with horseradish peroxidase (HRP) before decapitation, 6 h after reopening of the MCA. HRP-filled microvessels exhibited sausage-like segmental constrictions in ischemic areas on brain sections (*upper row*). The differential interference contrast (DIC) microscopy images illustrate frequent interruptions in the erythrocyte column in an ischemic capillary contrary to a continuous row of erythrocytes flowing through an intact capillary (*middle row*). The constricted segments colocalized with α-smooth muscle actin (α-SMA) immunoreactive pericytes (*bottom row*). IF denotes immunofluorescence. Scale bar for *upper* and *middle row*, 20 μm; *bottom row* 10 μm (Reproduced from Yemisci et al., 2009 with permission)

RBCs [19, 56] (Fig. 3 middle row). Entrapped erythrocytes trigger platelet and fibrin aggregation by impeding passage of blood cells [69, 79]. The failure of erythrocyte circulation within some of the microvessels and increased heterogeneity of RBC transit times through patent capillaries (due to varying degrees of capillary resistances) can catastrophically reduce O_2 delivery to the tissue struggling to recover from ischemia-induced perturbations [80]. Since the plasma flow in constricted capillaries is relatively less restricted compared to RBC flux, glucose supply to some parts of the tissue may exceed O_2 supply and stimulate anaerobic glycolysis,

hence, lactic acidosis ([56, 77], please also see supplementary movies 5–7 in [33]). Therefore, ischemia-induced pericyte contractions emerge as a viable target for restoring impaired microcirculatory reperfusion. Indeed, sustained release of adenosine within circulation from nano-assemblies (NA) (adenosine itself has only a few minutes of plasma residence time) has recently been shown to reduce ischemia-induced erythrocyte entrapments and improve microcirculatory reflow by relaxing contracted pericytes after 2 h of MCA occlusion [76] (Fig. 4). Unlike adenosine infusion or synthetic adenosine agonists, slow release from squalenoyladenosine NAs did not cause cardiotoxicity or hypotension in the mouse model used.

Since pericytes also play an important role in maintenance of the BBB integrity [4, 21, 49], the ischemia/reperfusion-induced pericyte dysfunction may contribute to BBB leakiness as well. This can be further aggravated by the death of capillary pericytes within 24 h after MCA occlusion as shown in mice and rats [18, 81]. Increased BBB permeability predisposes to intraparenchymal hemorrhage and brain swelling in about 6 % of patients receiving i.v. tissue plasminogen activator (tPA) [82]. Diabetic patients are more prone to hemorrhage perhaps due to dysfunctional microvascular pericytes, a well-known cause of diabetic retinopathy [83–85]. Interestingly, pericyte loss is increasingly reported for conditions that are risk factors for stroke, such as ageing, hypertension, and diabetes, the impact of which on stroke outcome needs to be clarified with future research [86]. Among many complex mechanisms, overproduction of oxygen and nitrogen radicals on the microvascular wall appears to contribute to both BBB leakiness and incomplete reflow during cerebral ischemia/reperfusion [56, 87]. Altogether, these findings bring about the exciting possibility that effective suppression of oxidative/nitrative stress during reopening of the occluded artery may improve the outcome of recanalization therapies by promoting microcirculatory reperfusion as well as by preventing hemorrhagic conversion and vasogenic edema [87]. Despite failure of an antioxidant agent in clinical trials, the experimental evidence still warrants pursuit of this goal [88, 89].

5 Clinical Evidence for “No-Reflow” After Recanalization Therapies for Stroke

A short therapeutic time window limits the use of recanalization therapies for majority of stroke patients [90, 91]. This brief therapeutic time window is attributed to rapid loss of neuronal viability in the ischemic penumbra [82, 92]. However, increasing clinical evidence suggests that an incomplete reperfusion plays a critical role in determining tissue survival after successful recanalization [93, 94]. Several recent imaging studies serially analyzing recanalization and reperfusion in ischemic stroke patients report that, on average, 26 % of recanalized patients with thrombolytics do not show reperfusion [95]. This incomplete reperfusion is observed after pharmacological (intravenous or intraarterial) as well as interventional recanalization [93, 96–98]. Clinical trials have repeatedly demonstrated that a good outcome was better correlated with reperfusion than recanalization in stroke patients treated

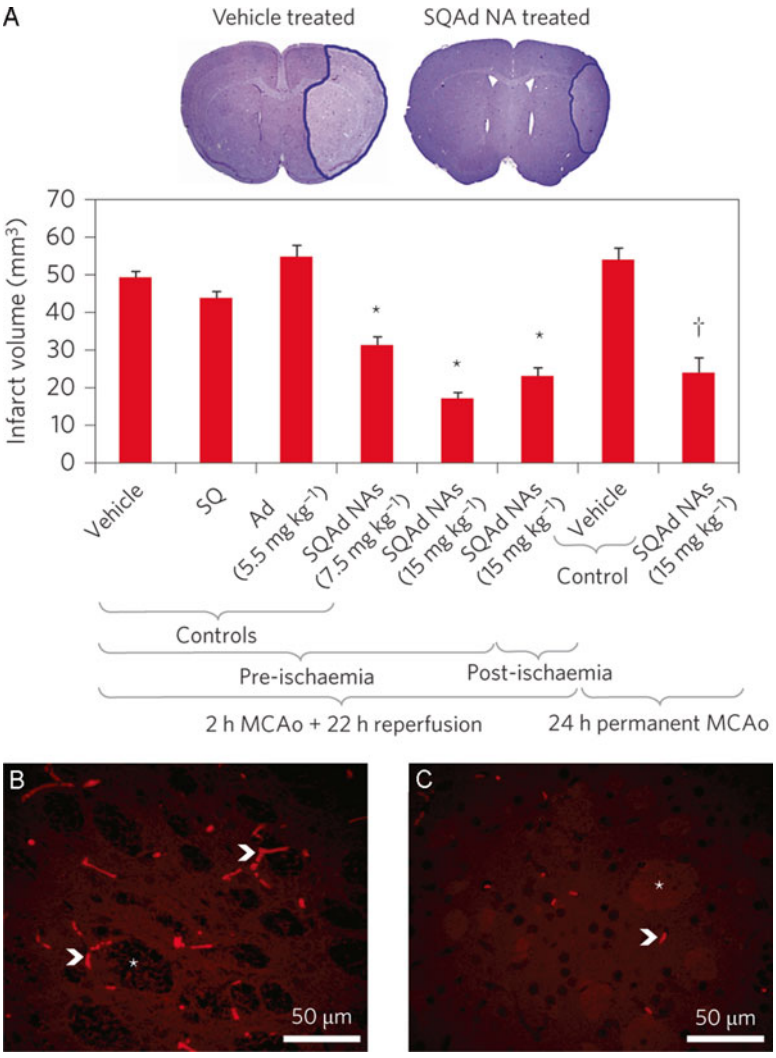

Fig. 4 Systemic administration of squalenoyladenosine (SQAd) nano-assemblies (NAs) provides significant neuroprotection in a mouse model of focal cerebral ischaemia. (**A**) Infarct areas in control and treated mice subjected to transient (2 h MCAo and 22 h reperfusion) and permanent (24 h MCAo) focal cerebral ischemia were identified by reduced Nissl staining under a light microscope (magnification ×10, insets) (data are presented as mean (mm3) ± S.D., $N = 6$ animals per group; † and * indicate $P < 0.05$ compared to respective controls). Intravenous administration of 7.5 or 15 mg kg^{-1} SQAd NAs just before ischemia or 2 h post-ischemia significantly decreased the infarct volume compared with control groups that received vehicle (dextrose 5 %), adenosine-unconjugated SQ NAs (9.45 mg kg^{-1}) or free adenosine equivalent to the amount in NAs (5.5 mg kg^{-1}). A significant therapeutic effect was also observed when SQAd NAs were administered 2 h post-ischemia in the permanent MCAo model. (**B**, **C**) In untreated mice, capillaries in the ischemic brain were filled with trapped erythrocytes, whose hemoglobin was rendered fluorescent by treating brain sections with $NaBH_4$ (**B**, *red*, *arrowheads*) 6 h after reopening of the MCA following 2 h of occlusion, whereas the majority of capillaries were not clogged in SQAd NAs-treated mice (**C**)

with tPA or interventional methods [93, 94, 96, 97, 99, 100]. Recent imaging studies show that increased capillary transit time heterogeneity (a measure of incomplete/impaired reperfusion) is a good predictor of the tissue destined to infarct [101].

6 Role of Pericytes in CADASIL

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is caused by mutations in the *NOTCH3* gene [102]. The protein encoded by the *NOTCH3* gene is expressed in pericytes as well as vascular SMCs. Recent studies in Notch3 transgenic mice expressing one of the human mutations have disclosed that Notch3 aggregated around microvascular pericytes, leading to pericyte loss or reduced coverage of capillaries by pericyte processes [103, 104]. These changes were associated with a leaky BBB, reduction in communication with endothelial cells and neurovascular dysfunction. Confirming the clinical significance of these findings, pericyte loss was also observed in skin and muscle biopsies of CADASIL patients [105].

7 Post-stroke Angioneurogenesis and Pericytes

Pericytes are essential especially for the early phase of neovascularization (angiogenic sprouting) [10, 11] (Fig. 5). Pericytes and endothelial cells communicate with each other for regulation of angiogenesis [10–12]. Platelet-derived growth factor-β (PDGFβ), transforming growth factor-β, notch, angiopoietin, and sphingosine-1-phosphate signaling mediate this crosstalk [2, 106]. Increasing evidence suggests that pericytes play an important role in post-stroke angiogenesis as well [107–111]. Typically, endothelial cells start to proliferate and give off vessel sprouts 12–24 h after brain ischemia, leading to formation of new vessels in the peri-infarct region 3 days after ischemic injury [107, 112, 113]. Following a similar time course, the PDGFRβ expression is upregulated in pericytes, which increase in number and start migrating from the microvessel wall to the newly formed vessel sprouts to foster their maturation after ischemic injury [114–117]. Renner et al. found that PDGFRβ increased in pericytes 48 h after permanent ischemia [117]. Similarly, NG2+ or PDGFRβ+ pericytes reportedly increase in peri-infarct areas 1–3 weeks after transient MCA occlusion [81, 118]. A proportion of locally proliferating pericytes give rise to microglial cells [119]. Interestingly, chronic administration of cilostazol, an antiplatelet drug, has been claimed to promote pericyte proliferation, which might decrease the final infarct size by promoting new vessel formation after naturally occurring stroke in spontaneously hypertensive rats [120]. Corroborating these studies, conditional knockout of PDGFβ/PDGFRβ signaling in adult mice that have normally developed brain vasculature, led to larger infarcts than controls when subjected to focal cerebral ischemia [121]. Similarly, Zechariah et al. showed that

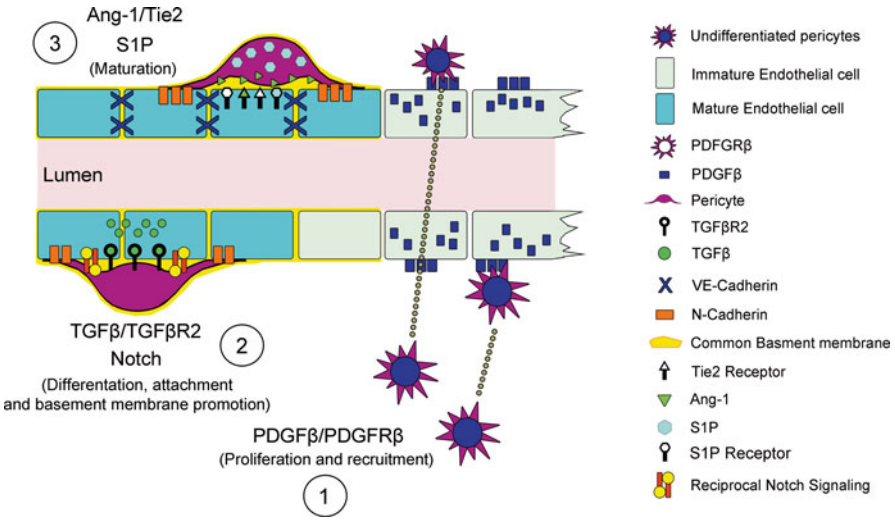

Fig. 5 Role of pericytes in angiogenesis. The interaction between PDGFβ secreted by the endothelium and its receptor localized on pericytes (PDGFRβ) is essential for recruitment of undifferentiated mesenchymal cells/pericytes to newly formed vessels. Once pericytes are at the vascular wall, reciprocal Notch signaling between the endothelia and pericytes as well as interactions between TGFβ secreted by endothelial cells and its receptor TGFβR2 located at pericytes differentiate mural cells and attach them to the newly formed vessels. The TGFβ/TGFβR2 interaction also promotes formation of the common basement membrane and stabilizes newly formed vessels by inhibiting endothelial proliferation. Ang-1, which is secreted by pericytes, activates its endothelial receptor Tie2 and promotes blood–brain barrier formation. Finally, S1P, whose receptor is abundantly expressed on pericytes downregulates genes related to vascular permeability and promotes both endothelial–endothelial (VE-cadherin) and pericyte-endothelial cell (N-cadherin) interconnections

pericytes did not appropriately cover the brain capillaries in hyperlipidemic mice exposed to ischemia and, this was associated with attenuation of post-stroke angiogenesis [111].

Albeit indirectly, further supporting a role for pericytes in post-stroke angiogenesis, intravenous injection of a combination of smooth muscle progenitor cells and endothelial progenitor cells 1 day after MCA occlusion enhanced the angiogenesis and vessel maturation in the peri-infarct areas [122]. Since the adult bone marrow is considered to be a rich reservoir of pericyte progenitor cells, bone marrow-derived pericytes may be involved in post-ischemic angiogenesis [109, 123, 124]. Indeed, Kokovay et al. showed that, following brain ischemia, bone marrow-derived cells with a pericytic phenotype and expressing angiogenic factors were recruited to cerebral capillaries [109].

Angiogenesis is also essential to promote neurogenesis after stroke [122, 125, 126]. In fact, newly formed neurons have been found located near to the remodeled vessels [127], probably because vascular cells recruit and form a niche for neural stem cells [126, 128]. Since pericytes are essential in post-stroke angiogenesis and express factors that can induce neurogenesis as well as angiogenesis, pericytes may

also be involved in post-stroke neurogenesis [25, 129]. In vitro studies have clearly shown that the brain-derived pericytes have a potential to differentiate into neurons in response to trophic factors such as basic fibroblast growth factor, Sox2 and Mash1 [22, 130–132]. Pericytes obtained from ischemic MCA tissue of adult animals or pericytes cultured under ischemic conditions also showed capability to differentiate to cells of neural as well as vascular lineage [133].

Acknowledgments Dr. Turgay Dalkara's research is supported by The Turkish Academy of Sciences. Dr. Luis Alarcon-Martinez's research is supported by the Co-funded Brain Circulation Scheme of Marie Curie Actions into the Seventh Framework Programme of European Union. Dr. Muge Yemisci's research is supported by The Scientific and Technological Research Council of Turkey (TÜBITAK) project no: 114S190. Dr. Luis Alarcon-Martinez prepared Figs. 1a and 5.

References

1. Abbott NJ, Ronnback L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci. 2006;7(1):41–53.
2. Armulik A, Genové G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. Dev Cell. 2011;21:193–215.
3. Attwell D, Buchan AM, Charpak S, Lauritzen M, Macvicar BA, Newman EA. Glial and neuronal control of brain blood flow. Nature. 2010;468:232–43.
4. Daneman R, Zhou L, Kebede AA, Barres BA. Pericytes are required for blood-brain barrier integrity during embryogenesis. Nature. 2010;468:562–6.
5. Iadecola C. Neurovascular regulation in the normal brain and in Alzheimer's disease. Nat Rev Neurosci. 2004;5:347–60.
6. Dohgu S, Banks WA. Brain pericytes increase the lipopolysaccharide-enhanced transcytosis of HIV-1 free virus across the in vitro blood-brain barrier: evidence for cytokine-mediated pericyte-endothelial cell crosstalk. Fluids Barriers CNS. 2013;10(1):23.
7. Leick M, Azcutia V, Newton G, Luscinskas FW. Leukocyte recruitment in inflammation: basic concepts and new mechanistic insights based on new models and microscopic imaging technologies. Cell Tissue Res. 2014;355(3):647–56.
8. Proebstl D, Voisin M-B, Woodfin A, Whiteford J, D'Acquisto F, Jones GE, et al. Pericytes support neutrophil subendothelial cell crawling and breaching of venular walls in vivo. J Exp Med. 2012;209(6):1219–34.
9. Stark K, Eckart A, Haidari S, Tirniceriu A, Lorenz M, von Brühl M-L, et al. Capillary and arteriolar pericytes attract innate leukocytes exiting through venules and "instruct" them with pattern-recognition and motility programs. Nat Immunol. 2013;14(1):41–51.
10. Gerhardt H, Betsholtz C. Endothelial-pericyte interactions in angiogenesis. Cell Tissue Res. 2003;314:15–23.
11. Ozerdem U, Stallcup WB. Early contribution of pericytes to angiogenic sprouting and tube formation. Angiogenesis. 2003;6:241–9.
12. Díaz-Flores L, Gutiérrez R, Madrid JF, Varela H, Valladares F, Acosta E, et al. Pericytes. Morphofunction, interactions and pathology in a quiescent and activated mesenchymal cell niche. Histol Histopathol. 2009;24(7):909–69.
13. Dalkara T, Alarcon-Martinez L. Cerebral microvascular pericytes and neurogliovascular signaling in health and disease. Brain Res. 2015;1623:3–17.
14. Zhang L, Zhang ZG, Chopp M. The neurovascular unit and combination treatment strategies for stroke. Trends Pharmacol Sci. 2012;33(8):415–22.
15. Rouget C. Memoire sur le developpement, la structure et les proprietes physiologiques des capillaries sanguins et lymphatiques. Arch Physiol Norm Path. 1873;5:603–63.

16. Zimmermann K. Der feinere Bau der Blutkapillaren. Z Anat Entwicklungsgesch. 1923;68:29–109.
17. Göritz C, Dias DO, Tomilin N, Barbacid M, Shupliakov O, Frisén J. A pericyte origin of spinal cord scar tissue. Science. 2011;333:238–42.
18. Hall CN, Reynell C, Gesslein B, Hamilton NB, Mishra A, Sutherland BA, O'Farrell FM, Buchan AM, Lauritzen M, Attwell D. Capillary pericytes regulate cerebral blood flow in health and disease. Nature. 2014;508:55–60.
19. Hamilton NB, Attwell D, Hall CN. Pericyte-mediated regulation of capillary diameter: a component of neurovascular coupling in health and disease. Front Neuroenergetics. 2010; 2. pii:5.
20. Krueger M, Bechmann I. CNS pericytes: concepts, misconceptions, and a way out. Glia. 2010;58:1–10.
21. Winkler EA, Bell RD, Zlokovic BV. Central nervous system pericytes in health and disease. Nat Neurosci. 2011;14(11):1398–405.
22. Dore-Duffy P, Katychev A, Wang X, Van Buren E. CNS microvascular pericytes exhibit multipotential stem cell activity. J Cereb Blood Flow Metab. 2006;26:613–24.
23. Frank RN, Dutta S, Mancini MA. Pericyte coverage is greater in the retinal than in the cerebral capillaries of the rat. Invest Ophthalmol Vis Sci. 1987;28(7):1086–91.
24. Shepro D, Morel NM. Pericyte physiology. FASEB J. 1993;7(11):1031–8.
25. Dore-Duffy P, Cleary K. Morphology and properties of pericytes. In: Nag S, editor. The blood-brain and other neural barriers [Internet]. Totowa: Humana Press; 2011 [cited 2015 Sep 6]. p. 49–68. http://link.springer.com/10.1007/978-1-60761-938-3_2
26. Sims DE. The pericyte—a review. Tissue Cell. 1986;18(2):153–74.
27. Hartmann DA, Underly RG, Grant RI, Watson AN, Lindner V, Shih AY. Pericyte structure and distribution in the cerebral cortex revealed by high-resolution imaging of transgenic mice. Neurophotonics. 2015;2(4):041402.
28. Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. Circ Res. 2005; 97(6):512–23.
29. Cuevas P, Gutierrez-Diaz JA, Reimers D, Dujovny M, Diaz FG, Ausman JI. Pericyte endothelial gap junctions in human cerebral capillaries. Anat Embryol (Berl). 1984;170(2):155–9.
30. Peppiatt CM, Howarth C, Mobbs P, Attwell D. Bidirectional control of CNS capillary diameter by pericytes. Nature. 2006;443:700–4.
31. Mathiisen TM, Lehre KP, Danbolt NC, Ottersen OP. The perivascular astroglial sheath provides a complete covering of the brain microvessels: an electron microscopic 3D reconstruction. Glia. 2010;58:1094–103.
32. Rodriguez-Baeza A, Reina-De La Torre F, Ortega-Sanchez M, Sahuquillo-Barris J. Perivascular structures in corrosion casts of the human central nervous system: a confocal laser and scanning electron microscope study. Anat Rec. 1998;252(2):176–84.
33. Hill RA, Tong L, Yuan P, Murikinati S, Gupta S, Grutzendler J. Regional blood flow in the normal and ischemic brain is controlled by arteriolar smooth muscle cell contractility and not by capillary pericytes. Neuron. 2015;87(1):95–110.
34. Nehls V, Drenckhahn D. Heterogeneity of microvascular pericytes for smooth muscle type alpha-actin. J Cell Biol. 1991;113(1):147–54.
35. Sharma V, Ling TW, Rewell SS, Hare DL, Howells DW, Kourakis A, et al. A novel population of α-smooth muscle actin-positive cells activated in a rat model of stroke: an analysis of the spatio-temporal distribution in response to ischemia. J Cereb Blood Flow Metab. 2012;32(11):2055–65.
36. Bandopadhyay R, Orte C, Lawrenson JG, Reid AR, De Silva S, Allt G. Contractile proteins in pericytes at the blood-brain and blood-retinal barriers. J Neurocytol. 2001;30(1):35–44.
37. Hartmann DA, Underly RG, Watson AN, Shih AY. A murine toolbox for imaging the neurovascular unit. Microcirculation. 2015;22(3):168–82.
38. Allt G, Lawrenson JG. Pericytes: cell biology and pathology. Cells Tissues Organs. 2001; 169(1):1–11.

39. Das A, Frank RN, Weber ML, Kennedy A, Reidy CA, Mancini MA. ATP causes retinal pericytes to contract in vitro. Exp Eye Res. 1988;46(3):349–62.
40. Fujimoto T, Singer SJ. Immunocytochemical studies of desmin and vimentin in pericapillary cells of chicken. J Histochem Cytochem. 1987;35(10):1105–15.
41. Herman IM, D'Amore PA. Microvascular pericytes contain muscle and nonmuscle actins. J Cell Biol. 1985;101(1):43–52.
42. Joyce NC, Haire MF, Palade GE. Contractile proteins in pericytes. I. Immunoperoxidase localization of tropomyosin. J Cell Biol. 1985;100(5):1379–86.
43. Joyce NC, Haire MF, Palade GE. Contractile proteins in pericytes. II. Immunocytochemical evidence for the presence of two isomyosins in graded concentrations. J Cell Biol. 1985;100(5):1387–95.
44. Kelley C, D'Amore P, Hechtman HB, Shepro D. Microvascular pericyte contractility in vitro: comparison with other cells of the vascular wall. J Cell Biol. 1987;104(3):483–90.
45. Wallow IH, Burnside B. Actin filaments in retinal pericytes and endothelial cells. Invest Ophthalmol Vis Sci. 1980;19(12):1433–41.
46. Puro DG. Physiology and pathobiology of the pericyte-containing retinal microvasculature: new developments. Microcirculation. 2007;14(1):1–10.
47. Fernandez-Klett F, Offenhauser N, Dirnagl U, Priller J, Lindauer U. Pericytes in capillaries are contractile in vivo, but arterioles mediate functional hyperemia in the mouse brain. Proc Natl Acad Sci U S A. 2010;107:22290–5.
48. Fernández-Klett F, Priller J. Diverse functions of pericytes in cerebral blood flow regulation and ischemia. J Cereb Blood Flow Metab. 2015;35(6):883–7.
49. Armulik A, Genove G, Mae M, Nisancioglu MH, Wallgard E, Niaudet C, He L, Norlin J, Lindblom P, Strittmatter K, Johansson BR, Betsholtz C. Pericytes regulate the blood-brain barrier. Nature. 2010;468:557–61.
50. Kim JH, Yu YS, Kim DH, Kim KW. Recruitment of pericytes and astrocytes is closely related to the formation of tight junction in developing retinal vessels. J Neurosci Res. 2009;87:653–9.
51. Kamouchi M, Kitazono T, Ago T, Wakisaka M, Ooboshi H, Ibayashi S, Iida M. Calcium influx pathways in rat CNS pericytes. Brain Res Mol Brain Res. 2004;126:114–20.
52. Kamouchi M, Kitazono T, Ago T, Wakisaka M, Kuroda J, Nakamura K, Hagiwara N, Ooboshi H, Ibayashi S, Iida M. Hydrogen peroxide-induced Ca2+ responses in CNS pericytes. Neurosci Lett. 2007;416:12–6.
53. Nakamura K, Kamouchi M, Kitazono T, Kuroda J, Shono Y, Hagiwara N, Ago T, Ooboshi H, Ibayashi S, Iida M. Amiloride inhibits hydrogen peroxide-induced Ca2+ responses in human CNS pericytes. Microvasc Res. 2009;77:327–34.
54. Kuroda J, Ago T, Nishimura A, Nakamura K, Matsuo R, Wakisaka Y, et al. Nox4 is a major source of superoxide production in human brain pericytes. J Vasc Res. 2014;51(6):429–38.
55. Manea A, Raicu M, Simionescu M. Expression of functionally phagocyte-type NAD(P)H oxidase in pericytes: effect of angiotensin II and high glucose. Biol Cell. 2005;97:723–34.
56. Yemisci M, Gursoy-Ozdemir Y, Vural A, Can A, Topalkara K, Dalkara T. Pericyte contraction induced by oxidative-nitrative stress impairs capillary reflow despite successful opening of an occluded cerebral artery. Nat Med. 2009;15:1031–7.
57. Armitage GA, Todd KG, Shuaib A, Winship IR. Laser speckle contrast imaging of collateral blood flow during acute ischemic stroke. J Cereb Blood Flow Metab. 2010;30(8):1432–6.
58. Baran U, Li Y, Wang RK. Vasodynamics of pial and penetrating arterioles in relation to arteriolo-arteriolar anastomosis after focal stroke. Neurophotonics. 2015;2(2):025006.
59. Schaffer CB, Friedman B, Nishimura N, Schroeder LF, Tsai PS, Ebner FF, et al. Two-photon imaging of cortical surface microvessels reveals a robust redistribution in blood flow after vascular occlusion. PLoS Biol. 2006;4(2):e22.
60. Dziennis S, Qin J, Shi L, Wang RK. Macro-to-micro cortical vascular imaging underlies regional differences in ischemic brain. Sci Rep. 2015;5:10051.
61. Shih AY, Friedman B, Drew PJ, Tsai PS, Lyden PD, Kleinfeld D. Active dilation of penetrating arterioles restores red blood cell flux to penumbral neocortex after focal stroke. J Cereb Blood Flow Metab. 2009;29(4):738–51.

62. Terpolilli NA, Kim S-W, Thal SC, Kataoka H, Zeisig V, Nitzsche B, et al. Inhalation of nitric oxide prevents ischemic brain damage in experimental stroke by selective dilatation of collateral arterioles. Circ Res. 2012;110(5):727–38.
63. Ames 3rd A, Wright RL, Kowada M, Thurston JM, Majno G. Cerebral ischemia. II. The no-reflow phenomenon. Am J Pathol. 1968;52(2):437–53.
64. Crowell RM, Olsson Y. Impaired microvascular filling after focal cerebral ischemia in the monkey. Modification by treatment. Neurology. 1972;22(5):500–4.
65. Belayev L, Pinard E, Nallet H, Seylaz J, Liu Y, Riyamongkol P, et al. Albumin therapy of transient focal cerebral ischemia: in vivo analysis of dynamic microvascular responses. Stroke. 2002;33(4):1077–84.
66. Garcia JH, Liu KF, Yoshida Y, Chen S, Lian J. Brain microvessels: factors altering their patency after the occlusion of a middle cerebral artery (Wistar rat). Am J Pathol. 1994; 145(3):728–40.
67. Little JR, Kerr FWL, Sundt TM. Microcirculatory obstruction in focal cerebral ischemia: an electron microscopic investigation in monkeys. Stroke. 1976;7:25–30.
68. Morris DC, Davies K, Zhang Z, Chopp M. Measurement of cerebral microvessel diameters after embolic stroke in rat using quantitative laser scanning confocal microscopy. Brain Res. 2000;876:31–6.
69. Zhang ZG, Chopp M, Goussev A, Lu D, Morris D, Tsang W, et al. Cerebral microvascular obstruction by fibrin is associated with upregulation of PAI-1 acutely after onset of focal embolic ischemia in rats. J Neurosci. 1999;19(24):10898–907.
70. del Zoppo GJ, Schmid-Schonbein GW, Mori E, Copeland BR, Chang CM. Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. Stroke. 1991;22(10):1276–83.
71. Ritter LS, Orozco JA, Coull BM, McDonagh PF, Rosenblum WI. Leukocyte accumulation and hemodynamic changes in the cerebral microcirculation during early reperfusion after stroke. Stroke. 2000;31(5):1153–61.
72. Choudhri TF, Hoh BL, Zerwes HG, Prestigiacomo CJ, Kim SC, Connolly Jr ES, Kottirsch G, Pinsky DJ. Reduced microvascular thrombosis and improved outcome in acute murine stroke by inhibiting GP IIb/IIIa receptor-mediated platelet aggregation. J Clin Invest. 1998; 102:1301–10.
73. Hallenbeck JM, Dutka AJ, Tanishima T, Kochanek PM, Kumaroo KK, Thompson CB, et al. Polymorphonuclear leukocyte accumulation in brain regions with low blood flow during the early postischemic period. Stroke. 1986;17(2):246–53.
74. Ishikawa M, Vowinkel T, Stokes KY, Arumugam TV, Yilmaz G, Nanda A, Granger DN. CD40/CD40 ligand signaling in mouse cerebral microvasculature after focal ischemia/reperfusion. Circulation. 2005;111:1690–6.
75. Mori E, del Zoppo GJ, Chambers JD, Copeland BR, Arfors KE. Inhibition of polymorphonuclear leukocyte adherence suppresses no-reflow after focal cerebral ischemia in baboons. Stroke. 1992;23(5):712–8.
76. Gaudin A, Yemisci M, Eroglu H, Lepetre-Mouelhi S, Turkoglu OF, Dönmez-Demir B, Caban S, Sargon MF, Garcia-Argote S, Pieters G, Loreau O, Rousseau B, Tagit O, Hildebrandt N, Le Dantec Y, Mougin J, Valetti S, Chacun H, Nicolas V, Desmaële D, Andrieux K, Capan Y, Dalkara T, Couvreur P. Squalenoyl adenosine nanoparticles provide neuroprotection after stroke and spinal cord injury. Nat Nanotechnol. 2014;9:1054–62.
77. Dalkara T, Gursoy-Ozdemir Y, Yemisci M. Brain microvascular pericytes in health and disease. Acta Neuropathol (Berl). 2011;122(1):1–9.
78. Attwell D, Mishra A, Hall C, O'Farrell F, Dalkara T. What is a pericyte? J Cereb Blood Flow Metab. 2016;36(2):451–55.
79. del Zoppo GJ, Hamann GF. The cerebral microvasculature and responses to ischemia. In: Mohr JP, Wolf PA, Grotta JC, Moskowitz MA, Mayberg MR, von Kummer R, editors. Stroke: pathophysiology, diagnosis and management [Internet]. 5th ed. Philadelphia: Elsevier; 2011. p. 16–28. http://www.sciencedirect.com/science/article/pii/B9781416054788100028

80. Jespersen SN, Østergaard L. The roles of cerebral blood flow, capillary transit time heterogeneity, and oxygen tension in brain oxygenation and metabolism. J Cereb Blood Flow Metab. 2012;32(2):264–77.
81. Fernández-Klett F, Potas JR, Hilpert D, Blazej K, Radke J, Huck J, et al. Early loss of pericytes and perivascular stromal cell-induced scar formation after stroke. J Cereb Blood Flow Metab. 2013;33(3):428–39.
82. Donnan GA, Davis SM, Parsons MW, Ma H, Dewey HM, Howells DW. How to make better use of thrombolytic therapy in acute ischemic stroke. Nat Rev Neurol. 2011;7:400–9.
83. Desilles J-P, Meseguer E, Labreuche J, Lapergue B, Sirimarco G, Gonzalez-Valcarcel J, et al. Diabetes mellitus, admission glucose, and outcomes after stroke thrombolysis: a registry and systematic review. Stroke. 2013;44(7):1915–23.
84. Wardlaw JM, Murray V, Berge E, Del Zoppo GJ. Thrombolysis for acute ischaemic stroke. Cochrane Database Syst Rev. 2009;4:CD000213.
85. Willard AL, Herman IM. Vascular complications and diabetes: current therapies and future challenges. J Ophthalmol. 2012;2012:209538.
86. Østergaard L, Engedal TS, Moreton F, Hansen MB, Wardlaw JM, Dalkara T, et al. Cerebral small vessel disease: capillary pathways to stroke and cognitive decline. J Cereb Blood Flow Metab. 2016;36(2):302–25.
87. Gursoy-Ozdemir Y, Yemisci M, Dalkara T. Microvascular protection is essential for successful neuroprotection in stroke. J Neurochem. 2012;123 Suppl 2:2–11.
88. Diener H-C, Lees KR, Lyden P, Grotta J, Davalos A, Davis SM, et al. NXY-059 for the treatment of acute stroke: pooled analysis of the SAINT I and II Trials. Stroke. 2008;39(6): 1751–8.
89. Amaro S, Chamorro Á. Translational stroke research of the combination of thrombolysis and antioxidant therapy. Stroke J Cereb Circ. 2011;42(5):1495–9.
90. Donnan GA, Baron JC, Ma H, Davis SM. Penumbral selection of patients for trials of acute stroke therapy. Lancet Neurol. 2009;8:261–9.
91. Lees KR, Bluhmki E, von Kummer R, Brott TG, Toni D, Grotta JC, Albers GW, Kaste M, Marler JR, Hamilton SA, Tilley BC, Davis SM, Donnan GA, Hacke W, Allen K, Mau J, Meier D, del Zoppo G, De Silva DA, Butcher KS, Parsons MW, Barber PA, Levi C, Bladin C, Byrnes G. Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials. Lancet. 2010; 375:1695–703.
92. Del Zoppo GJ, Sharp FR, Heiss WD, Albers GW. Heterogeneity in the penumbra. J Cereb Blood Flow Metab. 2011;31:1836–51.
93. De Silva DA, Fink JN, Christensen S, Ebinger M, Bladin C, Levi CR, Parsons M, Butcher K, Barber PA, Donnan GA, Davis SM. Assessing reperfusion and recanalization as markers of clinical outcomes after intravenous thrombolysis in the echoplanar imaging thrombolytic evaluation trial (EPITHET). Stroke. 2009;40:2872–4.
94. Soares BP, Tong E, Hom J, Cheng SC, Bredno J, Boussel L, Smith WS, Wintermark M. Reperfusion is a more accurate predictor of follow-up infarct volume than recanalization: a proof of concept using CT in acute ischemic stroke patients. Stroke. 2010;41:e34–40.
95. Dalkara T, Arsava EM. Can restoring incomplete microcirculatory reperfusion improve stroke outcome after thrombolysis? J Cereb Blood Flow Metab. 2012;32:2091–9.
96. Al-Ali F, Jefferson A, Barrow T, Cree T, Louis S, Luke K, Major K, Nemeth D, Smoker S, Walker S. The capillary index score: rethinking the acute ischemic stroke treatment algorithm. Results from the Borgess Medical Center Acute Ischemic Stroke Registry. J Neurointerv Surg. 2013;5(2):139–43.
97. Horsch AD, Dankbaar JW, Niesten JM, van Seeters T, van der Schaaf IC, van der Graaf Y, et al. Predictors of reperfusion in patients with acute ischemic stroke. AJNR Am J Neuroradiol. 2015;36(6):1056–62.
98. Berkhemer OA, Fransen PSS, Beumer D, van den Berg LA, Lingsma HF, Yoo AJ, et al. A randomized trial of intraarterial treatment for acute ischemic stroke. N Engl J Med. 2015;372(1):11–20.

99. Cho T-H, Nighoghossian N, Mikkelsen IK, Derex L, Hermier M, Pedraza S, et al. Reperfusion within 6 hours outperforms recanalization in predicting penumbra salvage, lesion growth, final infarct, and clinical outcome. Stroke. 2015;46(6):1582–9.
100. Eilaghi A, Brooks J, d'Esterre C, Zhang L, Swartz RH, Lee T-Y, et al. Reperfusion is a stronger predictor of good clinical outcome than recanalization in ischemic stroke. Radiology. 2013;269(1):240–8.
101. Mouridsen K, Hansen MB, Østergaard L, Jespersen SN. Reliable estimation of capillary transit time distributions using DSC-MRI. J Cereb Blood Flow Metab. 2014;34(9):1511–21.
102. Joutel A, Corpechot C, Ducros A, Vahedi K, Chabriat H, Mouton P, et al. Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. Nature. 1996;383(6602):707–10.
103. Ghosh M, Balbi M, Hellal F, Dichgans M, Lindauer U, Plesnila N. Pericytes are involved in the pathogenesis of CADASIL. Ann Neurol. 2015;78(6):887–900.
104. Gu X, Liu X-Y, Fagan A, Gonzalez-Toledo ME, Zhao L-R. Ultrastructural changes in cerebral capillary pericytes in aged Notch3 mutant transgenic mice. Ultrastruct Pathol. 2012;36(1):48–55.
105. Dziewulska D, Lewandowska E. Pericytes as a new target for pathological processes in CADASIL. Neuropathology. 2012;32(5):515–21.
106. Gaengel K, Genové G, Armulik A, Betsholtz C. Endothelial-mural cell signaling in vascular development and angiogenesis. Arterioscler Thromb Vasc Biol. 2009;29:630–8.
107. Beck H, Plate KH. Angiogenesis after cerebral ischemia. Acta Neuropathol (Berl). 2009;117:481–96.
108. Ergul A, Alhusban A, Fagan SC. Angiogenesis: a harmonized target for recovery after stroke. Stroke. 2012;43:2270–4.
109. Kokovay E, Li L, Cunningham LA. Angiogenic recruitment of pericytes from bone marrow after stroke. J Cereb Blood Flow Metab. 2006;26:545–55.
110. Zechariah A, ElAli A, Doeppner TR, Jin F, Hasan MR, Helfrich I, Mies G, Hermann DM. Vascular endothelial growth factor promotes pericyte coverage of brain capillaries, improves cerebral blood flow during subsequent focal cerebral ischemia, and preserves the metabolic penumbra. Stroke. 2013;44:1690–7.
111. Zechariah A, ElAli A, Hagemann N, Jin F, Doeppner TR, Helfrich I, Mies G, Hermann DM. Hyperlipidemia attenuates vascular endothelial growth factor-induced angiogenesis, impairs cerebral blood flow, and disturbs stroke recovery via decreased pericyte coverage of brain endothelial cells. Arterioscler Thromb Vasc Biol. 2013;33:1561–7.
112. Hayashi T, Noshita N, Sugawara T, Chan PH. Temporal profile of angiogenesis and expression of related genes in the brain after ischemia. J Cereb Blood Flow Metab. 2003;23(2):166–80.
113. Chopp M, Zhang ZG, Jiang Q. Neurogenesis, angiogenesis, and MRI indices of functional recovery from stroke. Stroke. 2007;38(2 Suppl):827–31.
114. Arimura K, Ago T, Kamouchi M, Nakamura K, Ishitsuka K, Kuroda J, et al. PDGF receptor β signaling in pericytes following ischemic brain injury. Curr Neurovasc Res. 2012;9(1):1–9.
115. Dore-Duffy P, Owen C, Balabanov R, Murphy S, Beaumont T, Rafols JA. Pericyte migration from the vascular wall in response to traumatic brain injury. Microvasc Res. 2000;60(1):55–69.
116. Marti HJ, Bernaudin M, Bellail A, Schoch H, Euler M, Petit E, et al. Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. Am J Pathol. 2000;156(3):965–76.
117. Renner O, Tsimpas A, Kostin S, Valable S, Petit E, Schaper W, et al. Time- and cell type-specific induction of platelet-derived growth factor receptor-beta during cerebral ischemia. Brain Res Mol Brain Res. 2003;113(1–2):44–51.
118. Yang Y, Thompson JF, Taheri S, Salayandia VM, McAvoy TA, Hill JW, et al. Early inhibition of MMP activity in ischemic rat brain promotes expression of tight junction proteins and angiogenesis during recovery. J Cereb Blood Flow Metab. 2013;33(7):1104–14.

119. Özen I, Deierborg T, Miharada K, Padel T, Englund E, Genové G, et al. Brain pericytes acquire a microglial phenotype after stroke. Acta Neuropathol (Berl). 2014;128(3):381–96.
120. Omote Y, Deguchi K, Kono S, Liu N, Liu W, Kurata T, et al. Neurovascular protection of cilostazol in stroke-prone spontaneous hypertensive rats associated with angiogenesis and pericyte proliferation. J Neurosci Res. 2014;92(3):369–74.
121. Shen J, Ishii Y, Xu G, Dang TC, Hamashima T, Matsushima T, Yamamoto S, Hattori Y, Takatsuru Y, Nabekura J, Sasahara M. PDGFR-β as a positive regulator of tissue repair in a mouse model of focal cerebral ischemia. J Cereb Blood Flow Metab. 2012;32:353–67.
122. Nih LR, Deroide N, Leré-Déan C, Lerouet D, Soustrat M, Levy BI, et al. Neuroblast survival depends on mature vascular network formation after mouse stroke: role of endothelial and smooth muscle progenitor cell co-administration. Eur J Neurosci. 2012;35(8):1208–17.
123. Kamouchi M, Ago T, Kitazono T. Brain pericytes: emerging concepts and functional roles in brain homeostasis. Cell Mol Neurobiol. 2011;31(2):175–93.
124. Lamagna C, Bergers G. The bone marrow constitutes a reservoir of pericyte progenitors. J Leukoc Biol. 2006;80(4):677–81.
125. Kamouchi M, Ago T, Kuroda J, Kitazono T. The possible roles of brain pericytes in brain ischemia and stroke. Cell Mol Neurobiol. 2012;32:159–65.
126. Palmer TD, Willhoite AR, Gage FH. Vascular niche for adult hippocampal neurogenesis. J Comp Neurol. 2000;425(4):479–94.
127. Okano H, Sakaguchi M, Ohki K, Suzuki N, Sawamoto K. Regeneration of the central nervous system using endogenous repair mechanisms. J Neurochem. 2007;102(5):1459–65.
128. Licht T, Keshet E. The vascular niche in adult neurogenesis. Mech Dev. 2015;138(Pt 1):56–62.
129. Wang L, Zhang Z, Wang Y, Zhang R, Chopp M. Treatment of stroke with erythropoietin enhances neurogenesis and angiogenesis and improves neurological function in rats. Stroke. 2004;35(7):1732–7.
130. Karow M. Mountaineering pericytes—a universal key to tissue repair? BioEssays News Rev Mol Cell Dev Biol. 2013;35(9):771–4.
131. Karow M, Sánchez R, Schichor C, Masserdotti G, Ortega F, Heinrich C, et al. Reprogramming of pericyte-derived cells of the adult human brain into induced neuronal cells. Cell Stem Cell. 2012;11(4):471–6.
132. Paul G, Özen I, Christophersen NS, Reinbothe T, Bengzon J, Visse E, et al. The adult human brain harbors multipotent perivascular mesenchymal stem cells. PloS One. 2012;7(4):e35577.
133. Nakagomi T, Kubo S, Nakano-Doi A, Sakuma R, Lu S, Narita A, et al. Brain vascular pericytes following ischemia have multipotential stem cell activity to differentiate into neural and vascular lineage cells. Stem Cells. 2015;33(6):1962–74.

Glial Support of Blood–Brain Barrier Integrity: Molecular Targets for Novel Therapeutic Strategies in Stroke

Patrick T. Ronaldson and Thomas P. Davis

Abbreviations

ABC	ATP-binding cassette
ALK	Activin receptor-like kinase
APC	Activated protein C
AQP	Aquaporin
BBB	Blood–brain barrier
BCRP	Breast cancer resistance protein
CNS	Central nervous system
COX	Cyclooxygenase
EAAT	Excitatory amino-acid transporter
EPCR	Endothelial protein C receptor
GSH	Glutathione
GSSG	Glutathione disulfide
H/R	Hypoxia/reperfusion
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
JAM	Junctional adhesion molecule
Keap1	Kelch-like ECH-associated protein 1
MAGUK	Membrane-associated guanylate kinase
MAPK	Mitogen-activated protein kinase
MCAO	Middle cerebral artery occlusion
MCP	Monocyte chemoattractant protein

P.T. Ronaldson, Ph.D. (✉) • T.P. Davis, Ph.D.
Department of Pharmacology, College of Medicine, University of Arizona,
1501 North Campbell Avenue, P.O. Box 245050, Tucson, AZ 85724-5050, USA

Department of Pharmacology, College of Medicine, University of Arizona,
1501 North Campbell Avenue, P.O. Box 245050, Tucson, AZ 85724-5050, USA
e-mail: pronald@email.arizona.edu; davistp@email.arizona.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_3

MDR	Multidrug resistance
MMP	Matrix metalloproteinase
MRP	Multidrug resistance protein
NO	Nitric oxide
NOS	Nitric oxide synthase
Nrf2	Nuclear factor E2-related factor-2
NVU	Neurovascular unit
OATP	Organic anion transporting polypeptide
PAR	Proteinase-activated receptor
P-gp	P-glycoprotein
RANTES	Regulated upon activation normal T-cell expressed and secreted
ROS	Reactive oxygen species
r-tPA	Recombinant tissue plasminogen activator
SGLT	Sodium–glucose cotransporter
SLC	Solute carrier
SOD	Superoxide dismutase
TEMPOL	4-Hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl
TGF	Transforming growth factor
TLR	Toll-like receptor
VEGF	Vascular endothelial growth factor
ZO	Zonula occluden

1 Introduction

The blood–brain barrier (BBB) is an essential barrier system that separates the central nervous system (CNS) from the circulation. It is formed by a monolayer of endothelial cells that limit brain uptake of xenobiotics in an effort to maintain cerebral homeostasis. The BBB is also a significant obstacle to CNS drug delivery. In fact, several currently marketed drugs have limited or no efficacy in the brain due to an inability to cross the BBB and attain therapeutic CNS concentrations [1]. Brain microvascular endothelial cells are not intrinsically capable of forming a "barrier." Formation of the BBB requires coordinated cell–cell interactions and signaling from adjacent glial cells (i.e., astrocytes, microglia), pericytes, neurons, and extracellular matrix [2]. Such an intricate relationship implies existence of a "neurovascular unit (NVU)." During ischemic stroke, various NVU cell types are triggered by pathological stimuli. Understanding endothelial and glial cell responses that are involved in modifying the NVU/BBB in the context of stroke provides an opportunity not only to protect BBB integrity but also to target these mechanisms for effective CNS drug delivery. In this chapter, we summarize BBB physiology including interactions with associated cell types/structures (i.e., glial cells, pericytes, neurons, extracellular matrix) of the NVU. Additionally, we highlight endothelial and glial cell mechanisms that contribute to BBB/NVU dysfunction. Finally, we provide insights on how such mechanisms can be targeted in an effort to protect BBB integrity and/or optimize CNS drug delivery for stroke pharmacotherapy.

2 The Neurovascular Unit

The NVU consists of multiple CNS cell types/structures including brain microvascular endothelial cells, astrocytes, microglia, pericytes, neurons, and extracellular matrix [3]. The concept of the NVU emphasizes that the brain response to ischemic stroke occurs via coordinated interactions between these cell types [4, 5]. Decreased BBB functional integrity occurs prior to neuronal injury, which suggests that NVU dysfunction is directly linked to the BBB disruption observed in the setting of stroke [6, 7].

2.1 Components of the Neurovascular Unit

2.1.1 Endothelial Cells and the Blood–Brain Barrier

CNS function requires precise regulation of the brain extracellular space. Additionally, metabolic demands of brain tissue are considerable and account for approximately 20 % of total oxygen consumption [8]. The interface between CNS and systemic circulation must possess mechanisms that can facilitate nutrient transport, exactly regulate ion balance, and provide a barrier to potential toxins. This emphasizes a critical need for both physical and biochemical mechanisms that contribute to BBB barrier properties.

The current understanding of BBB structure is based upon studies by Reese, Karnovsky, and Brightman in the late 1960s [9, 10]. Anatomically, BBB endothelial cells are demarcated by a lack of fenestrations, minimal pinocytotic activity, and presence of tight junctions [11]. Cerebral endothelial cells have elevated mitochondrial content that is required for transport of solutes into and out of the brain thereby contributing to maintenance of CNS homeostasis [12]. Several receptors, ion channels, and influx/efflux transport proteins are expressed in brain microvascular endothelial cells. Transporters are a critical BBB biochemical mechanism that are prominently involved in permitting brain entry of some substances while excluding accumulation of other substances in brain parenchyma [13].

2.2 Molecular Characteristics of the BBB

2.2.1 Tight Junction Protein Complexes

BBB endothelial cells are interconnected by tight junctions (Fig. 1), which are large multi-protein complexes maintained by direct contact with astrocytes [14]. Physiologically, tight junctions form a continuous, almost impermeable barrier that limits paracellular diffusion of blood-borne substances with the exception of small, lipid soluble molecules [11]. The high BBB transendothelial resistance (1500–2000 $\Omega\ cm^2$) further restricts free flow of water and solutes [15]. BBB tight

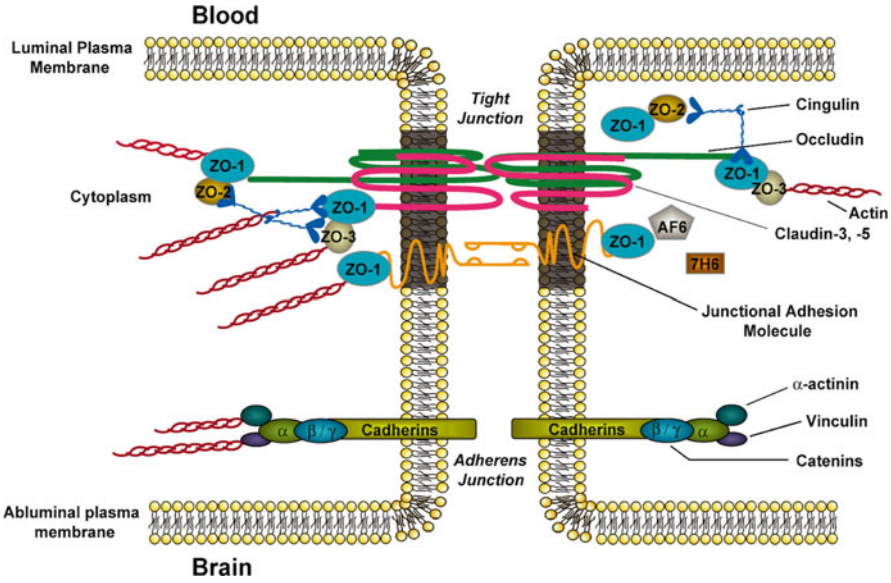

Fig. 1 Basic molecular organization of tight junction protein complexes at the blood–brain barrier. Adapted from Ronaldson & Davis. *Curr Pharm Des.* 18(25): 3624–3644 (2012)

junction formation primarily involves specific transmembrane proteins (i.e., junction adhesion molecules (JAMs), occludin, and claudins (i.e., claudin-1, -3, and -5)) that are linked to cytoskeletal filaments by interactions with accessory proteins (i.e., zonula occluden (ZO)-1, -2, and -3) [16].

Several JAM isoforms have been identified at the mammalian BBB including JAM-1, JAM-2, and JAM-3 [3, 16]. JAMs regulate transendothelial migration of neutrophils and monocytes/macrophages [17, 18]. Loss of JAM protein expression is directly correlated with BBB disruption and injury [19]. Additionally, studies in an immortalized human brain endothelial cell line (hCMEC/d3) showed that inflammatory stimulation led to increased JAM movement away from the tight junction and increased paracellular solute diffusion, which further suggests a central role for JAMs in maintaining BBB integrity [20].

Monomeric occludin is a 60- to 65-kDa protein that is highly expressed along endothelial cell margins in brain vasculature [21, 22]. Our group has shown that occludin is a critical regulator of BBB permeability in vivo [21, 22]. Occludin assembles into dimers and higher-order oligomers, a characteristic that is required for restriction of paracellular permeability. Altered occludin expression is associated with BBB dysfunction in several pathologies including hypoxia/aglycemia [23], hypoxia/reoxygenation stress [22], and focal cerebral ischemia [24].

Claudins are 20- to 24-kDa proteins that contribute to the physiological "seal" of the tight junction [25]. In cerebral microvascular endothelial cells, various claudin isoforms have been detected including claudin-1, -3, and -5 [24, 26–28]. In experimental stroke models [19, 29], altered expression of claudin-5 and an associated

enhancement in paracellular permeability has been reported. Claudin-1 expression was shown to decrease at the in vivo BBB following exposure to human amyloid-β ($A\beta_{40}$) peptide [30]. Aβ deposition at the BBB is characteristic of cerebral amyloid angiopathy, a pathological condition that contributes to microvascular injury including hemorrhages and ischemia.

Proper physiological functioning of the BBB, particularly restriction of paracellular solute transport, requires association of JAMs, occludin, and claudins with cytoplasmic accessory proteins. In brain microvascular endothelial cells, membrane-associated guanylate kinase-like (MAGUK) proteins are involved in clustering of tight junction protein complexes to the cell membrane [31]. Three MAGUK proteins have been identified at the tight junction: ZO-1, -2, and -3. ZO-1 links transmembrane tight junction proteins (i.e., JAMs, occludin, claudins) to the actin cytoskeleton [32]. Previous studies have shown that dissociation of ZO-1 from the junction complex is associated with increased permeability, suggesting that the ZO-1-transmembrane protein interaction is critical to tight junction stability and function [33–35]. ZO-1 has been shown to localize to the endothelial cell nucleus under conditions of proliferation or injury [36], following Ca^{2+} depletion [37], and in response to nicotine [27]. Similarly, ZO-2 binds tight junction constituents, signaling molecules and transcription factors [38]. In fact, ZO-2 may act redundantly with ZO-1 as it has been shown to facilitate formation of morphologically intact tight junctions in cultured cells lacking ZO-1 [39]. ZO-3 is expressed at the BBB but its exact role in the formation of tight junctions and/or maintenance of tight junction integrity has not been elucidated [40]. CNS pathologies including hypoxia, cerebral ischemia/reperfusion injury, and focal cerebral ischemia are associated with reduced ZO-1 expression at the BBB [41–43].

2.2.2 Adherens Junctions

Adherens junctions are specialized cell–cell interactions, which are formed by cadherins and associated proteins that are directly linked to actin filaments [44]. Cadherins regulate endothelial function by activation of phosphoinositide 3-kinase signaling, an intracellular pathway that organizes the cytoskeleton and enables complex formation with vascular endothelial growth factor receptor 2. Therefore, cadherin-mediated signaling is essential for endothelial cell layer integrity and for the spatial organization of new microvessels [45]. Barrier-forming endothelium has been shown to express higher levels of cadherin-10 relative to VE-cadherin [46]. In contrast, circumventricular organs and choroid plexus capillaries (i.e., brain microvasculature that is devoid of BBB properties) primarily express VE-cadherin [46]. Optimal cadherin function requires direct association with catenins. At least four catenin isoforms (i.e., β, α, χ, and p120) are expressed at the BBB, with β-catenin linking cadherin to α-catenin, which binds this protein complex to the actin cytoskeleton [47]. In vitro studies by Steiner and colleagues demonstrated that the heparin sulfate proteoglycan agrin also contributes to barrier properties of adherens junction by promoting localization of VE-cadherin and β-catenin to endothelial cell

junctions [48]. Paracellular expression of VE-cadherin and/or β-catenin is associated with BBB repair following focal astrocyte loss in vivo [49]. VE-cadherin expression was also decreased at the BBB in mice subjected to focal cerebral ischemia, suggesting that adherens junction disruption contributes to BBB dysfunction in the context of stroke [50].

2.3 Transporters

For many endogenous and exogenous substances, proteins expressed at the BBB determine their ability to traverse biological membranes. Such transport proteins include ATP-binding cassette (ABC) transporters and solute carrier (SLC) transporters [13]. In order to target transporters for optimization of pharmacotherapy, it is critical to understand localization (i.e., luminal versus abluminal), expression, and activity of endogenous transporters at the BBB endothelium (Fig. 2).

ABC Transporters. ABC transporters require biological energy via ATP hydrolysis to transport drugs and their metabolites against their concentration gradient. ABC drug transporters, specifically P-glycoprotein (P-gp), Multidrug Resistance Proteins (MRPs in humans; Mrps in rodents) and Breast Cancer Resistance Protein (BCRP in

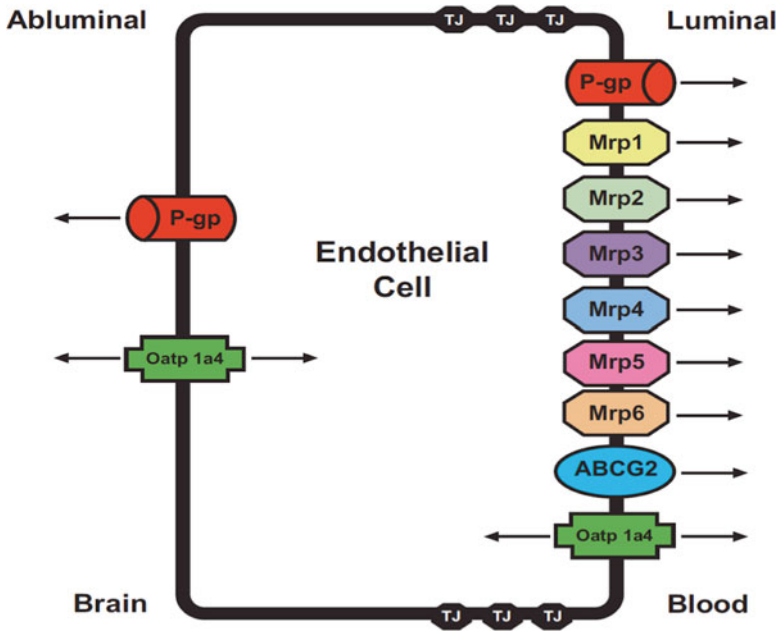

Fig. 2 Endothelial localization of drug transporters known to be involved in transport of therapeutic agents at the blood–brain barrier. Adapted from Ronaldson & Davis. *Curr Pharm Des.* **18**(25): 3624–3644 (2012)

humans; Bcrp in rodents) are involved in cellular extrusion of drugs and greatly contribute to limiting effective therapeutic delivery to the brain. In general, P-gp transports cationic or basic and neutral compounds, whereas MRPs/Mrps are involved in cellular efflux of anionic drugs as well as their glucuronidated, sulfated, and glutathione-conjugated metabolites [13, 51]. BCRP/Bcrp has significant overlap in substrate specificity profile with P-gp and has been shown to recognize a vast array of sulfoconjugated organic anions, hydrophobic, and amphiphilic compounds [52].

P-gp is a 170-kDa ATP-dependent transporter that primarily functions as a defense mechanism against potentially toxic xenobiotics [53]. P-gp orthologues from different species have greater than 70 % sequence identity [53] and are encoded by closely related genes (i.e., multidrug resistance (MDR) genes), which have two isoforms in humans (MDR1, MDR2) and three isoforms in both mice (i.e., mdr1, mdr2, mdr3) and rats (i.e., mdr1a, mdr1b, mdr2). The human MDR2 gene and the murine/rodent mdr2 gene products are exclusively involved in hepatic transport of phosphatidylcholine. In contrast, human MDR1, murine mdr1/mdr3, and rodent mdr1a/mdr1b are involved in drug transport at the BBB endothelium. P-gp has been localized to both the luminal and abluminal membrane of brain microvascular endothelial cells [54]. Abluminal localization of P-gp has also been identified on perivascular astrocyte foot processes [54–56]. Increased expression and/or activity of P-gp has been reported in hippocampal microvessels isolated from stroke-prone spontaneously hypertensive rats [57], which suggests that alterations in P-gp expression and/or activity may be a component of the BBB response to pathological stressors.

The mammalian MRP family belongs to the ABCC group of proteins [58]. Many of the functionally characterized MRP isoforms (i.e., MRP1/Mrp1, MRP2/Mrp2, MRP4/Mrp4, Mrp5, Mrp6) that have the potential to determine CNS drug delivery have been localized to the mammalian BBB [13, 16]. Additionally, the ability of Mrp isoforms to actively efflux the endogenous antioxidant glutathione (GSH) may have significant implications in stroke. GSH is responsible for maintenance of cellular redox balance and antioxidant defense in the brain. It has been demonstrated that functional expression of Mrps is upregulated in response to oxidative stress, a primary component of stroke pathophysiology, which leads to increased cellular efflux of GSH [59]. Enhanced functional expression of Mrp isoforms at the BBB can cause reduced brain and/or endothelial cell concentrations of GSH, an alteration in cellular redox status, and increased potential for cell injury and death.

Several recent studies have demonstrated localization of BCRP at the brain microvasculature, particularly along the luminal side of the BBB [60, 61]. In terms of transport activity, data from recent in vitro and in vivo studies are controversial. Although some studies have suggested that BCRP is not functional at the BBB [61, 62] or plays a minimal role in xenobiotic efflux from the brain [63], more detailed analyses have confirmed that BCRP is a critical determinant of drug permeation across the BBB [64, 65]. More recently, oxygen/glucose deprivation was shown to increase the endothelial expression of Bcrp at the mRNA level in an in vitro BBB model [66]. In vivo, Bcrp mRNA expression was observed to be upregulated in the peri-infarct region following reversible middle cerebral artery occlusion (MCAO) [67]. The functional implications of increased Bcrp molecular expression at the BBB/NVU have yet to be determined in the context of stroke.

Solute Carrier (SLC) Transporters: In contrast to ABC transporters, membrane transport of circulating solutes by SLC family members is governed by either an electrochemical gradient utilizing an inorganic or organic solute as a driving force or the transmembrane concentration gradient of the substance actually being transported. Perhaps the most viable SLC candidates for transporter targeting are members of the SLC21A/SLCO family, which includes organic anion transporting polypeptides (OATPs in humans; Oatps in rodents). OATPs/Oatps have distinct substrate preferences for amphipathic solutes [68]. OATPs/Oatps are well known to transport 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (i.e., statins), which have been shown to exhibit neuroprotective and antioxidant properties [69, 70]. Studies in Oatp1a4(−/−) mice demonstrated reduced blood-to-brain transport of pitavastatin and rosuvastatin as compared to wild-type controls, which suggests that Oatp1a4 is involved in statin transport across the BBB [71]. Although OATP isoforms are expressed in several tissues, not all exist at the BBB. Immunofluorescence staining of human brain frontal cortex demonstrated OATP1A2 localization at the level of the brain microvascular endothelium [72]. Expression of Oatp1a4, Oatp1c1, and Oatp2a1 has been reported at the rodent BBB [73–78]. Oatp1c1 is selectively expressed at the BBB [74], has relatively narrow substrate specificity, and primarily transports thyroxine and conjugated sterols [75, 76]. Oatp2a1 regulates BBB transport of prostaglandins [73]. It has been proposed that Oatp1a4, a rodent homologue of OATP1A2, is the primary drug transporting Oatp isoform expressed at the rat BBB [79, 80]. Recently, our laboratory demonstrated that Oatp1a4 is a BBB transporter that can be effectively targeted for facilitation of effective CNS drug delivery [77, 78].

2.4 Astrocytes

Astrocytes are the most abundant cell type in the brain and cover over 99 % of cerebral capillaries with their end-feet [3, 81]. Astrocytes are critical in development and/or maintenance of BBB characteristics [14, 82, 83]. Astrocytes may be involved in transient regulation of cerebral microvascular permeability [84], in particular via dynamic Ca^{2+} signaling between astrocytes and the endothelium via gap junctions and purinergic transmission [85, 86]. Additionally, astrocytes play an essential role in regulating water and ion exchange across the brain microvascular endothelium [87]. Astrocytes possess two high-affinity transporters for uptake of glutamate, termed excitatory amino-acid transporter 1 and 2 (i.e., EAAT1 (i.e., GLAST) and EAAT2 (i.e., GLT-1)) that remove excess glutamate from the synapse [88]. Elevated brain levels of glutamate may lead to a pathological condition known as excitotoxicity, which has been implicated in neuronal damage in ischemic stroke [89]. Additionally, astrocytes are known to express volume-regulated anion channels. These channels are involved in Ca^{2+}-independent release of anionic amino acids (i.e., glutamate, aspartate, taurine) during conditions that cause astrocyte swelling such as cerebral hypoxia [90]. Astrocytes are also known to express transport

proteins including P-gp [91], MRP/Mrp isoforms [13, 59], and Bcrp [92]. The expression of multiple drug transporters in astrocytes suggests that these glial cells may act as a secondary barrier to CNS drug permeation. That is, the balance of transporters in astrocytes may either sequester drugs within the astrocyte cytoplasm, thereby preventing these compounds from reaching their site of action in the brain, or concentrate drugs in brain extracellular fluid. Pharmacological agents within brain extracellular space can be effluxed by active transport mechanisms at brain barrier sites or via "sink" effects of the CSF [51].

2.5 Microglia

Microglia are derived from the monocyte lineage that represents approximately 20 % of the total glial cell population within the CNS [93]. Under normal physiological conditions, microglia exist in a quiescent state lacking endocytotic and phagocytotic activity. These microglia possess a ramified morphology characterized by a small (5–10 μm) cell body and multiple radial cell processes extending from the cell body. Ramified microglia are thought to contribute to CNS homeostasis by participating in extracellular fluid cleansing and neurotransmitter deactivation [51]. During disease or trauma, microglia may become activated and the degree of this activation is directly correlated to the type and severity of brain injury [94]. Activated microglia are identified by their larger cell body and short cytoplasmic processes as well as upregulation of cell surface receptors such as CD14 and Toll-like receptors (TLRs) [95, 96]. During an immune response, activated microglia may be further converted into a reactive state, which is characterized by a spheroid or rod-like morphology and the presence of phagocytotic activity. Microglia activation and proliferation has been implicated in the development of neuronal death in ischemic stroke and cerebral hypoxia [97–99]. Furthermore, activation of microglia is associated with dysfunction of the BBB characterized by changes in TJ protein expression and enhanced paracellular permeability [100]. When activated, microglia produce high levels of neurotoxic mediators such as nitric oxide and peroxide as well as inflammatory cytokines (i.e., TNF-α), proteases, and complement components [94]. Excessive production of these substances may further lead to cell injury in the CNS characterized by astrocyte activation, further microglia activation, and neuronal cell death.

Microglia express several ion channels including multiple potassium, calcium, sodium, and chloride channels [101]. The expression patterns of these ion channels depend on the microglial functional state and are involved in a variety of physiological functions including proliferation, ramification, and maintenance of membrane potential, intracellular pH regulation, and cell volume regulation [102]. Glutamate receptors [103] and nutrient carrier systems such as GLUT-1 [104] are expressed in microglia. These cells also express membrane proteins involved in drug transport. Studies in a continuous rat microglia cell line (i.e., MLS-9) demonstrated functional expression of P-gp [91], Mrp1 [105], and Mrp4/Mrp5 [106].

2.6 Pericytes

In addition to glia, pericytes also play a crucial role in the maintenance of BBB homeostasis [107]. Pericytes are flat, undifferentiated, contractile cells that attach at irregular intervals along the capillary walls and communicate with the other cell types of the NVU [108]. These cells, via secretion of pericyte-derived angiopoetin, induce expression of occludin at the BBB, which suggests that pericytes are directly involved in induction and/or maintenance of barrier properties [109]. Involvement of pericytes in induction of BBB properties is also exemplified by the observation that proper localization of endothelial proteins (i.e., P-gp, utrophin) requires co-culture with pericytes [110]. Studies using adult-viable pericyte-deficient mouse mutants demonstrated that pericytes are critical in maintaining expression of BBB-specific genes in endothelial cells (i.e., transferrin receptor) and by inducing polarization of astrocyte end-feet adjacent to the cerebral microvasculature [111]. Additionally, MRP isoforms (MRP1, MRP4, MRP5) have been identified in pericytes in vitro, which implies that pericytes may contribute to regulation of BBB xenobiotic permeability and to CNS drug delivery [112].

2.7 Neurons

Noradrenergic [113], serotonergic [114], cholinergic [115], and GABAergic [116] neurons have been shown to make distinct connections with other cell types of the NVU. The need for direct innervation of brain microvasculature comes from the dynamic nature of neural activity and the metabolic requirements of nervous tissue, implying that the cerebral microcirculation must be highly responsive to the needs of CNS tissue. Interestingly, disruption of BBB integrity induced by pathophysiological factors (i.e., inflammation, hypertension, ischemia) often accompanies changes in cerebral blood flow and perfusion pressure [117–119] and there is evidence that such BBB opening may be a selective, compensatory event rather than a simple anatomical disruption. This implies that communication between neurons and the brain microvasculature may not simply regulate blood flow, but BBB permeability as well.

2.8 Extracellular Matrix

In addition to cellular components of the NVU, the extracellular matrix of the basal lamina also interacts with the BBB endothelium. Disruption of extracellular matrix is strongly associated with increased BBB permeability in pathological states including stroke [120, 121]. The extracellular matrix serves as an anchor for the endothelium via interaction of laminin and other matrix proteins with endothelial integrin receptors [122]. Matrix proteins can also influence expression of TJ proteins [123, 124]. Proteolysis of extracellular matrix proteins is well known to occur

in response to stroke, an effect that greatly contributes to BBB disruption [125]. Additionally, protein fragments generated from extracellular matrix proteolysis (i.e., perlecan domain V) may have beneficial effects in neuroprotection and post-stroke brain repair [125, 126].

3 Ischemic Stroke

3.1 Overview of Ischemic Stroke

Stroke is a leading cause of death and long-term disability in the United States. Every year, more than 795,000 Americans suffer from either a new or recurrent stroke, which averages one incidence of stroke every 40 s [127]. Of all strokes, 87 % are ischemic [127]. The annual cost of stroke rehabilitation in the United States is an estimated $34 billion [127]. Several factors have been identified that increase risk of stroke including history of transient ischemic attacks, hypertension, impaired glucose tolerance and diabetes mellitus, atrial fibrillation, cigarette smoking, and low serum concentrations of HDL cholesterol [127].

Stroke is characterized by a heterogeneous spectrum of conditions caused by interruption of blood flow supplying the brain [128]. Such a deficit in cerebral blood flow causes an irreversibly damaged ischemic core and salvageable surrounding neural tissue known as the penumbra [129]. CNS energy requirements are met by brain uptake of oxygen and glucose, which are metabolized to enable phosphorylation of ADP to ATP. When blood flow to the brain is interrupted during stroke, the ischemic core is quickly deprived of oxygen and glucose. Inability to provide sufficient quantities of ATP causes collapse of ion gradients and subsequent release of neurotransmitters (i.e., dopamine, glutamate), an event that causes neuronal cell death and development of an infarction [130]. Excess release of glutamate is particularly deleterious to the CNS due to overstimulation of glutamate receptors, activation of phospholipases/sphingomyelinases, phospholipid hydrolysis, release of arachidonic acid and ceramide, and disruption of CNS calcium homeostasis [4]. Oxidative stress is also observed in the CNS at early time points following ischemic injury and is well known to contribute to cell death in the ischemic core [131]. As neuronal cell damage extends to the ischemic penumbra, neuroinflammation and apoptosis become more prevalent and dramatically affect viability of salvageable brain tissue within the penumbra [131].

Cell death processes in the ischemic core occur extremely rapidly (i.e., within minutes) thereby rendering this region difficult to protect using pharmacological approaches [4]. In contrast, cells within the ischemic penumbra die more slowly by active cell death mechanisms [4]. The primary goal of drug therapy for acute ischemic stroke is to salvage the penumbra as much as possible and as early as possible [129]. Currently, there is only one therapeutic agent approved by the FDA for acute ischemic stroke treatment, recombinant tissue plasminogen activator (r-tPA) [132]. The primary goal of r-tPA therapy is to restore the blood flow and oxygen supply to

ischemic brain tissue; however, most cellular damage to the brain occurs when cerebral perfusion is re-established (i.e., reoxygenation) [132]. Such hypoxia/reoxygenation (H/R) stress/injury is associated with neuronal apoptosis characterized by cytochrome *c* release, caspase-3 activation, and internucleosomal DNA fragmentation [80]. Pathophysiological mechanisms that can cause neuronal apoptosis during H/R include increased production of reactive oxygen species (ROS) [16, 80]. ROS contribute to brain injury by interacting with proteins, lipids, and nucleic acids as well as via activation of redox-sensitive signaling pathways. Such responses are characterized by increased CNS production of hydrogen peroxide, upregulation of the cellular stress marker heat shock protein-70, and increased nuclear expression of hypoxia-sensitive transcription factors such as hypoxia-inducible factor-1 and nuclear factor-κB [80]. The H/R component of stroke is also associated with decreased brain concentrations of GSH [133], an effect that is further indicative of oxidative stress. These molecular events associated with H/R injury emphasize a critical need in stroke therapy for discovery of new drugs that can be administered alone or in conjunction with r-tPA for "rescue" of neural tissue.

3.2 The Neurovascular Unit in Ischemic Stroke

3.2.1 Disruption of the Blood–Brain Barrier

BBB homeostasis is dependent on discrete interactions between NVU components [134, 135]. Perturbation of extracellular matrix (i.e., type IV collagen, heparan sulfate proteoglycan, laminin, fibronectin, perlecan) disrupts cell-matrix and cell–cell signaling mechanisms critical to NVU function [121, 125]. Proteinases such as matrix metalloproteinases (MMPs) contribute to breakdown of the extracellular matrix and BBB disruption in stroke [136]. This includes MMPs that are activated by HIF-1α-dependent mechanisms (i.e., MMP2) and MMPs whose activation is triggered by pro-inflammatory cytokines (i.e., TNF-α, IL-1β) such as MMP3 and MMP9 [135]. Involvement of MMPs in BBB disruption following ischemic stroke has been reported in experimental stroke models [137, 138]. Furthermore, a recent clinical study demonstrated that MMP9 levels were elevated in stroke patients [139]. MMPs directly compromise the BBB by degrading tight junction constituent proteins such as claudin-5 and occludin [135]. MMP-mediated opening of the BBB in ischemic stroke may be regulated by nitric oxide (NO) signaling [140]. Overall, damage and subsequent opening of the BBB is a key event in development of intracerebral hemorrhage and brain edema following ischemic stroke.

Experimental models of stroke have provided considerable information on solute leak across the BBB. Using the transient MCAO rodent model, Pfefferkorn and Rosenberg demonstrated increased leak of sucrose, a vascular marker that does not typically cross the BBB, in the ischemic hemisphere [141]. However, BBB disruption following an ischemic insult is much more profound than to allow leak of small molecules only. Recently, it was shown that BBB disruption following focal cerebral

ischemia was sufficient to allow blood-to-brain leak of Evan's blue dye [41]. Evan's blue dye, when unconjugated to plasma proteins, is a relatively small molecule with a molecular weight of 960.8 Da. It is well established that Evan's blue dye irreversibly binds to serum albumin in vivo. This leads to the formation of a very large, solute-protein complex (i.e., in excess of 60,000 Da) that can only traverse the BBB under considerable pathological stress such as that observed during an ischemic stroke [142]. Jiao and colleagues observed redistribution of various tight junction proteins following 2 h of focal cerebral ischemia, an event that correlated with increased blood-to-brain flux of Evan's blue-albumin [41]. Reorganization of tight junction proteins following focal cerebral ischemia is also mediated by vascular endothelial growth factor (VEGF) [143] and NO [144].

BBB dysfunction and subsequent leak across the microvascular endothelium following focal ischemia enables considerable movement of vascular fluid across the microvascular endothelium and development of vasogenic edema [145]. Recent studies using the MCAO model have shown that water movement across the BBB is exacerbated by enhanced blood-to-brain movement of sodium. Alterations in sodium gradients across the microvascular endothelium dramatically alter oncotic pressure and are facilitated by increased functional expression of Na–K–Cl cotransporter [146], as well as Na–H exchangers NHE1 and/or NHE2 [147]. MCAO studies in spontaneously hypertensive rats demonstrated that the NHE1 transporter is a critical regulator of ischemic-induced infarct volume [148]. Disruption of sodium gradients across the BBB during ischemic stroke can also involve upregulation of sodium-dependent glucose transporters such as sodium–glucose cotransporters (SGLTs). Specifically, pharmacological inhibition of SGLT in MCAO rats significantly reduced infarct and edema ratios, which implies that this transporter may be a critical determinant of stroke outcome [149].

Functional BBB integrity is disrupted by production of ROS and subsequent oxidative stress (Fig. 3). Production of superoxide anion, a potent ROS generated when molecular oxygen is reduced by only one electron, is a known mediator of cellular damage following stroke [150, 151]. Superoxide dismutase (SOD) enzymes tightly control biological activity of superoxide anion, a by-product of normal physiological processes. Under oxidative stress conditions, superoxide is produced at high levels that overwhelm the metabolic capacity of SOD. This phenomenon is supported by the observation that infarct size and cerebral edema were markedly reduced in mice engineered to overexpress SOD as compared to wild-type controls [150]. Increased levels of superoxide also contribute to BBB endothelial dysfunction [152, 153]. BBB damage can be intensified by conjugation of superoxide and NO to form peroxynitrite, a cytotoxic and pro-inflammatory molecule. Peroxynitrite causes injury to cerebral microvessels through lipid peroxidation, consumption of endogenous antioxidants (i.e., reduced GSH), and induction of mitochondrial failure [80, 154]. Peroxynitrite is known to induce endothelial damage by its ability to nitrosylate tyrosine, leading to functional modifications of critical proteins [155]. Peroxynitrite formation at the BBB becomes more likely with activation of endothelial nitric oxide synthase (eNOS) and inducible NOS (iNOS) because NO rapidly diffuses through membranes and reacts with superoxide anion [154].

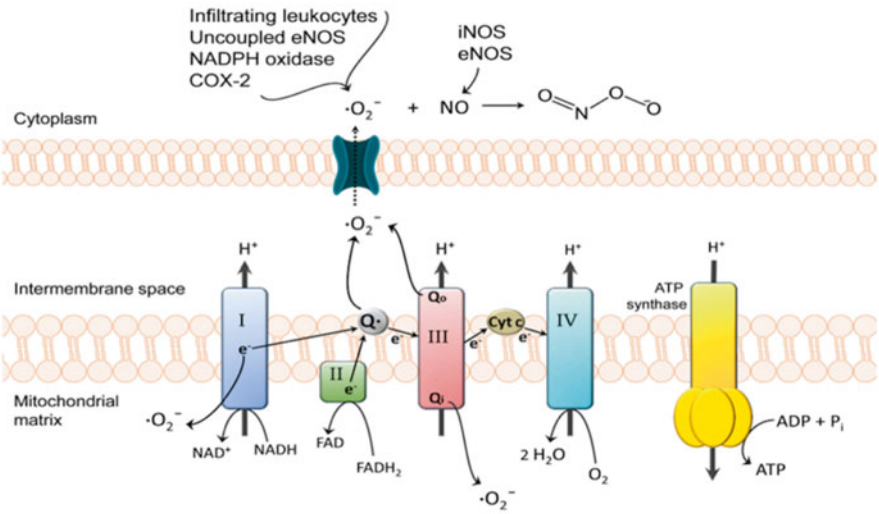

Fig. 3 Generation of reactive oxygen species (ROS) in brain microvascular endothelial cells. During disease, mitochondrial superoxide levels increase via NO inhibition of cytochrome complexes and oxidation of reducing equivalents in the electron transport chain. Complex I as well as both sides of complex III (i.e., Qi and Qo sites) are the most common sources of mitochondrial superoxide. Superoxide generated within the intermembrane space of mitochondria can reach the cytosol through voltage-dependent mitochondrial anion channels. Superoxide levels further increase via cyclooxygenase-2, NADPH oxidase, uncoupled eNOS, and infiltrating leukocytes. The resulting high levels of superoxide coupled with the activation of NO-producing eNOS and iNOS increases the probability of peroxynitrite formation. Peroxynitrite-induced cellular damage includes protein oxidation, tyrosine nitration, DNA damage, poly(ADP-ribose) polymerase activation, lipid peroxidation, and mitochondrial dysfunction. Adapted from Thompson and Ronaldson (2014). *Adv Pharmacol.* **71**: 165–209

Inflammatory stimuli are critical mediators of BBB dysfunction in the setting of stroke. Previous research has demonstrated that inflammatory mechanisms in focal cerebral ischemia are mediated through pro-inflammatory cytokines TNF-α and IL-1β, which appear within 2–6 h following ischemic insult [156]. Pro-inflammatory signaling induces adhesion molecules and subsequent transmigration of activated neutrophils, lymphocytes, or monocytes into brain parenchyma [157]. Physiologically, expression of vascular adhesion molecules such as ICAM-1 and VCAM-1 are minimally detectable at the BBB; however, their expression is dramatically increased in response to diseases such as stroke [157, 158]. Pro-inflammatory mediators also alter functional expression of endogenous BBB transporters and tight junction proteins. For example, TNF-α increased expression of P-gp but decreased BCRP expression in hCMEC/d3 cells [159]. Production and secretion of TNF-α and IL-1β has been observed to alter the expression of occludin and ZO-1 [160], suggesting involvement of inflammation in exacerbating paracellular leak during stroke.

3.3 Ischemic Stroke and Glial Support of the BBB

It is well established that the glial response to an ischemic insult is highly complex and multifaceted; however, it is known that injury and/or activation of astrocytes at the NVU leads to compromise of the BBB. In the inferior colliculus, focal astrocyte loss demarcated by reduced GFAP immunoreactivity directly corresponded with decreased paracellular localization of critical tight junction proteins claudin-5, occludin, and ZO-1 [83]. At the same time points that tight junction proteins were downregulated, an increase in leak of dextran (10 kDa) and fibrinogen was observed [83], which suggests a significant disruption of the BBB due to astrocyte cell death. The results of this study and others [161, 162] illustrate the requirement of intercellular communication between astrocytes and endothelial cells for maintenance of BBB integrity. Additionally, astrocytes have many other features that contribute to BBB physiology. For example, astrocytes are well known to express the water channel aquaporin 4 (AQP4) at end-feet localized adjacent to brain microvascular endothelium, which contributes to endothelial cell polarity and brain water volume [163]. Astrocytes secrete VEGF and fibroblast growth factor-2 (FGF-2), which promote angiogenesis and regulate biological transport at the BBB [164].

Astrocytes are essential contributors to the brain immunological response during ischemic stroke. Astrocytes maintain focal contacts with neighboring microglia and maintain these cells in a dormant, ramified state [94]. Regulation of microglia by astrocytes is prevented by inflammatory signaling, thus enabling microglia to elicit an immune response [94]. Astrocytes can directly contribute to brain immunological responses via upregulation of adhesion molecules (i.e., ICAM-1, VCAM) at their cell surface, an event that contributes to CNS targeting of leukocytes [165]. This enhancement is also facilitated by astrocytic secretion of chemokines such as macrophage inflammatory protein-1α/β (MIP-1 α/β), monocyte chemoattractant protein-1 (MCP-1), and regulated upon activation normal T-cell expressed and secreted (RANTES). Production and secretion of these chemokines by astrocytes is well known to occur in response to ischemic stroke [166, 167] and to increased brain parenchymal concentrations of tPA [168]. Of particular note, MCP-1 secretion by astrocytes was shown to coincide with a significant increase in FITC-albumin leak in an in vitro co-culture of endothelial cells and astrocytes subjected to 5 h oxygen–glucose deprivation, suggesting that MCP-1 may be a critical factor involved in BBB opening following an ischemic stroke [166]. Astrocytes also synthesize several pro- and anti-inflammatory cytokines including interleukins (i.e., IL-1α, IL-1β, IL-4-8, IL-10), TNF-α, and interferon-γ [169] as well as transforming growth factor-β (TGF-β) [170]. Cytokines such as TNF-α, IL-1α, IL-1β, and interferon-γ can trigger the endothelium and activate processes involved in BBB disruption. Although secretion of TGF-β1 may play a neuroprotective role in brain parenchyma [170], its effects on the endothelium are much more deleterious. Specifically, excessive endothelial stimulation by TGF-β1 affects the angiogenic response to ischemic stroke by causing formation of capillaries that lack pericytes, contain fewer endothelial cells and are shorter in length [84]. Indeed, pharmacological

targeting of TGF-β signaling at the level of the brain microvascular endothelium may be an efficacious approach for the protection of BBB integrity and/or preservation of angiogenic responses to stroke.

Inflammatory signaling by astrocytes is a critical event in exacerbation of CNS oxidative stress during ischemic stroke. Previous studies have shown that astrocytic production of pro-inflammatory cytokines can induce deleterious processes in astrocytes themselves via upregulation of iNOS [171]. Upregulation of iNOS leads to a significant enhancement in NO production, which can react with superoxide to produce peroxynitrite. Increased exposure of astrocytes to peroxynitrite can lead to rapid astrocyte proliferation and hypertrophy (i.e., reactive astrocytosis) and astrocyte apoptosis [172, 173]. Reactive astrocytosis is associated with disruption of tight junctions between adjacent brain microvessel endothelial cells and increased BBB permeability [174].

Although astrocyte injury is a critical determinant of BBB dysfunction in the setting of ischemic stroke, endothelial damage can also be induced via immune stimulated microglia [175]. This is supported by the observation that minocycline, a pharmacological inhibitor of activated microglia, dramatically reduced cell death in cultures of murine endothelial cells exposed to activated microglia in vitro [175]. Activated microglia produce pro-inflammatory cytokines in response to cerebral ischemia [176], all of which can trigger BBB disruption. In the setting of ischemic stroke, cytokine production in microglia is mediated by NF-kB signaling [177]. Inflammatory signaling in microglia may also involve cyclooxygenase-2 (COX2), which is inducible in response to ischemic injury and contributes to opening of the BBB [135]. In neuroinflammation, COX2 activates sphingomyelinases leading to release of ceramides, an event that leads to activation of p38 mitogen-activated protein kinase (MAPK) and subsequent secretion of pro-inflammatory cytokines [178–180]. Taken together, these observations point to a critical role for microglia in the inflammatory response to ischemic stroke.

3.4 Targeting the Neurovascular Unit in Ischemic Stroke

3.4.1 Targeting the Tight Junction

The BBB and associated glial support network of the NVU are clearly compromised in response to ischemic stroke. A critical "component" of ischemic stroke is cerebral hypoxia and subsequent brain injury resulting from reoxygenation/reperfusion (i.e., H/R stress). Over the past several years, our laboratory has studied BBB changes associated with H/R stress in an in vivo rodent model [21, 22, 26, 78, 148, 181]. Changes in BBB integrity under H/R conditions were demarcated by enhanced brain accumulation of ^{14}C-sucrose [22, 26, 181], a vascular marker that does not typically cross the brain microvascular endothelium. Additionally, H/R stress also increased vascular leak to dextrans (molecular weight range 4–10 kDa) in hippocampal and cortical microvessels [182]. These alterations in BBB permeability in animals subjected to H/R stress were directly associated with an increase in the

expression of HIF-1α and NF-kB in nuclear fractions isolated from intact microvessels [183]. In our studies, changes in brain solute uptake is not likely attributed to altered cerebral blood flow because we have previously shown that blood flow changes are negligible in our in vivo H/R model [26]. Changes in BBB permeability to ^{14}C-sucrose and dextrans were directly correlated with modified organization and/or expression of constituent TJ proteins including occludin, claudin-5, and ZO-1 [22, 26, 182]. Of paramount significance was the observation that H/R stress disrupted disulfide-bonded occludin oligomeric assemblies, thereby preventing monomeric occludin from forming an impermeable physical barrier to paracellular transport [21]. These changes in tight junction organization and BBB solute leak also correlated with a significant increase in brain water content following H/R, providing further evidence that disruption of the BBB under conditions of cerebral ischemia contributes to vasogenic edema [181].

Production of ROS and subsequent oxidative stress has been shown to alter the BBB expression of claudin-5 and occludin leading to increased paracellular solute leak [184]. Therefore, we hypothesized that oxidative stress associated changes in BBB permeability and occludin expression could be attenuated with the use of an antioxidant drug. In order to conduct these studies, we utilized 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPOL), a stable, membrane-permeable, water-soluble nitroxide antioxidant. TEMPOL shows SOD-like activity towards the superoxide anion as well as reactivity with hydroxyl radicals, nitrogen dioxide, and the carbonate radical. TEMPOL readily crosses the BBB and has been previously shown to provide neuroprotection as a free radical scavenger in several models of brain injury and ischemia [185, 186]. Using the dual artery in situ brain perfusion technique, we demonstrated that administration of TEMPOL 10 min before H/R treatment significantly attenuated CNS uptake of ^{14}C-sucrose as compared to animals subjected to H/R only [22]. This reduction in ^{14}C-sucrose leak was associated with a preservation of occludin localization and occludin oligomerization at the TJ [22]. Specifically, TEMPOL inhibits breakage of disulfide bonds on occludin monomers and thus prevents breakdown of occludin oligomeric assemblies and subsequent blood-to-brain leak of circulating solutes (Fig. 4). Restoration of BBB functional integrity coincided with a decrease in nuclear translocation of HIF-1α and a decrease in microvascular expression of the cellular stress marker heat shock protein 70 (hsp70) in rats subjected to H/R stress and administered TEMPOL [22]. Taken together, these observations provide evidence that the tight junction can be targeted pharmacologically during ischemic stroke for the purpose of reducing both oxidative stress associated injury to the brain microvascular endothelium and blood-to-brain solute leak (i.e., vascular protection).

3.5 Targeting Endogenous BBB Transporters

The ability of a drug to elicit a pharmacological effect at the level of the BBB requires achievement of efficacious concentrations within CNS. This therapeutic objective is dependent upon multiple mechanisms of transport that may include uptake into the brain by an influx transporter and/or extrusion by an efflux

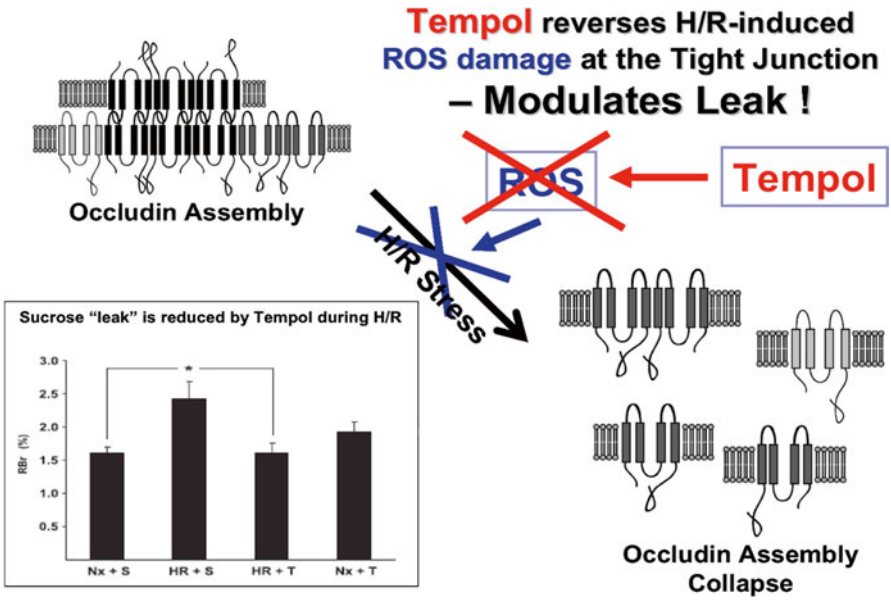

Fig. 4 Effect of TEMPOL on H/R-mediated disruption of the tight junction. ROS and subsequent oxidative stress are known to disrupt assembly of critical TJ proteins such as occludin. Our results show that administration of TEMPOL, by scavenging ROS, prevents disruption of occludin oligomeric assemblies. Furthermore, TEMPOL attenuates the increase in sucrose leak across the BBB observed in animals subjected to H/R stress. Taken together, our studies with TEMPOL demonstrate that the TJ can be targeted pharmacologically in an effort to preserve BBB functional integrity during ischemic stroke. Adapted from Ronaldson & Davis. *Curr Pharm Des.* **18(25)**: 3624–3644 (2012)

transporter. For many drugs, it is this balance between influx and efflux that determines if a drug will elicit a therapeutic effect in the brain or at the BBB. The complexity of drug transporter biology is further underscored by the observation that functional expression of transporters can be dramatically altered by oxidative stress [59, 187, 188]. A thorough understanding of regulation and functional expression of endogenous BBB transporters in both health and disease is essential for effective pharmacotherapy. Furthermore, such information will enable effective targeting of transporters and/or transporter regulatory mechanisms, thus allowing endogenous BBB transport systems to be exploited for purposes of improving CNS drug delivery and/or conferring BBB protection.

Considerable research has focused on studying mechanisms that limit endothelial membrane transport by describing the role of P-gp in restricting drug uptake from the systemic circulation [1, 189–191]; however, clinical trials targeting P-gp with small molecule inhibitors have been unsuccessful in improving pharmacotherapy due to inhibitor toxicity and/or enhanced tissue penetration of drugs [192, 193]. An alternative approach for optimizing delivery of drugs is to focus on BBB transporters that are involved in blood-to-brain transport. One intriguing candidate is Oatp1a4 (Fig. 5), which is known to transport HMG-CoA reductase inhibitors

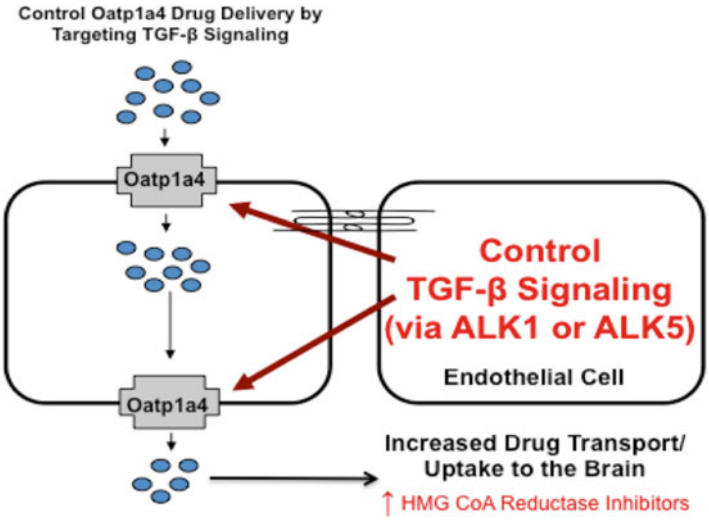

Fig. 5 Targeting Oatp transporters at the BBB for optimization of CNS drug delivery. Results from our studies demonstrate that targeting Oatp transporters during pathophysiological stress can modify CNS drug delivery. Oatp1a4 facilitates brain delivery of drugs that may exhibit efficacy in treatment of peripheral inflammatory pain or cerebral hypoxia such as statins and opioid peptide analgesics. The TGF-β signaling pathway enables control of Oatp isoforms by targeting TGF-β receptors (i.e., ALK1, ALK5) with small molecule therapeutics

(i.e., statins). Recent evidence suggests that statins can act as ROS scavengers independent of their well-documented effects on cholesterol biosynthesis [70]. Specifically, studies in dogs demonstrated that atorvastatin reduced the expression of oxidative and nitrosative stress markers (i.e., protein carbonyls, 4-hydroxy-2-noneal, 3-nitrotyrosine) and increased brain GSH levels [70, 194]. Interestingly, Cui and colleagues showed, in vivo, that atorvastatin administration during the acute phase of cerebral ischemia prevented increases in BBB permeability [195]. More recently, simvastatin was demonstrated to preserve barrier function following experimental intracerebral hemorrhage in an in vivo study involving MRI measurements of T_{1sat}, a marker of BBB integrity [196]. Taken together, these studies suggest that targeted delivery of statins may be an effective strategy for neuroprotection and/or BBB protection in the setting of stroke. We have shown, in vivo, that Oatp1a4 is a BBB transporter target that can be exploited to optimize CNS delivery of drugs, including statins [77, 78].

Although pathophysiological stressors can modulate BBB transporters, such changes must be controlled to provide optimal delivery of drugs. For example, we have demonstrated increased functional expression of Oatp1a4 only after 1 h hypoxia followed by up to 1 h reoxygenation [78]. If Oatp1a4 is to facilitate effective delivery of drugs (i.e., statins), its functional expression must be reliably controlled over a more desirable time course than is possible by relying solely on disease mechanisms. This objective can be accomplished by pharmacological targeting of Oatp regulatory pathways such as the TGF-β system [77, 78, 80]. TGF-βs

are cytokines that signal by binding to a heterotetrameric complex of type I and type II receptors [197]. The type I receptors, also known as activin receptor-like kinases (ALKs) propagate intracellular signals through phosphorylation of receptor-specific Smad proteins (i.e., (R)-Smads). At the BBB, only two ALK receptors (ALK1, ALK5) have been identified [28]. We have shown that pharmacological inhibition of TGF-β/ALK5 signaling can increase Oatp1a4 functional expression [77, 78]. This observation suggests that targeting of the TGF-β/ALK5 pathway may enable control of BBB Oatp1a4 expression and/or activity, thereby providing novel strategies for improved CNS drug delivery and/or BBB protection in stroke.

Optimization of drug delivery is not the only benefit that can be achieved from targeting transporters. BBB transporters mediate the flux of endogenous substrates, many of which are essential to the cellular response to pathological insult. One such substance is the endogenous antioxidant GSH. During oxidative stress, GSH is rapidly oxidized to glutathione disulfide (GSSG). Therefore, the redox state of a cell is represented by the ratio of GSH to GSSG [198]. In vitro studies using human and rodent brain microvascular endothelial cells have demonstrated that hypoxia reduces intracellular GSH levels and decreases the GSH:GSSG ratio, suggesting significant oxidative stress at the level of the BBB [199–201]. Using an in vivo model, oxidative stress was shown to cause BBB disruption characterized by altered expression/assembly of tight junction proteins occludin, claudin-5, and ZO-1 [21–23, 26, 35, 182]. These tight junction modifications correlated with increased BBB permeability to sucrose, an established vascular marker [22, 181], and dextrans [182]. Such increases in BBB permeability can result in leak of neurotoxic substances from blood into brain and/or contribute to vasogenic edema. BBB protection and/or repair in stroke are paramount to protecting the brain from neurological damage. One approach that can accomplish this therapeutic objective is to prevent cellular loss of GSH from endothelial cells by targeting endogenous BBB transporters (Fig. 6). BBB transporters that can transport GSH and GSSG include MRPs/Mrps. Both GSH and GSSG are substrates for MRP1/Mrp1 [59, 202, 203], MRP2/Mrp2 [204], and MRP4/Mrp4 [205]. It is well known that increased cellular concentrations of GSH are cytoprotective while processes that promote GSH loss from cells are damaging [206]. Therefore, it stands to reason that pharmacological targeting of Mrps during oxidative stress may have profound therapeutic benefits including vascular protection at the level of the BBB. Using the known Mrp transport inhibitor MK571, Tadepalle and colleagues showed that inhibition of Mrp1-mediated GSH transport resulted prevented GSH depletion in primary cultures of rat astrocytes [203]. Indeed, the effect of Mrp transport inhibition at the BBB and its effect on endothelial redox status and barrier integrity require further study.

Previous studies have shown that Mrp expression and/or activity can change in response to oxidative stress [59, 207]. Altered BBB expression of Mrps may prevent endothelial cells from retaining effective GSH concentrations. A thorough understanding of signaling pathways involved in Mrp regulation during oxidative stress will enable development of pharmacological approaches to target Mrp-mediated efflux (i.e., GSH transport) for the purpose of preventing BBB dysfunction in diseases with an oxidative stress component. One intriguing pathway is signaling mediated by nuclear factor E2-related factor-2 (Nrf2), a sensor of oxidative stress [188, 208]. In the presence of ROS, the cytosolic Nrf2 repressor Kelch-like ECH-associated

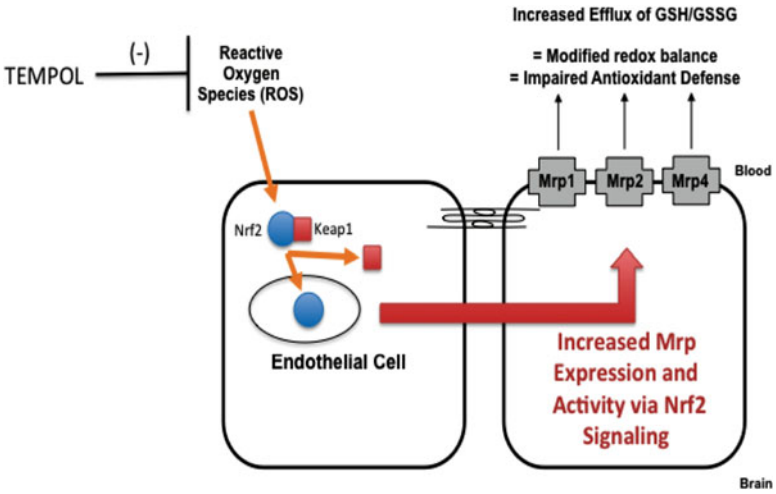

Fig. 6 Prevention of BBB Dysfunction by Targeting Mrp Isoforms in Cerebral Endothelial Cells. Results from our laboratory demonstrate increased expression of Mrp1, Mrp2, and Mrp4 at the BBB following an H/R insult. Furthermore, H/R stress is known to suppress GSH levels and increase GSSG concentrations in the brain. We propose that changes in GSH/GSSG transport occur during H/R as a result of altered functional expression of at least one Mrp isoform. Since Nrf2, a ROS sensitive transcription factor, is known to regulate Mrps, we hypothesize that this pathway is a critical regulatory mechanism for Mrps at the BBB. TEMPOL, a ROS scavenging antioxidant, is a pharmacological tool that can be utilized in order to understand how targeting activation of the Nrf2 pathway can control Mrp expression/activity

protein 1 (Keap1) undergoes structural alterations that cause dissociation from the Nrf2-Keap1 complex. This enables Nrf2 to translocate to the nucleus and induce transcription of genes that possess an antioxidant response element at their promoter [209, 210]. It has been demonstrated that activation of Nrf2 signaling induces expression of Mrp1, Mrp2, and Mrp4 [188, 207, 209, 211]. An emerging concept is that Nrf2 acts as a double-edged sword [210]: on one hand, Nrf2 is required for protecting tissues from oxidative stress; on the other, its activation can lead to deleterious effects. Therefore, an alteration in the balance of Mrp isoforms via activation of Nrf2 signaling may adversely affect redox balance and antioxidant defense at the brain microvascular endothelium. Indeed, this points towards a need for rigorous study of pharmacological approaches (i.e., use of antioxidant drugs such as TEMPOL) that can modulate Nrf2 signaling and control expression of Mrp isoforms and/or GSH transport at the BBB.

3.6 Targeting Glial Support of the BBB

In addition to the BBB endothelium, glial cells (i.e., astrocytes, microglia) are potential therapeutic targets in treatment of stroke. As noted above, glia play a crucial role in regulating BBB functional integrity in health and disease through release

of trophic factors that maintain tight junction protein complexes, release of factors that promote angiogenesis, pro-inflammatory signaling, and production of ROS. Pharmacological manipulation of glial cell biology represents a therapeutic approach that may enable control of BBB/NVU pathophysiological mechanisms during ischemic stroke and/or H/R injury.

An opportunity for cellular protection of glia in ischemic stroke involves targeting the proteinase-activated receptor (PAR) pathway. To date, four members of the PAR family (i.e., PAR-1, PAR-2, PAR-3, PAR-4) have been cloned and characterized [212]. Both PAR-1 and PAR-2 are expressed on the cell surface of astrocytes [213] and microglia [214, 215] as well as on the endothelial cell surface [216]. PAR-1 has been implicated in cytoprotective mechanisms [217, 218] while PAR-2 is involved in regulation of inflammatory responses [219]. Recent research has focused on pharmaceutical development of agonists targeted to the PAR-1 receptor such as activated protein C (APC) [216]. In a mouse model of transient cerebral ischemia, APC was shown to reduce ischemic brain damage and promote neovascularization and neurogenesis, suggesting that pharmacological targeting of the PAR-1 receptor may be an efficacious approach for the treatment of ischemic stroke [220]. Brain vascular perfusion studies demonstrated that brain accumulation of APC was reduced by 64 % in mice lacking the endothelial protein-C receptor (EPCR), suggesting that CNS delivery of APC is dependent upon saturable EPCR-mediated transport at the BBB [221]. Although native APC exhibits cytoprotection in stroke models, its use is limited by bleeding complications [222]; however, a mutant form of APC termed 3K3A-APC has been discovered that exhibits considerable cytoprotective efficacy without complications of bleeding [218]. Specifically, studies in human brain endothelial cells in vitro showed that 3K3A-APC protected these cells from oxygen–glucose deprivation to a significantly greater degree than APC [218]. Furthermore, 3K3A-APC improved the functional outcome and reduced the infarction size at a level that was significantly better than APC in the in vivo murine distal MCAO model [218], which implies that 3K3A-APC offers a safer and more efficacious alternative to APC in pharmacological targeting of the PAR-1 receptor. In the presence of r-tPA, 3K3A-APC reduced significant reduced infarct volume following focal cerebral ischemia in mice and embolic stroke in rats by up to 65 % [223]. More recently, a phase I clinical trial of 3K3A-APC demonstrated that this therapeutic was well tolerated in healthy adult volunteers [224], which provides an impetus to study the effects of 3K3A-APC in stroke patients. In the case of the PAR-2 receptor, a small molecule PAR-2 antagonist (i.e., N1-3-methylbutyryl-N4-6-aminohexanoyl-piperazine; ENMD-1068) has been shown to attenuate inflammatory responses in a dose-dependent manner [225].

Minocycline is a tetracycline with anti-inflammatory properties that directly inhibit microglial activation. Minocycline easily crosses the BBB, has a good safety profile, and a delayed therapeutic window thus rendering it an ideal candidate drug for treatment of ischemic stroke [226]. Blocking microglial activation may limit BBB disruption and reduce vasogenic edema in the context of ischemic stroke. For example, Yenari and colleagues reported that, in vivo, minocycline reduced infarction volume and neurological deficits as well as prevented BBB disruption and hem-

orrhage in a murine experimental stroke model [175]. In vitro, inhibition of microglial activation with minocycline limited ischemic damage in cultured endothelial cells and reduced superoxide release following oxygen–glucose deprivation [175]. More recently, combination therapy of minocycline and candesartan, a pro-angiogenic drug, was shown to improve long-term recovery in Wistar rats subjected to MCAO [227]. In vivo, minocycline was observed to reduce the frequency of hemorrhage in a murine model of cerebral amyloid angiopathy [228]. Currently, minocycline has been incorporated into clinical trials involving stroke patients. Results of these studies demonstrated that minocycline administration, both alone and in combination with r-tPA, improved functional neurological outcome following ischemic stroke [226].

TLRs are highly expressed in human CNS tissue, particularly by astrocytes and microglia [229]. Targeting these receptors has emerged as a promising goal for therapeutic control of ischemic stroke, primarily because TLRs are involved in BBB dysfunction and NVU ischemic injury [230]. While mRNA for TLRs 1–10 have been detected in murine microglia [231], all except TLR10 have been reported in human microglia [232]. Astrocytes possess a much more limited complement of TLRs since mRNA for TLRs 2, 4, 5, and 9 have been detected in murine astrocytes [233] and only TLR3 mRNA in human astrocytes [234]. While the large number of TLR receptors expressed on glial cells suggests a plethora of potential therapeutic targets for modification of glial pathology in the ischemic brain, much work needs to be done on understanding pharmacokinetics of TLR ligand binding and interactions between the TLR and the Toll/IL-1 receptor before TLR-based stroke therapeutics can reach development [230]. This field has shown promise as demonstrated by recent data in a murine model of cerebral ischemia where CNS delivery of TAT-hsp70 was shown to confer BBB protection via reduction of microglial activation, an effect that may be due to targeting of hsp70 to TLR2/4 [235].

4 Conclusion

The field of BBB biology, particularly the study of tight junction protein complexes and endogenous transport systems, has rapidly advanced over the past two decades. It is now well established that tight junction protein complexes are dynamic in nature and can organize and reorganize in response to ischemic stroke. These changes in tight junctions can lead to increased BBB permeability to small molecule drugs via the paracellular route. Additionally, many previous studies reported on the controversial ability of transporters (i.e., Oatp1a4) to act as facilitators of brain drug uptake. Now, it is beginning to be appreciated that endogenous BBB transporters can facilitate uptake of therapeutics from blood to the brain, thereby rendering these proteins, potential molecular targets for pharmacotherapy. Additionally, MRPs may represent viable molecular targets for BBB vascular protection in the setting of ischemic stroke. Molecular machinery involved in regulating these endogenous BBB transport systems (i.e., TGF-β/ALK5 signaling, Nrf2

pathway) are just now being fully characterized. These crucial discoveries have identified multiple targets that can be exploited for optimization of CNS delivery of therapeutic agents or for protection against BBB dysfunction. Perhaps targeting of currently marketed or novel drugs to influx transporters such as Oatp1a4 or to efflux transporters such as Mrp1, Mrp2, or Mrp4 will lead to significant advancements in ischemic stroke treatment. Identification and characterization of intracellular signaling pathways that can regulate the functional expression of uptake or efflux transporters provides yet another approach for pharmacological control of transporter systems in an effort to precisely deliver therapeutics to the CNS. Additionally, identification and characterization of novel targets on glial cells (i.e., astrocytes, microglia) provide yet another opportunity for the design and development of therapeutics aimed at protecting the BBB/NVU during ischemic injury and, by extension, controlling CNS drug delivery. Future work will continue to provide more insight on the interplay of tight junction protein complexes, transporters, and intracellular signaling pathways at the BBB/NVU and how these systems can be effectively targeted for improved stroke therapy.

Acknowledgments This work was supported by grants from the National Institutes of Health to P.T.R. (R01 NS084941) and to T.P.D. (R01 NS42652 and R01 DA11271).

*Conflict of Interest Statement***:** The authors have no conflict of interest to declare.

References

1. Hartz AM, Bauer B. Regulation of ABC transporters at the blood-brain barrier: new targets for CNS therapy. Mol Interv. 2010;10(5):293–304.
2. Blanchette M, Daneman R. Formation and maintenance of the BBB. Mech Dev. 2015;138 Pt 1:8–16. pii: S0925-4773(15)30009-5. doi:10.1016/j.mod.2015.07.007.
3. Ronaldson PT, Davis TP. Blood-brain barrier integrity and glial support: mechanisms that can be targeted for novel therapeutic approaches in stroke. Curr Pharm Des. 2012;18(25):3624–44.
4. Arai K, Lok J, Guo S, Hayakawa K, Xing C, Lo EH. Cellular mechanisms of neurovascular damage and repair after stroke. J Child Neurol. 2011;26(9):1193–8.
5. Posada-Duque RA, Barreto GE, Cardona-Gomez GP. Protection after stroke: cellular effectors of neurovascular unit integrity. Front Cell Neurosci. 2014;8:231.
6. DiNapoli VA, Huber JD, Houser K, Li X, Rosen CL. Early disruptions of the blood-brain barrier may contribute to exacerbated neuronal damage and prolonged functional recovery following stroke in aged rats. Neurobiol Aging. 2008;29(5):753–64.
7. Sakadzic S, Lee J, Boas DA, Ayata C. High-resolution in vivo optical imaging of stroke injury and repair. Brain Res. 2015;1623:174–92. pii: S0006-8993(15)00340-6. doi:10.1016/j.brainres.2015.04.044.
8. Rolfe DF, Brown GC. Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol Rev. 1997;77(3):731–58.
9. Reese TS, Karnovsky MJ. Fine structural localization of a blood-brain barrier to exogenous peroxidase. J Cell Biol. 1967;34(1):207–17.
10. Brightman MW, Reese TS. Junctions between intimately apposed cell membranes in the vertebrate brain. J Cell Biol. 1969;40(3):648–77.

11. Abbott NJ. Blood-brain barrier structure and function and the challenges for CNS drug delivery. J Inherit Metab Dis. 2013;36(3):437–49.
12. Oldendorf WH, Cornford ME, Brown WJ. The large apparent work capability of the blood-brain barrier: a study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat. Ann Neurol. 1977;1(5):409–17.
13. Sanchez-Covarrubias L, Slosky LM, Thompson BJ, Davis TP, Ronaldson PT. Transporters at CNS barrier sites: obstacles or opportunities for drug delivery? Curr Pharm Des. 2014;20(10):1422–49.
14. Janzer RC, Raff MC. Astrocytes induce blood-brain barrier properties in endothelial cells. Nature. 1987;325(6101):253–7.
15. Butt AM, Jones HC, Abbott NJ. Electrical resistance across the blood-brain barrier in anesthetized rats: a developmental study. J Physiol. 1990;429:47–62.
16. Ronaldson PT, Davis TP. Targeting transporters: promoting blood-brain barrier repair in response to oxidative stress injury. Brain Res. 2015;1623:39–52. pii:S0006-8993(15)00200-0. doi:10.1016/j.brainres.2015.03.018.
17. Williams DW, Calderon TM, Lopez L, Carvallo-Torres L, Gaskill PJ, Eugenin EA, Morgello S, Berman JW. Mechanisms of HIV entry into the CNS: increased sensitivity of HIV infected CD14+CD16+ monocytes to CCL2 and key roles of CCR2, JAM-A, and ALCAM in diapedesis. PLoS One. 2013;8:e69270.
18. Sladojevic N, Stamatovic SM, Keep RF, Grailer JJ, Sarma JV, Ward PA, Andjelkovic AV. Inhibition of junctional adhesion molecule-A/LFA interaction attenuates leukocyte trafficking and inflammation in brain ischemia/reperfusion injury. Neurobiol Dis. 2014; 67:57–70.
19. Wang XS, Fang HL, Chen Y, Liang SS, Zhu ZG, Zeng QY, et al. Idazoxan reduces blood-brain barrier damage during experimental autoimmune encephalomyelitis in mouse. Eur J Pharmacol. 2014;736:70–6.
20. Haarmann A, Deiss A, Prochaska J, Foerch C, Weksler B, Romero I, et al. Evaluation of soluble junctional adhesion molecule-A as a biomarker of human brain endothelial barrier breakdown. PLoS One. 2010;5:e13568.
21. McCaffrey G, Willis CL, Staatz WD, Nametz N, Quigley CA, Hom S, et al. Occludin oligomeric assemblies at tight junctions of the blood-brain barrier are altered by hypoxia and reoxygenation stress. J Neurochem. 2009;110(1):58–71.
22. Lochhead JJ, McCaffrey G, Quigley CE, Finch J, DeMarco KM, Nametz N, et al. Oxidative stress increases blood-brain barrier permeability and induces alterations in occludin during hypoxia-reoxygenation. J Cereb Blood Flow Metab. 2010;29(6):1625–36.
23. Brown RC, Davis TP. Hypoxia/aglycemia alters expression of occludin and actin in brain endothelial cells. Biochem Biophys Res Commun. 2005;327(4):1114–23.
24. Liang J, Qi Z, Liu W, Wang P, Shi W, Dong W, et al. Normobaric hyperoxia slows blood-brain barrier damage and expands the therapeutic time window for tissue-type plasminogen activator treatment in cerebral ischemia. Stroke. 2015;46(5):1344–51.
25. Ueno M. Molecular anatomy of the brain endothelial barrier: an overview of the distributional features. Curr Med Chem. 2007;14(11):1199–206.
26. Witt KA, Mark KS, Hom S, Davis TP. Effects of hypoxia-reoxygenation on rat blood-brain barrier permeability and tight junctional protein expression. Am J Physiol Heart Circ Physiol. 2003;285(6):H2820–31.
27. Hawkins BT, Abbruscato TJ, Egleton RD, Brown RC, Huber JD, Campos CR, et al. Nicotine increases in vivo blood-brain barrier permeability and alters cerebral microvascular tight junction protein distribution. Brain Res. 2004;1027(1–2):48–58.
28. Ronaldson PT, DeMarco KM, Sanchez-Covarrubias L, Solinsky CM, Davis TP. Transforming growth factor-beta signaling alters substrate permeability and tight junction protein expression at the blood-brain barrier during inflammatory pain. J Cereb Blood Flow Metab. 2009;29(6):1084–98.
29. Gibson CL, Srivastava K, Sprigg N, Bath PM, Bayraktutan U. Inhibition of rho-kinase protects cerebral barrier from ischemia-evoked injury through modulations of endothelial cell oxidative stress and tight junctions. J Neurochem. 2014;129:816–26.

30. Hartz AM, Bauer B, Soldner EL, Wolf A, Boy S, Backhaus R, et al. Amyloid-β contributes to blood-brain barrier leakage in transgenic human amyloid precursor protein mice and in humans with cerebral amyloid angiopathy. Stroke. 2012;43:514–23.
31. Gonzalez-Mariscal L, Betanzos A, Avila-Flores A. MAGUK proteins: structure and role in the tight junction. Semin Cell Dev Biol. 2000;11(4):315–24.
32. Fanning AS, Jameson BJ, Jesaitis LA, Anderson JM. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. J Biol Chem. 1998;273(45):29745–53.
33. Abbruscato TJ, Lopez SP, Mark KS, Hawkins BT, Davis TP. Nicotine and cotinine modulate cerebral microvascular permeability and protein expression of ZO-1 through nicotinic acetylcholine receptors expressed on brain endothelial cells. J Pharm Sci. 2002;91(12):2525–38.
34. Fischer S, Wobben M, Marti HH, Renz D, Schaper W. Hypoxia-induced hyperpermeability in brain microvessel endothelial cells involves VEGF-mediated changes in the expression of zonula occludens-1. Microvasc Res. 2002;63(1):70–80.
35. Mark KS, Davis TP. Cerebral microvascular changes in permeability and tight junctions induced by hypoxia-reoxygenation. Am J Physiol Heart Circ Physiol. 2002;282(4): H1485–94.
36. Gottardi CJ, Arpin M, Fanning AS, Louvard D. The junction-associated protein, zonula occludens-1, localizes to the nucleus before the maturation and during the remodeling of cell-cell contacts. Proc Natl Acad Sci U S A. 1996;93(20):10779–84.
37. Riesen FK, Rothen-Rutishauser B, Wunderli-Allenspach H. A ZO1-GFP fusion protein to study the dynamics of tight junctions in living cells. Histochem Cell Biol. 2002;117(4): 307–15.
38. Betanzos A, Huerta M, Lopez-Bayghen E, Azuara E, Amerena J, Gonzalez-Mariscal L. The tight junction protein ZO-2 associates with Jun, Fos and C/EBP transcription factors in epithelial cells. Exp Cell Res. 2004;292(1):51–66.
39. Umeda K, Matsui T, Nakayama M, Furuse K, Sasaki H, Furuse M, et al. Establishment and characterization of cultured epithelial cells lacking expression of ZO-1. J Biol Chem. 2004;279(43):44785–94.
40. Takenaga Y, Takagi N, Murotomi K, Tanonaka K, Takeo S. Inhibition of Src activity decreases tyrosine phosphorylation of occludin in brain capillaries and attenuates increase in permeability of the blood-brain barrier after transient focal cerebral ischemia. J Cereb Blood Flow Metab. 2009;29(6):1099–108.
41. Jiao H, Wang Z, Liu Y, Wang P, Xue Y. Specific role of tight junction proteins claudin-5, occludin, and ZO-1 of the blood-brain barrier in a focal cerebral ischemic insult. J Mol Neurosci. 2011;44(2):130–9.
42. Zehendner CM, Librizzi L, Hedrich J, Bauer NM, Angamo EA, de Curtis M, et al. Moderate hypoxia followed by reoxygenation results in blood-brain barrier breakdown via oxidative stress-dependent tight-junction protein disruption. PLoS One. 2013;8:e82823.
43. Li H, Gao A, Feng D, Wang Y, Zhang L, Cui Y, et al. Evaluation of the protective potential of brain microvascular endothelial cell autophagy on blood-brain barrier integrity during experimental cerebral ischemia-reperfusion injury. Transl Stroke Res. 2014;5:618–26.
44. Redzic Z. Molecular biology of the blood-brain and the blood-cerebrospinal fluid barriers: similarities and differences. Fluids Barriers CNS. 2011;8(1):3.
45. Lampugnani MG, Dejana E. The control of endothelial cell functions by adherens junctions. Novartis Found Symp. 2007;283:4–13.
46. Williams MJ, Lowrie MB, Bennett JP, Firth JA, Clark P. Cadherin-10 is a novel blood-brain barrier adhesion molecule in human and mouse. Brain Res. 2005;1058:62–72.
47. Meng W, Takeichi M. Adherens junction: molecular architecture and regulation. Cold Spring Harb Perspect Biol. 2009;1:a002899.
48. Steiner E, Enzmann GU, Lyck R, Lin S, Ruegg MA, Kroger S, et al. The heparin sulphate proteoglycan agrin contributes to barrier properties of mouse brain endothelial cells by stabilizing adherens junctions. Cell Tissue Res. 2014;358:465–79.

49. Willis CL, Camire RB, Brule SA, Ray DE. Partial recovery of the damaged rat blood-brain barrier is mediated by adherens junction complexes, extracellular matrix remodelling and macrophage infiltration following focal astrocyte loss. Neuroscience. 2013;250:773–85.
50. Wacker BK, Freie AB, Perfater JL, Gidday JM. Junction protein regulation by sphingosine kinase 2 contributes to blood-brain barrier protection in hypoxic preconditioning-induced cerebral ischemic tolerance. J Cereb Blood Flow Metab. 2012;32:1014–23.
51. Ronaldson PT, Persidsky Y, Bendayan R. Regulation of ABC membrane transporters in glial cells: relevance to the pharmacotherapy of brain HIV-1 infection. Glia. 2008;56(16): 1711–35.
52. Robey RW, To KK, Polgar O, Dohse M, Fetsch P, Dean M, et al. ABCG2: a perspective. Adv Drug Deliv Rev. 2009;61(1):3–13.
53. Gottesman MM, Hrycyna CA, Schoenlein PV, Germann UA, Pastan I. Genetic analysis of the multidrug transporter. Annu Rev Genet. 1995;29:607–49.
54. Bendayan R, Ronaldson PT, Gingras D, Bendayan M. In situ localization of P-glycoprotein (ABCB1) in human and rat brain. J Histochem Cytochem. 2006;54(10):1159–67.
55. Golden PL, Pardridge WM. P-glycoprotein on astrocyte foot processes of unfixed isolated human brain capillaries. Brain Res. 1999;819(1–2):143–6.
56. Schlachetzki F, Pardridge WM. P-glycoprotein and caveolin-1alpha in endothelium and astrocytes of primate brain. Neuroreport. 2003;14(16):2041–6.
57. Ueno M, Nakagawa T, Huang CL, Ueki M, Kusaka T, Hosomi N, et al. The expression of P-glycoprotein is increased in vessels with blood-brain barrier impairment in a stroke-prone hypertensive model. Neuropathol Appl Neurobiol. 2009;35(2):147–55.
58. Dallas S, Miller DS, Bendayan R. Multidrug resistance-associated proteins: expression and function in the central nervous system. Pharmacol Rev. 2006;58(2):140–61.
59. Ronaldson PT, Bendayan R. HIV-1 viral envelope glycoprotein gp120 produces oxidative stress and regulates the functional expression of multidrug resistance protein-1 (Mrp1) in glial cells. J Neurochem. 2008;106(3):1298–313.
60. Hori S, Ohtsuki S, Tachikawa M, Kimura N, Kondo T, Watanabe M, et al. Functional expression of rat ABCG2 on the luminal side of brain capillaries and its enhancement by astrocyte-derived soluble factor(s). J Neurochem. 2004;90(3):526–36.
61. Lee YJ, Kusuhara H, Jonker JW, Schinkel AH, Sugiyama Y. Investigation of efflux transport of dehydroepiandrosterone sulfate and mitoxantrone at the mouse blood-brain barrier: a minor role of breast cancer resistance protein. J Pharmacol Exp Ther. 2005;312(1):44–52.
62. van Herwaarden AE, Jonker JW, Wagenaar E, Brinkhuis RF, Schellens JH, Beijnen JH, et al. The breast cancer resistance protein (Bcrp1/Abcg2) restricts exposure to the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. Cancer Res. 2003;63(19):6447–52.
63. Zhao R, Raub TJ, Sawada GA, Kasper SC, Bacon JA, Bridges AS, et al. Breast cancer resistance protein interacts with various compounds in vitro, but plays a minor role in substrate efflux at the blood-brain barrier. Drug Metab Dispos. 2009;37(6):1251–8.
64. Zhou L, Schmidt K, Nelson FR, Zelesky V, Troutman MD, Feng B. The effect of breast cancer resistance protein and P-glycoprotein on the brain penetration of flavopiridol, imatinib mesylate (Gleevec), prazosin, and 2-methoxy-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl)propanoic acid (PF-407288) in mice. Drug Metab Dispos. 2009;37(5):946–55.
65. Agarwal S, Sane R, Ohlfest JR, Elmquist WF. The role of the breast cancer resistance protein (ABCG2) in the distribution of sorafenib to the brain. J Pharmacol Exp Ther. 2011;336(1):223–33.
66. Neuhaus W, Gaiser F, Mahringer A, Franz J, Riethmuller C, Forster C. The pivotal role of astrocytes in an in vitro stroke model of the blood-brain barrier. Front Cell Neurosci. 2014;8:352.
67. Dazert P, Suofu Y, Grube M, Popa-Wagner A, Kroemer HK, Jedlitschky G, et al. Differential regulation of transport proteins in the periinfarct region following reversible middle cerebral artery occlusion in rats. Neuroscience. 2006;142(4):1071–9.

68. Hagenbuch B, Meier PJ. Organic anion transporting polypeptides of the OATP/SLC21 family: phylogenetic classification as OATP/SLCO superfamily, new nomenclature and molecular/functional properties. Pflugers Arch. 2004;447(5):653–65.
69. Wood WG, Eckert GP, Igbavboa U, Muller WE. Statins and neuroprotection: a prescription to move the field forward. Ann N Y Acad Sci. 2010;1199:69–76.
70. Barone E, Cenini G, Di Domenico F, Martin S, Sultana R, Mancuso C, et al. Long-term high-dose atorvastatin decreases brain oxidative and nitrosative stress in a preclinical model of Alzheimer's disease: a novel mechanism of action. Pharmacol Res. 2011;63(3):172–80.
71. Ose A, Kusuhara H, Endo C, Tohyama K, Miyajima M, Kitamura S, et al. Functional characterization of mouse organic anion transporting polypeptide 1a4 in the uptake and efflux of drugs across the blood-brain barrier. Drug Metab Dispos. 2010;38(1):168–76.
72. Gao B, Hagenbuch B, Kullak-Ublick GA, Benke D, Aguzzi A, Meier PJ. Organic anion-transporting polypeptides mediate transport of opioid peptides across blood-brain barrier. J Pharmacol Exp Ther. 2000;294(1):73–9.
73. Kis B, Isse T, Snipes JA, Chen L, Yamashita H, Ueta Y, et al. Effects of LPS stimulation on the expression of prostaglandin carriers in the cells of the blood-brain and blood-cerebrospinal fluid barriers. J Appl Physiol. 2006;100(4):1392–9.
74. Chu C, Li JY, Boado RJ, Pardridge WM. Blood-brain barrier genomics and cloning of a novel organic anion transporter. J Cereb Blood Flow Metab. 2008;28(2):291–301.
75. Westholm DE, Salo DR, Viken KJ, Rumbley JN, Anderson GW. The blood-brain barrier thyroxine transporter organic anion-transporting polypeptide 1c1 displays atypical transport kinetics. Endocrinology. 2009;150(11):5153–62.
76. Westholm DE, Stenehjem DD, Rumbley JN, Drewes LR, Anderson GW. Competitive inhibition of organic anion transporting polypeptide 1c1-mediated thyroxine transport by the fenamate class of nonsteroidal antiinflammatory drugs. Endocrinology. 2009;150(2): 1025–32.
77. Ronaldson PT, Finch JD, DeMarco KM, Quigley CE, Davis TP. Inflammatory pain signals an increase in functional expression of organic anion transporting polypeptide 1a4 at the blood-brain barrier. J Pharmacol Exp Ther. 2011;336(3):827–39.
78. Thompson BJ, Sanchez-Covarrubias L, Slosky LM, Zhang Y, Laracuente ML, Ronaldson PT. Hypoxia/reoxygenation stress signals an increase in organic anion transporting polypeptide 1a4 (Oatp1a4) at the blood-brain barrier: relevance to CNS drug delivery. J Cereb Blood Flow Metab. 2014;34(4):699–707.
79. Ronaldson PT, Davis TP. Targeted drug delivery to treat pain and cerebral hypoxia. Pharmacol Rev. 2013;65(1):291–314.
80. Thompson BJ, Ronaldson PT. Drug delivery to the ischemic brain. Adv Pharmacol. 2014;71:165–202.
81. Mathiisen TM, Lehre KP, Danbolt NC, Ottersen OP. The perivascular astroglial sheath provides a complete covering of the brain microvessels: an electron microscopic 3D reconstruction. Glia. 2010;58(9):1094–103.
82. Hayashi Y, Nomura M, Yamagishi S, Harada S, Yamashita J, Yamamoto H. Induction of various blood-brain barrier properties in non-endothelial cells by close apposition to co-cultured astrocytes. Glia. 1997;19(1):13–26.
83. Willis CL, Nolan CC, Reith SN, Lister T, Prior MJ, Guerin CJ, et al. Focal astrocyte loss is followed by microvascular damage, with subsequent repair of the blood-brain barrier in the apparent absence of direct astrocytic contact. Glia. 2004;45(4):325–37.
84. Ballabh P, Braun A, Nedergaard M. The blood-brain barrier: an overview: structure, regulation, and clinical implications. Neurobiol Dis. 2004;16(1):1–13.
85. Goldberg M, De Pitta M, Volman V, Berry H, Ben Jacob E. Nonlinear gap junctions enable long-distance propagation of pulsating calcium waves in astrocyte networks. PLoS Comput Biol. 2010;6(8).
86. Pelligrino DA, Vetri F, Xu HL. Purinergic mechanisms in gliovascular coupling. Semin Cell Dev Biol. 2011;22(2):229–36.

87. Abbott NJ, Ronnback L, Hansson E. Astrocyte-endothelial interactions at the blood-brain barrier. Nat Rev Neurosci. 2006;7(1):41–53.
88. Bak LK, Schousboe A, Waagepetersen HS. The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. J Neurochem. 2006; 98(3):641–53.
89. Anderson CM, Swanson RA. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia. 2000;32(1):1–14.
90. Kimelberg HK, Goderie SK, Higman S, Pang S, Waniewski RA. Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. J Neurosci. 1990;10(5):1583–91.
91. Ronaldson PT, Bendayan M, Gingras D, Piquette-Miller M, Bendayan R. Cellular localization and functional expression of P-glycoprotein in rat astrocyte cultures. J Neurochem. 2004;89(3):788–800.
92. Nies AT, Jedlitschky G, Konig J, Herold-Mende C, Steiner HH, Schmitt HP, et al. Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain. Neuroscience. 2004;129(2):349–60.
93. Raivich G, Bohatschek M, Kloss CU, Werner A, Jones LL, Kreutzberg GW. Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. Brain Res Brain Res Rev. 1999;30(1):77–105.
94. Speth C, Dierich MP, Sopper S. HIV-infection of the central nervous system: the tightrope walk of innate immunity. Mol Immunol. 2005;42(2):213–28.
95. Bsibsi M, Ravid R, Gveric D, van Noort JM. Broad expression of Toll-like receptors in the human central nervous system. J Neuropathol Exp Neurol. 2002;61(11):1013–21.
96. Rivest S. Molecular insights on the cerebral innate immune system. Brain Behav Immun. 2003;17(1):13–9.
97. Deierborg T, Roybon L, Inacio AR, Pesic J, Brundin P. Brain injury activates microglia that induce neural stem cell proliferation ex vivo and promote differentiation of neurosphere-derived cells into neurons and oligodendrocytes. Neuroscience. 2010;171(4):1386–96.
98. Faustino JV, Wang X, Johnson CE, Klibanov A, Derugin N, Wendland MF, et al. Microglial cells contribute to endogenous brain defenses after acute neonatal focal stroke. J Neurosci. 2011;31(36):12992–3001.
99. Wei Z, Chigurupati S, Arumugam TV, Jo DG, Li H, Chan SL. Notch activation enhances the microglia-mediated inflammatory response associated with focal cerebral ischemia. Stroke. 2011;42(9):2589–94.
100. Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 2008;57(2):178–201.
101. Eder C. Ion channels in microglia (brain macrophages). Am J Physiol. 1998;275(2 Pt 1):C327–42.
102. Eder C. Regulation of microglial behavior by ion channel activity. J Neurosci Res. 2005;81(3):314–21.
103. Noda M, Nakanishi H, Nabekura J, Akaike N. AMPA-kainate subtypes of glutamate receptor in rat cerebral microglia. J Neurosci. 2000;20(1):251–8.
104. Maher F. Immunolocalization of GLUT1 and GLUT3 glucose transporters in primary cultured neurons and glia. J Neurosci Res. 1995;42(4):459–69.
105. Dallas S, Zhu X, Baruchel S, Schlichter L, Bendayan R. Functional expression of the multidrug resistance protein 1 in microglia. J Pharmacol Exp Ther. 2003;307(1):282–90.
106. Dallas S, Schlichter L, Bendayan R. Multidrug resistance protein (MRP) 4- and MRP 5-mediated efflux of 9-(2-phosphonylmethoxyethyl)adenine by microglia. J Pharmacol Exp Ther. 2004;309(3):1221–9.
107. Dalkara T, Alarcon-Martinez L. Cerebral microvascular pericytes and neurogliovascular signalling in health and disease. Brain Res. 2015;1632:3–17. pii:S0006-8993(15)00282-6. doi:10.1016/j.brainres.2015.03.047.
108. Dore-Duffy P, Cleary K. Morphology and properties of pericytes. Methods Mol Biol. 2011;686:49–68.

109. Hori S, Ohtsuki S, Hosoya K, Nakashima E, Terasaki T. A pericyte-derived angiopoietin-1 multimeric complex induces occludin gene expression in brain capillary endothelial cells through Tie-2 activation in vitro. J Neurochem. 2004;89(2):503–13.
110. Al Ahmad A, Taboada CB, Gassmann M, Ogunshola OO. Astrocytes and pericytes differentially modulate blood-brain barrier characteristics during development and hypoxic insult. J Cereb Blood Flow Metab. 2011;31(2):693–705.
111. Armulik A, Genove G, Mae M, Nisancioglu MH, Wallgard E, Niaudet C, et al. Pericytes regulate the blood-brain barrier. Nature. 2010;468(7323):557–61.
112. Berezowski V, Landry C, Dehouck MP, Cecchelli R, Fenart L. Contribution of glial cells and pericytes to the mRNA profiles of P-glycoprotein and multidrug resistance-associated proteins in an in vitro model of the blood-brain barrier. Brain Res. 2004;1018(1):1–9.
113. Cohen Z, Molinatti G, Hamel E. Astroglial and vascular interactions of noradrenaline terminals in the rat cerebral cortex. J Cereb Blood Flow Metab. 1997;17(8):894–904.
114. Cohen Z, Bonvento G, Lacombe P, Hamel E. Serotonin in the regulation of brain microcirculation. Prog Neurobiol. 1996;50(4):335–62.
115. Tong XK, Hamel E. Regional cholinergic denervation of cortical microvessels and nitric oxide synthase-containing neurons in Alzheimer's disease. Neuroscience. 1999;92(1):163–75.
116. Vaucher E, Tong XK, Cholet N, Lantin S, Hamel E. GABA neurons provide a rich input to microvessels but not nitric oxide neurons in the rat cerebral cortex: a means for direct regulation of local cerebral blood flow. J Comp Neurol. 2000;421(2):161–71.
117. Cucullo L, Couraud PO, Weksler B, Romero IA, Hossain M, Rapp E, et al. Immortalized human brain endothelial cells and flow-based vascular modeling: a marriage of convenience for rational neurovascular studies. J Cereb Blood Flow Metab. 2008;28(2):312–28.
118. Vital SA, Terao S, Nagai M, Granger DN. Mechanisms underlying the cerebral microvascular responses to angiotensin II-induced hypertension. Microcirculation. 2010;17(8):641–9.
119. Shen Q, Du F, Huang S, Duong TQ. Spatiotemporal characteristics of postischemic hyperperfusion with respect to changes in T1, T2, diffusion, angiography, and blood-brain barrier permeability. J Cereb Blood Flow Metab. 2011;31(10):2076–85.
120. Sood RR, Taheri S, Candelario-Jalil E, Estrada EY, Rosenberg GA. Early beneficial effect of matrix metalloproteinase inhibition on blood-brain barrier permeability as measured by magnetic resonance imaging countered by impaired long-term recovery after stroke in rat brain. J Cereb Blood Flow Metab. 2008;28(2):431–8.
121. Del Zoppo GJ. The neurovascular unit, matrix proteases, and innate inflammation. Ann N Y Acad Sci. 2010;1207:46–9.
122. Hynes RO, Lander AD. Contact and adhesive specificities in the associations, migrations, and targeting of cells and axons. Cell. 1992;68(2):303–22.
123. Tilling T, Korte D, Hoheisel D, Galla HJ. Basement membrane proteins influence brain capillary endothelial barrier function in vitro. J Neurochem. 1998;71(3):1151–7.
124. Savettieri G, Di Liegro I, Catania C, Licata L, Pitarresi GL, D'Agostino S, et al. Neurons and ECM regulate occludin localization in brain endothelial cells. Neuroreport. 2000;11(5):1081–4.
125. Roberts J, Kahle MP, Bix GJ. Perlecan and the blood-brain barrier: beneficial proteolysis? Front Pharmacol. 2012;3:155.
126. Guell K, Bix GJ. Brain endothelial cell specific integrins and ischemic stroke. Expert Rev Neurother. 2014;14(11):1287–92.
127. Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, et al. Heart disease and stroke statistics—2015 update: a report from the American Heart Association. Circulation. 2015;131(4):e29–322.
128. Moskowitz MA, Lo EH, Iadecola C. The science of stroke: mechanisms in search of treatments. Neuron. 2010;67(2):181–98.
129. Liu S, Levine SR, Winn HR. Targeting ischemic penumbra: part I—from pathophysiology to therapeutic strategy. J Exp Stroke Transl Med. 2010;3(1):47–55.
130. Adibhatla RM, Hatcher JF, Larsen EC, Chen X, Sun D, Tsao FH. CDP-choline significantly restores phosphatidylcholine levels by differentially affecting phospholipase A2 and CTP: phosphocholine cytidylyltransferase after stroke. J Biol Chem. 2006;281(10):6718–25.

131. Candelario-Jalil E. Injury and repair mechanisms in ischemic stroke: considerations for the development of novel neurotherapeutics. Curr Opin Investig Drugs. 2009;10(7):644–54.
132. Moretti A, Ferrari F, Villa RF. Pharmacological therapy of acute ischemic stroke: achievements and problems. Pharmacol Ther. 2015;153:79–89.
133. Schild L, Reiser G. Oxidative stress is involved in the permeabilization of the inner membrane of brain mitochondria exposed to hypoxia/reoxygenation and low micromolar Ca2+. FEBS J. 2005;272(14):3593–601.
134. Iadecola C, Nedergaard M. Glial regulation of the cerebral microvasculature. Nat Neurosci. 2007;10(11):1369–76.
135. Yang Y, Rosenberg GA. Blood-brain barrier breakdown in acute and chronic cerebrovascular disease. Stroke. 2011;42(11):3323–8.
136. Zhao LR, Navalitloha Y, Singhal S, Mehta J, Piao CS, Guo WP, et al. Hematopoietic growth factors pass through the blood-brain barrier in intact rats. Exp Neurol. 2007;204(2):569–73.
137. Bauer AT, Burgers HF, Rabie T, Marti HH. Matrix metalloproteinase-9 mediates hypoxia-induced vascular leakage in the brain via tight junction rearrangement. J Cereb Blood Flow Metab. 2010;30(4):837–48.
138. Kumari R, Willing LB, Patel SD, Baskerville KA, Simpson IA. Increased cerebral matrix metalloprotease-9 activity is associated with compromised recovery in the diabetic db/db mouse following a stroke. J Neurochem. 2011;119(5):1029–40.
139. Brouns R, Wauters A, De Surgeloose D, Marien P, De Deyn PP. Biochemical markers for blood-brain barrier dysfunction in acute ischemic stroke correlate with evolution and outcome. Eur Neurol. 2011;65(1):23–31.
140. Gu Y, Zheng G, Xu M, Li Y, Chen X, Zhu W, et al. Caveolin-1 regulates nitric oxide-mediated matrix metalloproteinases activity and blood-brain barrier permeability in focal cerebral ischemia and reperfusion injury. J Neurochem. 2012;120(1):147–56.
141. Pfefferkorn T, Rosenberg GA. Closure of the blood-brain barrier by matrix metalloproteinase inhibition reduces rtPA-mediated mortality in cerebral ischemia with delayed reperfusion. Stroke. 2003;34(8):2025–30.
142. Nagaraja TN, Keenan KA, Fenstermacher JD, Knight RA. Acute leakage patterns of fluorescent plasma flow markers after transient focal cerebral ischemia suggest large openings in blood-brain barrier. Microcirculation. 2008;15(1):1–14.
143. Yeh WL, Lu DY, Lin CJ, Liou HC, Fu WM. Inhibition of hypoxia-induced increase of blood-brain barrier permeability by YC-1 through the antagonism of HIF-1alpha accumulation and VEGF expression. Mol Pharmacol. 2007;72(2):440–9.
144. Yamauchi A, Dohgu S, Nishioku T, Shuto H, Naito M, Tsuruo T, et al. An inhibitory role of nitric oxide in the dynamic regulation of the blood-brain barrier function. Cell Mol Neurobiol. 2007;27(3):263–70.
145. Sandoval KE, Witt KA. Blood-brain barrier tight junction permeability and ischemic stroke. Neurobiol Dis. 2008;32(2):200–19.
146. Wallace BK, Jelks KA, O'Donnell ME. Ischemia-induced stimulation of cerebral microvascular endothelial cell Na-K-Cl cotransport involves p38 and JNK MAP kinases. Am J Physiol Cell Physiol. 2011;302(3):C505–17.
147. Lam TI, Wise PM, O'Donnell ME. Cerebral microvascular endothelial cell Na/H exchange: evidence for the presence of NHE1 and NHE2 isoforms and regulation by arginine vasopressin. Am J Physiol Cell Physiol. 2009;297(2):C278–89.
148. Hom S, Fleegal MA, Egleton RD, Campos CR, Hawkins BT, Davis TP. Comparative changes in the blood-brain barrier and cerebral infarction of SHR and WKY rats. Am J Physiol Regul Integr Comp Physiol. 2007;292(5):R1881–92.
149. Vemula S, Roder KE, Yang T, Bhat GJ, Thekkumkara TJ, Abbruscato TJ. A functional role for sodium-dependent glucose transport across the blood-brain barrier during oxygen glucose deprivation. J Pharmacol Exp Ther. 2009;328(2):487–95.
150. Kim GW, Lewen A, Copin J, Watson BD, Chan PH. The cytosolic antioxidant, copper/zinc superoxide dismutase, attenuates blood-brain barrier disruption and oxidative cellular injury after photothrombotic cortical ischemia in mice. Neuroscience. 2001;105(4):1007–18.

151. Heo JH, Han SW, Lee SK. Free radicals as triggers of brain edema formation after stroke. Free Radic Biol Med. 2005;39:51–70.
152. Nito C, Kamada H, Endo H, Niizuma K, Myer DJ, Chan PH. Role of the p38 mitogen-activated protein kinase/cytosolic phospholipase A2 signaling pathway in blood-brain barrier disruption after focal cerebral ischemia and reperfusion. J Cereb Blood Flow Metab. 2008;28(10):1686–96.
153. Lochhead JJ, McCaffrey G, Sanchez-Covarrubias L, Finch JD, DeMarco KM, Quigley CE, et al. Tempol modulates changes in xenobiotic permeability and occludin oligomeric assemblies at the blood-brain barrier during inflammatory pain. Am J Physiol Heart Circ Physiol. 2012;302(3):H582–93.
154. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev. 2007;87:315–424.
155. Salvemini D, Doyle TM, Cuzzocrea S. Superoxide, peroxynitrite and oxidative/nitrative stress in inflammation. Biochem Soc Trans. 2006;34(Pt 5):965–70.
156. Wang X, Barone FC, Aiyar NV, Feuerstein GZ. Interleukin-1 receptor and receptor antagonist gene expression after focal stroke in rats. Stroke. 1997;28(1):155–61.
157. Petty MA, Lo EH. Junctional complexes of the blood-brain barrier: permeability changes in neuroinflammation. Prog Neurobiol. 2002;68(5):311–23.
158. Staykova M, Maxwell L, Willenborg D. Kinetics and polarization of the membrane expression of cytokine-induced ICAM-1 on rat brain endothelial cells. J Neuropathol Exp Neurol. 2000;59(2):120–8.
159. Poller B, Drewe J, Krahenbuhl S, Huwyler J, Gutmann H. Regulation of BCRP (ABCG2) and P-glycoprotein (ABCB1) by cytokines in a model of the human blood-brain barrier. Cell Mol Neurobiol. 2010;30(1):63–70.
160. Ahishali B, Kaya M, Kalayci R, Uzun H, Bilgic B, Arican N, et al. Effects of lipopolysaccharide on the blood-brain barrier permeability in prolonged nitric oxide blockade-induced hypertensive rats. Int J Neurosci. 2005;115(2):151–68.
161. Didier N, Romero IA, Creminon C, Wijkhuisen A, Grassi J, Mabondzo A. Secretion of interleukin-1beta by astrocytes mediates endothelin-1 and tumour necrosis factor-alpha effects on human brain microvascular endothelial cell permeability. J Neurochem. 2003;86(1):246–54.
162. Haseloff RF, Blasig IE, Bauer HC, Bauer H. In search of the astrocytic factor(s) modulating blood-brain barrier functions in brain capillary endothelial cells in vitro. Cell Mol Neurobiol. 2005;25(1):25–39.
163. Kaur C, Sivakumar V, Zhang Y, Ling EA. Hypoxia-induced astrocyte reaction and increased vascular permeability in the rat cerebellum. Glia. 2006;54(8):826–39.
164. Vangilder RL, Rosen CL, Barr TL, Huber JD. Targeting the neurovascular unit for treatment of neurological disorders. Pharmacol Ther. 2011;130(3):239–47.
165. Woodman SE, Benveniste EN, Nath A, Berman JW. Human immunodeficiency virus type 1 TAT protein induces adhesion molecule expression in astrocytes. J Neurovirol. 1999;5(6):678–84.
166. Dimitrijevic OB, Stamatovic SM, Keep RF, Andjelkovic AV. Effects of the chemokine CCL2 on blood-brain barrier permeability during ischemia-reperfusion injury. J Cereb Blood Flow Metab. 2006;26(6):797–810.
167. Strecker JK, Minnerup J, Gess B, Ringelstein EB, Schabitz WR, Schilling M. Monocyte chemoattractant protein-1-deficiency impairs the expression of IL-6, IL-1beta and G-CSF after transient focal ischemia in mice. PLoS One. 2011;6(10):e25863.
168. Lee SR, Guo SZ, Scannevin RH, Magliaro BC, Rhodes KJ, Wang X, et al. Induction of matrix metalloproteinase, cytokines and chemokines in rat cortical astrocytes exposed to plasminogen activators. Neurosci Lett. 2007;417(1):1–5.
169. Tuttolomondo A, Di Raimondo D, di Sciacca R, Pinto A, Licata G. Inflammatory cytokines in acute ischemic stroke. Curr Pharm Des. 2008;14(33):3574–89.
170. Doyle KP, Cekanaviciute E, Mamer LE, Buckwalter MS. TGFbeta signaling in the brain increases with aging and signals to astrocytes and innate immune cells in the weeks after stroke. J Neuroinflammation. 2010;7:62.

171. Mander P, Borutaite V, Moncada S, Brown GC. Nitric oxide from inflammatory-activated glia synergizes with hypoxia to induce neuronal death. J Neurosci Res. 2005;79(1–2): 208–15.
172. Yasuda Y, Tateishi N, Shimoda T, Satoh S, Ogitani E, Fujita S. Relationship between S100beta and GFAP expression in astrocytes during infarction and glial scar formation after mild transient ischemia. Brain Res. 2004;1021(1):20–31.
173. Jiang J, Wang W, Sun YJ, Hu M, Li F, Zhu DY. Neuroprotective effect of curcumin on focal cerebral ischemic rats by preventing blood-brain barrier damage. Eur J Pharmacol. 2007;561(1–3):54–62.
174. Dallasta LM, Pisarov LA, Esplen JE, Werley JV, Moses AV, Nelson JA, et al. Blood-brain barrier tight junction disruption in human immunodeficiency virus-1 encephalitis. Am J Pathol. 1999;155(6):1915–27.
175. Yenari MA, Xu L, Tang XN, Qiao Y, Giffard RG. Microglia potentiate damage to blood-brain barrier constituents: improvement by minocycline in vivo and in vitro. Stroke. 2006; 37(4):1087–93.
176. Clausen BH, Lambertsen KL, Babcock AA, Holm TH, Dagnaes-Hansen F, Finsen B. Interleukin-1beta and tumor necrosis factor-alpha are expressed by different subsets of microglia and macrophages after ischemic stroke in mice. J Neuroinflammation. 2008;5:46.
177. Kaushal V, Schlichter LC. Mechanisms of microglia-mediated neurotoxicity in a new model of the stroke penumbra. J Neurosci. 2008;28(9):2221–30.
178. Akundi RS, Candelario-Jalil E, Hess S, Hull M, Lieb K, Gebicke-Haerter PJ, et al. Signal transduction pathways regulating cyclooxygenase-2 in lipopolysaccharide-activated primary rat microglia. Glia. 2005;51(3):199–208.
179. Bhat NR, Zhang P, Lee JC, Hogan EL. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. J Neurosci. 1998;18(5):1633–41.
180. Piao CS, Kim JB, Han PL, Lee JK. Administration of the p38 MAPK inhibitor SB203580 affords brain protection with a wide therapeutic window against focal ischemic insult. J Neurosci Res. 2003;73(4):537–44.
181. Witt KA, Mark KS, Sandoval KE, Davis TP. Reoxygenation stress on blood-brain barrier paracellular permeability and edema in the rat. Microvasc Res. 2008;75(1):91–6.
182. Willis CL, Meske DS, Davis TP. Protein kinase C activation modulates reversible increase in cortical blood-brain barrier permeability and tight junction protein expression during hypoxia and posthypoxic reoxygenation. J Cereb Blood Flow Metab. 2010;30(11):1847–59.
183. Witt KA, Mark KS, Huber J, Davis TP. Hypoxia-inducible factor and nuclear factor kappa-B activation in blood-brain barrier endothelium under hypoxic/reoxygenation stress. J Neurochem. 2005;92(1):203–14.
184. Schreibelt G, Kooij G, Reijerkerk A, van Doorn R, Gringhuis SI, van der PS, et al. Reactive oxygen species alter brain endothelial tight junction dynamics via RhoA, PI3 kinase, and PKB signaling. FASEB J. 2007;21(13):3666–76.
185. Cuzzocrea S, McDonald MC, Mazzon E, Filipe HM, Costantino G, Caputi AP, et al. Beneficial effects of tempol, a membrane-permeable radical scavenger, in a rodent model of splanchnic artery occlusion and reperfusion. Shock. 2000;14(2):150–6.
186. Rak R, Chao DL, Pluta RM, Mitchell JB, Oldfield EH, Watson JC. Neuroprotection by the stable nitroxide Tempol during reperfusion in a rat model of transient focal ischemia. J Neurosurg. 2000;92(4):646–51.
187. Hong H, Lu Y, Ji ZN, Liu GQ. Up-regulation of P-glycoprotein expression by glutathione depletion-induced oxidative stress in rat brain microvessel endothelial cells. J Neurochem. 2008;98:1465–73.
188. Wang X, Campos CR, Peart JC, Smith LK, Boni JL, Cannon RE, et al. Nrf2 upregulates ATP binding cassette transporter expression and activity at the blood-brain barrier and blood-spinal cord barriers. J Neurosci. 2014;34:8585–93.

189. Tournier N, Decleves X, Saubamea B, Schermann JM, Cisternino S. Opioid transport by ATP-binding cassette transporters at the blood-brain barrier: implications for neuropsychopharmacology. Curr Pharm Des. 2011;17:2829–42.
190. Slosky LM, Thompson BJ, Sanchez-Covarrubias L, Zhang Y, Laracuente ML, Vanderah TW, et al. Acetaminophen modulates P-glycoprotein functional expression at the blood-brain barrier by a constitutive androstane receptor-dependent mechanism. Mol Pharmacol. 2013;84:774–86.
191. Sanchez-Covarrubias L, Slosky LM, Thompson BJ, Zhang Y, Laracuente ML, DeMarco KM, et al. P-glycoprotein modulates morphine uptake into the CNS: a role for the non-steroidal anti-inflammatory drug diclofenac. PLoS One. 2014;9:e88516.
192. Potschka H. Modulating P-glycoprotein regulation: future perspectives for pharmacoresistant epilepsies? Epilepsia. 2010;51:1333–47.
193. Kalvass JC, Polli JW, Bourdet DL, Feng B, Huang SM, Liu X, et al. Why clinical modulation of efflux transport at the human blood-brain barrier is unlikely: the ITC evidence-based position. Clin Pharmacol Ther. 2013;94:80–94.
194. Butterfield DA, Barone E, Mancuso C. Cholesterol-independent neuroprotective and neurotoxic activities of statins: perspectives for statin use in Alzheimer disease and other age-related neurodegenerative disorders. Pharmacol Res. 2011;64(3):180–6.
195. Cui L, Zhang X, Yang R, Wang L, Liu L, Li M, et al. Neuroprotection of early and short-time applying atorvastatin in the acute phase of cerebral ischemia: down-regulated 12/15-LOX, p38MAPK and cPLA2 expression, ameliorated BBB permeability. Brain Res. 2010;1325:164–73.
196. Yang D, Knight RA, Han Y, Karki K, Zhang J, Chopp M, et al. Statins protect the blood-brain barrier acutely after experimental intracerebral haemorrhage. J Behav Brain Sci. 2013;3:100–6.
197. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature. 2003;425(6958):577–84.
198. Li W, Busu C, Circu ML, Aw TY. Glutathione in cerebral microvascular endothelial biology and pathobiology: implications for brain homeostasis. Int J Cell Biol. 2012;2012:434971.
199. Plateel M, Dehouck MP, Torpier G, Cecchelli R, Tessier E. Hypoxia increases the susceptibility to oxidant stress and the permeability of the blood-brain barrier endothelial cell monolayer. J Neurochem. 1995;65:2138–45.
200. Muruganandam A, Smith C, Ball R, Herring T, Stanimirovic D. Glutathione homeostasis and leukotriene-induced permeability in human blood-brain barrier endothelial cells subjected to in vitro ischemia. Acta Neurochir Suppl. 2000;76:29–34.
201. Namba K, Takeda Y, Sunami K, Hirakawa M. Temporal profiles of the levels of endogenous antioxidants after four-vessel occlusion in rats. J Neurosurg Anesthesiol. 2001;13:131–7.
202. Hirrlinger J, Dringen R. Multidrug resistance protein 1-mediated export of glutathione and glutathione disulphide from brain astrocytes. Methods Enzymol. 2005;400:395–409.
203. Tadepalle N, Koehler Y, Brandmann M, Meyer N, Dringen R. Arsenite stimulates glutathione export and glycolytic flux in viable primary rat brain astrocytes. Neurochem Int. 2014;76:1–11.
204. Paulusma CC, van Geer MA, Evers R, Heijn M, Ottenhoff R, Borst P, et al. Canalicular multispecific organix transporter/multidrug resistance protein 2 mediates low-affinity transport of reduced glutathione. Biochem J. 1999;338:393–401.
205. Rius M, Hummel-Eisenbeiss J, Hofmann AF, Keppler D. Substrate specificity of human ABCC4 (MRP4)-mediated cotransport of bile acids and reduced glutathione. Am J Physiol Gastrointest Liver Physiol. 2006;290:G640–9.
206. Ballatori N, Krance SM, Marchan R, Hammond CL. Plasma membrane glutathione transporters and their roles in cell physiology and pathophysiology. Mol Aspects Med. 2009;30:13–28.
207. Maher JM, Dieter MZ, Aleksunes LM, Slitt AL, Guo G, Tanaka Y, et al. Oxidative and electrophilic stress induces multidrug resistance-associated protein transporters via the nuclear factor-E2-related factor-2 transcriptional pathway. Hepatology. 2007;46:1597–610.

208. Alfieri A, Srivastava S, Slow RC, Modo M, Fraser PA, Mann GE. Targeting the Nrf2-Keap1 antioxidant defense pathway for neurovascular protection in stroke. J Physiol. 2011;58: 4125–36.
209. Hayashi A, Suzuki H, Itoh K, Yamamoto M, Sugiyama Y. Transcription factor Nrf2 is required for the constitutive and inducible expression of multidrug resistance-associated protein 1 in mouse embryo fibroblasts. Biochem Biophys Res Commun. 2003;310:824–9.
210. Ma Q. Role of Nrf2 in oxidative stress and toxicity. Annu Rev Pharmacol Toxicol. 2013;53:401–26.
211. Aleksunes LM, Slitt AL, Maher JM, Augustine LM, Goedken MJ, Chan JY, et al. Induction of Mrp3 and Mrp4 transporters during acetaminophen hepatotoxicity is dependent on Nrf2. Toxicol Appl Pharmacol. 2008;226:74–83.
212. Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. Pharmacol Rev. 2001;53(2):245–82.
213. McCoy KL, Traynelis SF, Hepler JR. PAR1 and PAR2 couple to overlapping and distinct sets of G proteins and linked signaling pathways to differentially regulate cell physiology. Mol Pharmacol. 2010;77(6):1005–15.
214. Noorbakhsh F, Tsutsui S, Vergnolle N, Boven LA, Shariat N, Vodjgani M, et al. Proteinase-activated receptor 2 modulates neuroinflammation in experimental autoimmune encephalomyelitis and multiple sclerosis. J Exp Med. 2006;203(2):425–35.
215. Pompili E, Fabrizi C, Nori SL, Panetta B, Geloso MC, Corvino V, et al. Protease-activated receptor-1 expression in rat microglia after trimethyltin treatment. J Histochem Cytochem. 2011;59(3):302–11.
216. Zlokovic BV, Griffin JH. Cytoprotective protein C pathways and implications for stroke and neurological disorders. Trends Neurosci. 2011;34(4):198–209.
217. Mosnier LO, Zlokovic BV, Griffin JH. The cytoprotective protein C pathway. Blood. 2007;109(8):3161–72.
218. Guo H, Singh I, Wang Y, Deane R, Barrett T, Fernandez JA, et al. Neuroprotective activities of activated protein C mutant with reduced anticoagulant activity. Eur J Neurosci. 2009; 29(6):1119–30.
219. Fabrizi C, Pompili E, Panetta B, Nori SL, Fumagalli L. Protease-activated receptor-1 regulates cytokine production and induces the suppressor of cytokine signaling-3 in microglia. Int J Mol Med. 2009;24(3):367–71.
220. Thiyagarajan M, Fernandez JA, Lane SM, Griffin JH, Zlokovic BV. Activated protein C promotes neovascularization and neurogenesis in postischemic brain via protease-activated receptor 1. J Neurosci. 2008;28(48):12788–97.
221. Deane R, LaRue B, Sagare AP, Castellino FJ, Zhong Z, Zlokovic BV. Endothelial protein C receptor-assisted transport of activated protein C across the mouse blood-brain barrier. J Cereb Blood Flow Metab. 2009;29(1):25–33.
222. Mendioroz M, Fernandez-Cadenas I, Alvarez-Sabin J, Rosell A, Quiroga D, Cuadrado E, et al. Endogenous activated protein C predicts hemorrhagic transformation and mortality after tissue plasminogen activator treatment in stroke patients. Cerebrovasc Dis. 2009;28(2): 143–50.
223. Wang Y, Zhang Z, Chow N, Davis TP, Griffin JH, Chopp M, et al. An activated protein C analog with reduced anticoagulant activity extends the therapeutic window of tissue plasminogen activator for ischemic stroke in rodents. Stroke. 2012;43(9):2444–9.
224. Lyden P, Levy H, Weymer S, Pryor K, Kramer W, Griffin JH, et al. Phase 1 safety, tolerability and pharmacokinetics of 3K3A-APC in healthy adult volunteers. Curr Pharm Des. 2013;19(42):7479–85.
225. Kelso EB, Ferrell WR, Lockhart JC, Elias-Jones I, Hembrough T, Dunning L, et al. Expression and proinflammatory role of proteinase-activated receptor 2 in rheumatoid synovium: ex vivo studies using a novel proteinase-activated receptor 2 antagonist. Arthritis Rheum. 2007;56(3): 765–71.
226. Fagan SC, Cronic LE, Hess DC. Minocycline development for acute ischemic stroke. Transl Stroke Res. 2011;2(2):202–8.

227. Sollman S, Ishrat T, Fouda AY, Patel A, Pillai B, Fagan SC. Sequential therapy with minocycline and candesartan improves long-term recovery after experimental stroke. Transl Stroke Res. 2015;6(4):309–22.
228. Yan P, Zhu A, Liao F, Xiao Q, Kraft AW, Gonzales E, et al. Minocycline reduces spontaneous haemorrhage in mouse models of cerebral amyloid angiopathy. Stroke. 2015;46(6): 1633–40.
229. Kielian T. Toll-like receptors in central nervous system glial inflammation and homeostasis. J Neurosci Res. 2006;83(5):711–30.
230. Downes CE, Crack PJ. Neural injury following stroke: are Toll-like receptors the link between the immune system and the CNS? Br J Pharmacol. 2010;160(8):1872–88.
231. Olson JK, Miller SD. Microglia initiate central nervous system innate and adaptive immune responses through multiple TLRs. J Immunol. 2004;173(6):3916–24.
232. Jack CS, Arbour N, Manusow J, Montgrain V, Blain M, McCrea E, et al. TLR signaling tailors innate immune responses in human microglia and astrocytes. J Immunol. 2005;175(7): 4320–30.
233. Bowman CC, Rasley A, Tranguch SL, Marriott I. Cultured astrocytes express Toll-like receptors for bacterial products. Glia. 2003;43(3):281–91.
234. Farina C, Krumbholz M, Giese T, Hartmann G, Aloisi F, Meinl E. Preferential expression and function of Toll-like receptor 3 in human astrocytes. J Neuroimmunol. 2005;159(1–2):12–9.
235. Doeppner TR, Kaltwasser B, Fengyan J, Hermann DM, Bahr M. TAT-Hsp70 induces neuroprotection against stroke via anti-inflammatory actions providing appropriate cellular microenvironment for transplantation of neural precursor cells. J Cereb Blood Flow Metab. 2013;33(11):1778–88.

Barrier Mechanisms in Neonatal Stroke

Zinaida S. Vexler

1 Introduction

The concept that the fetal and newborn blood–brain barrier (BBB) is "immature," implying that the BBB is not formed, leaky or even absent has dominated the field for a long time. However, such a simplistic idea has been challenged in multiple species, demonstrating in naïve brain that many functional barrier elements are already in place in fetal brain and, together with placenta, are effectively preserving brain metabolism and limiting brain edema that is associated with entrance of peripheral components. It has also become apparent that BBB integrity does not linearly change during postnatal brain maturation and that infection, inflammation, brain trauma, or stroke during postnatal development exert age-specific "susceptibility signatures" of BBB disturbances, processes that we will discuss in this chapter.

2 Development of the BBB and Other Brain Barriers

There are multiple brain barriers, including the BBB, blood–CSF barrier across choroid plexus (BCSFB) and the pia-arachnoid barrier [1]. There is also a barrier only present in the embryo, the CSF-ventricular zone interface lining the ventricular system. Cumulatively, these barriers limit exchange of solutes and various molecules between compartments as well as establish efflux/influx mechanisms that control ionic, nutrient, glucose, and other gradients. In the adult, the BBB exists at all levels

Z.S. Vexler, Ph.D. (✉)
Department of Neurology, University of California, San Francisco,
675 Nelson Rising Lane, San Francisco, CA 94158-0663, USA
e-mail: Zena.Vexler@ucsf.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_4

of the vasculature within the CNS, including the penetrating arteries and arterioles, the capillary bed, the postcapillary venules and the draining venules and veins [2, 3].

The development of the BBB is believed to be a multi-step process. Both the development and maintenance of the BBB are governed by intracellular endothelial mechanisms in conjunction with multiple extracellular mechanisms that link endothelial cells to the extracellular matrix (ECM), thereby supporting their proper positioning and functionality. Astrocytes, pericytes, and ECM components provide both structural and functional support to the BBB. BBB development starts mid-gestation and temporally occurs after establishment of a local microglial pool in the brain and before pericytes and astrocytes take their respective positions surrounding the vessels [4–7]. Thus, endothelial sprouts are guided by microglia/macrophages in fetal brain, largely via VEGF-dependent mechanisms [8]. Lack of or reduced number of PU.1 or SDF-1 microglia/macrophage leads to distorted vasculature and embryonic lethality [8]. Mice deficient in VEGF or VEGF receptor 2 (Flk-1−/−) fail vessel formation, which leads to embryonic lethality [9]. Lack of a single VEGF allele also leads to embryonic lethality despite partial blood vessel development [10]. Several VEGF-independent mechanisms that regulate embryonic angiogenesis and BBB formation were identified. Integrin/TGFβ signaling, for example, suppresses vascular branching/sprouting and prevents germinal matrix hemorrhage [11]. Excessive vascular sprouting, in turn, underlies cerebral hemorrhage in mice lacking αVβ8-TGFβ signaling in the brain [11].

Wnt-β-catenin signaling has been shown to be important in brain angiogenesis and BBB formation, but not in barrier formation in peripheral vessels [12]. The canonical Wnt pathway was demonstrated to play a key role in BBB formation via induction of the BBB genes, such as the nutrient transporter Glut-1 [13], while β-catenin was shown to regulate angiogenesis in the developing brain, in part by inducing expression of the death receptors Dr6 and Troy. Endothelial β-catenin was also shown to play a key role in embryonic and postnatal BBB maturation by regulating the formation of tight junctions (TJs) [14].

Pericytes contribute to BBB development and function in a number of ways, primarily by regulating capillary diameter and CBF and controlling BBB integrity [15]. They also contribute to vessel stability, possibly by affecting both TJs and transcytosis routes [5]. However, the existence and the role of multiple phenotypes are still poorly understood. Pericyte coverage gradually increases starting late gestation and continues through postnatal ages [4, 5]. Astrocytes are another cell population central to the maturing BBB in several ways [15]. Perivascular astrocytic end-feet at the abluminal side of brain vessels are polarized structures that are enriched with water channel aquaporin-4 (AQP-4) and several ion pumps. Astrocytes modulate TJs and are also an essential part of antioxidative mechanisms in the brain, recycling GSSG, which, as we discuss later, is of particular importance after stroke and other diseases. They also provide nutrition for neurons and neurotransmitter clearance and recycling.

Cells of the BBB/neurovascular unit do not exist in isolation. The ECM plays a key role both in the development and maintenance of the BBB under normal and disease conditions. Mutant ECM proteins, such as collagen-IVα1 isoform and, to a

lesser extent, α2 isoform adversely affect endothelial communications with the matrix, leading to mortality or hemorrhagic transformation [16]. During embryonic angiogenesis, TGFβ signaling suppresses vascular branching/sprouting and prevents hemorrhages [11]. Loss of TGFbR2/SMAD3 enhances vascular sprouting, branching, and hemorrhages during embryonic brain development but these effects are independent of BBB dysfunction [11]. Multiple lines of evidence show an important role for endothelial TGFβ/TGFbR2 signaling for vascular integrity during embryonic brain development [17]. Endothelial specific depletion of Alk5 leads to a similar cerebral hemorrhagic phenotype and embryonic lethality [17, 18], consistent with a requirement for both TGFbR1 and TGFbR2 proteins as a heteromeric receptor complex in endothelial cells. Mutations in other members of the TGFβ family, endoglin, (ENG) and activin receptor-like kinase 1(ALK1), were demonstrated as major contributors to intracerebral hemorrhages of brain arteriovenous malformations in children [19].

Although individual neurovascular unit components form at different paces during gestation, likely contributing to age-dependent susceptibility to infection, inflammatory stress and stroke, the fetal brain has surprisingly effective influx/efflux mechanisms. Efflux mechanisms are active across brain barriers in the developing brain. Multiple transcription factors involved in metabolic- or cell stress-induced detoxifying enzymes and transporters are expressed throughout embryonic brain development. For example, several proteins of the ATP-binding cassette (ABC) family, which reduce the entry of compounds from blood into the brain by active efflux, are already expressed in cerebral blood vessels and in choroid plexus in the fetal and neonatal rat brain [20]. Among ABC-transporters, Pgp/ABCB1 expression is lower in the fetus, whereas MRP1/ABCC1, MRP4/ABCC4, and BCRP/ABCG2 are expressed at comparable levels in fetal and adult brains. Data on amino acid transporters in endothelial cells in the developing brain are sparse but available data indicate that the function of several amino acid and inward transporters is higher than in the adult. For example, transporter mannose 6-phosphate receptor is expressed in developing rodent and rabbit brain but is progressively lost with age [21].

The BCSFB plays a prominent role in fetal and newborn brain. Ion pumps are active in the BCSFB. Essential nutrients and other molecules important for growth and differentiation of the brain enter the brain via uptake from the CSF. A recent transcriptome approach allowed insights into transport mechanisms at the developing mouse blood–CSF interface [22].

3 Blood–Brain Barrier and Adult Stroke

There is ample evidence of BBB disruption after acute stroke. Several recent reviews comprehensively discussed various mechanistic aspects of neurovascular state in adult stroke, including contribution of the ECM, its communication with endothelial cells, interaction between individual cell types, including endothelial cells,

astrocytes and pericytes, as well as various individual intracellular and intercellular mechanisms of adult stroke [23–25]; these aspects will not be discussed here. It is, however, important to mention that several long-term concepts in the stroke field are currently being revisited and reconsidered. Three most notable examples of revisited questions are the relative role of the paracellular compared to the transcellular route as paths for leukocyte trafficking [26], the ability of neutrophils to reach injured parenchyma and signal from within, rather than signal from the perivascular space [27], and beneficial, rather than purely toxic role of inflammation and microglial involvement in particular [25].

4 Maturation-Dependent Susceptibility of the BBB to Inflammation

Neuroinflammation is a characteristic feature of stroke progression in the adult and is a major contributor to brain injury [25]. Parenchymal, perivascular, and peripheral circulating cells independently and in concert contribute to stroke-induced production of inflammatory mediators and neuroinflammation [25] and activation of endothelial cells [28, 29]. Perivascular macrophages, microglial cells and mast cells, which are strategically positioned around brain vessels, further contribute to BBB disruption by induction and release of signaling molecules and proteases that promote vascular permeability.

A comparative study of the effects of recombinant interleukin-1 beta (IL-1β) injected into the brain parenchyma of adult, juvenile and newborn rats showed that while in the adult intense meningitis and disruption of the BCSFB, but not leukocyte recruitment, occur within 4 h, in the juvenile rats, a 500-fold lower IL-1β dose causes a large BBB leakage and neutrophil recruitment into the tissue around the injection site within the same time frame [30]. In the newborn rat (2-h-old rat), a similar low IL-1β dose gives rise to an increase in permeability in the meninges, but no increase in BBB permeability. The IL-1β-induced increases in vessel permeability in the meninges, parenchyma, and choroid plexus are neutrophil-dependent [30]. These findings demonstrate clear differences in the age-specific response of the BBB and barrier-type specific responsiveness to IL-1β. Another study utilizing prolonged systemic inflammation in early development in the rat showed induction of long-term changes in BBB permeability that ultimately led to white matter lesions [31]. LPS given at postnatal day 0 (P0)-P8 induced BBB permeability to small (sucrose and inulin) and large (protein) molecules during and immediately after the inflammatory response. Permeability to protein was increased only transiently whereas increased permeability to ^{14}C-sucrose and ^{14}C-inulin was more persistant, demonstrating size selective increases in BBB in the early postnatal period in response to prolonged systemic inflammation [31].

5 Models of Neonatal Focal Stroke and Hypoxia–Ischemia

While models of stroke in adult rodents have existed for several decades, models of perinatal focal arterial stroke have been established relatively recently. These models include permanent MCA ligation [32] and suture tMCAO in P7–P10 rats [33, 34] and P9 mice [35]. Varying the duration of tMCAO has allowed for induction of injuries of different severity [35] and MRI-based confirmation of recirculation following suture retraction in the tMCAO model [36] has allowed for studies of reperfusion, which frequently occurs in arterial stroke in term infants. The vast majority of experimental data related to ischemia-induced brain injury in neonates this far have been obtained using a hypoxia–ischemia (HI) model in P7–P9 rats and mice, a group of models that more closely mimic hypoxic-ischemic encephalopathy in term human babies than focal arterial stroke [37–40]. We and others recently reviewed the most important pathophysiological findings in the neonatal HI models [39, 41–43]. Thus, we will limit our discussion to available data on stroke and HI-induced changes in BBB permeability in the neonate [44, 45] and effects of neurovascular changes for injury and repair [46–48].

6 BBB Integrity After Neonatal Focal Stroke and Hypoxia–Ischemia

The data on BBB function after ischemia-related brain injury in the neonate are rather scant. Considering that endothelial TJs are already present during early embryonic development [49], specific BBB transporters are present in the brain endothelium during mid-gestation, with no fenestrations are observed at birth [50], that astrocytes and pericytes cover brain vessels in postnatal brain [5], at least to some extent, we examined whether neonatal stroke disrupts functional integrity of the BBB. To test if compared to adult stroke, the assumed more extensive magnitude of BBB disruption after stroke occurs in neonates, we determined BBB leakage in adult and P7 rats subjected to a 3-h tMCAO. We administered Evans Blue or fluorescent intravascular trace 3, 70 kDa dextran or TRITC-albumin at 24 after reperfusion and observed significant extravasation of Evans Blue, 70kD and TRITC-albumin in injured regions of adult rats. Extravasation of these molecules was significantly lower following acute tMCAO in P7 rats, showing a strikingly better-preserved BBB integrity in injured neonatal brains [44]. We also used Gd-DTPA-enhanced T1W to further test BBB permeability in neonates and showed that contrast enhancement in injured regions was negligible at 24 h, within 10 % [44]. Fig. 1a demonstrates increased leakage of 70 kDa dextran in injured adult brain and only minor leakage in injured neonatal rats. In contrast to tMCAO, permanent MCAO in P7 rats resulted in rapid BBB disruption and leukocyte extravasation [51], suggesting that

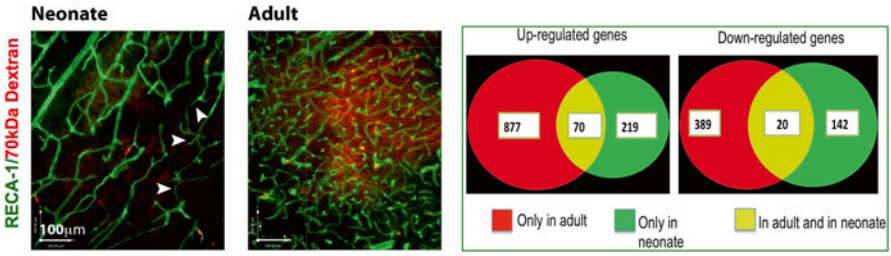

Fig. 1 Acute focal arterial stroke differentially affects BBB integrity in the adult and neonate. (**a**) Representative examples of the spatial distribution of intravenously administered TRITC-conjugated 70 kDa dextran in injured neonatal (*left*) and adult (*right*) brain regions 24 h after a 3-h tMCAO. (**b**) Differential effect of acute focal stroke on gene expression in endothelial cells of adult and neonatal rats. The endothelial transcriptome data were obtained in endothelial cells purified by sequential negative and positive selection from injured and contralateral tissue of adults and neonates 24 h after reperfusion. Shown are genes with >2-fold change in expression compared to that in contralateral hemisphere

persistent lack of cerebral microcirculation contributes to BBB collapse. Two studies that utilized tMCAO model in P10 rats observed increased BBB permeability during a sub-chronic injury phase, 72 h following tMCAO [52, 53]. The affected region was larger in spontaneously hypertensive pups [52] than in normotensive pups of the same age [53]. A recent study studied changes to the BBB in conjunction with CBF in a neonatal mouse HI model [45]. HI increased BBB permeability to small and large molecules, peaking at 6 h after the insult followed by normalization by 24 h. The opening of the BBB was associated with changes to BBB protein expression. Brain pathology was closely related to reductions in CBF during the hypoxia as well as the areas with compromised BBB [45]. Taken together, these data demonstrate a limited extent of BBB opening after ischemia-related injury in neonatal brain.

While the phenomenon of a better-preserved BBB integrity in neonatal stroke has been established, the underlying mechanisms are poorly understood. Comparative endothelial transcriptome data obtained in adult and neonatal rats subjected to tMCAO provided some mechanistic insight of age differences in BBB susceptibility to stroke. It appeared that, strikingly, the patterns of up- and down-regulated endothelial genes are largely non-overlapping between the two ages (Fig. 1b) [44]. Transcript levels of several adhesion molecules and ECM components were differentially affected by injury in immature and adult brain, including E-selectin and P-selectin. Gene expression of Mmp-9 was significantly upregulated in injured adults and, while high transcript levels of collagen type IV α1 (Col4a1) and Col4a2 remained unaltered in neonates, a significant increase of these two genes was evident in injured adult rats. Interestingly, transcripts of angiogenic regulators Vegfr-2 and Angpt2 were increased after stroke in adults but not in neonates [44]. Comparisons of protein expression of occludin, claudin-5, and ZO-1 between

adult and neonatal rats after tMCAO showed better-preserved expression in neonates than in adults [44]. Endothelial-ECM interaction via β1 integrins regulates the expression of claudin-5 and BBB tightness whereas other ECM proteins, like galectin-3, mediate integrin-induced stabilization of focal adhesions and activate cytokine receptors to enhance actions of growth factors [54]. Laminin degradation occurs after focal stroke in adults and causes detachment of astrocytic end-feet, disrupts BBB, and induces ICH [55], while in neonates, expression of this ECM protein is not reduced acutely [44]. The role of other ECM proteins in injured neonates is less studied but the opposite effects of galectin-3 in adult stroke and HI have been demonstrated [56, 57]. Together, these data suggest that intrinsic developmental differences in basement membrane and ECM formation may contribute to a better-preserved BBB integrity after acute neonatal arterial stroke.

Astrocytes and pericytes may contribute differently to neonatal and adult stroke. In adults, astrocyte swelling, mediated by water channels (aquaporin-4, AQP-4) present in astrocyte end-feet, is one of the mechanisms involved in the generation of cytotoxic edema during the early phase after stroke [58]. Retraction of astrocyte end-feet from the parenchymal basal lamina of vessels and cellular redistribution and/or degradation of AQP-4 at later stroke stages are involved in BBB leakage and formation of vasogenic edema [59]. While coverage of vessels with astrocytic end-feet begins before birth, it continues to increase during the first postnatal week [5]. However, these particular mechanisms have not yet been specifically characterized after neonatal stroke.

7 Leukocyte and BBB Integrity After Neonatal Stroke

Interactions between activated endothelial cells and peripheral leukocytes contribute to BBB disruption after stroke. Compared to the adult, neutrophil infiltration in neonates is negligible after tMCAO [44]. Following HI, neutrophil infiltration was shown limited [60] or brief [61]. Neuropenia was shown beneficial when induced before, not after, HI [60]. The exact mechanisms that restrict neutrophil infiltration in the injured neonatal brain are not well understood. The different or uncoordinated patterns of expression of adhesion molecules and MMPs between neonatal and adult rodents may be of critical importance. Particulars of chemokine gradients between the brain and the blood may affect neutrophil extravasation in the neonate [44]. For example, cytokine-induced neutrophil chemoattractant (CINC-1) is a major chemoattractant for neutrophils. Neutralization of peripheral CINC-1 following tMCAO in the adult halted neutrophil transmigration, reduced brain edema and protected [62] but CINC-1 neutralization in neonatal rats subjected to tMCAO promoted neutrophil infiltration. Alterations in the blood–brain gradient of CINC-1 not only led to increased presence of neutrophils in the brain parenchyma but also increased BBB permeability and injury volume [44], suggesting that neutrophils mediate BBB damage in association with transmigration.

Compared to adult stroke, infiltration of circulating monocytes across the BBB was also low during the acute phase after stroke in the neonate [63]. The exact mechanisms of low monocyte infiltration in the ischemic neonatal brain are not completely understood, and it remains unclear whether the higher resistance of the neonatal BBB to stroke is a cause or a consequence of reduced transmigration. Monocytes have been recently shown to have a dual role, elicit both inflammatory effects and maintain BBB integrity following cerebral ischemia [64]. T and B cell infiltration may be less profound [61] or transient [51] in injured neonates than in adults [65, 66] but there have been no studies that examined effects for BBB integrity.

8 BBB Integrity, Angiogenesis and Brain Repair After Neonatal Stroke

Physiological angiogenesis continues during the first two postnatal weeks in the rat brain [44]. Endothelial cell proliferation and endothelial tip cells are abundantly present [67, 68]. Interestingly, following stroke in P7 rats, angiogenesis is essentially arrested in injured brain regions up to 14 days after injury, and only subtle angiogenic response is detected in the ischemic boundaries in the cortex [69]. Figure 2 summarizes these findings. Considering that in adults endothelial cell proliferation and vascular outgrowth have been reported as soon as 24 h after stroke [70–72],

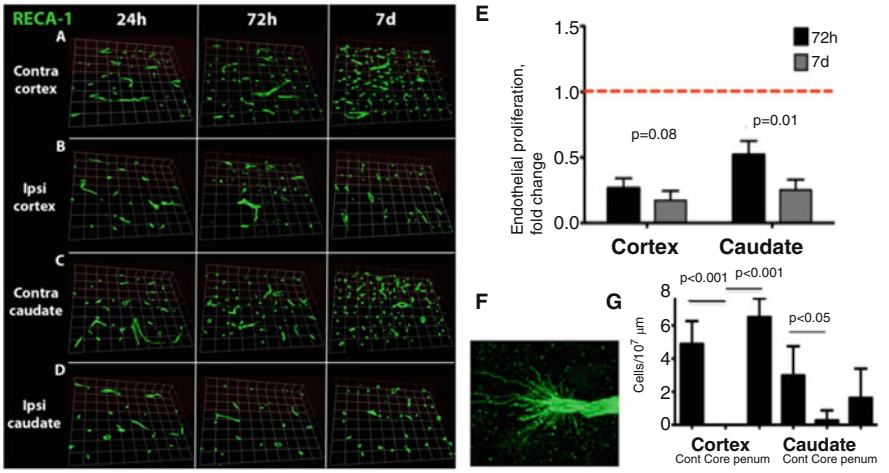

Fig. 2 Reduced vascular density and endothelial proliferation in sub-chronic injury phase after stroke in neonatal rats. (**a-d**) Examples of RECA-1+ vessel distribution in the ischemic core in the cortex (**a**, **b**) and caudate (**c**, **d**) at 24, 72 h, and 7 days after reperfusion. (**e**) Quantification of proliferating endothelial cells (BrdU$^+$/RECA-1$^+$) in the ischemic cortex and caudate. (**f**) Examples of PECAM-1$^+$ endothelial tip cells (defined by extended filopodia). (**g**) Quantification of the number of PECAM-1$^+$ endothelial tip cells in the contralateral and ischemic cortex and caudate

the response of neonatal brain to stroke differs to that in the adult in this aspect as well. Gain- and loss-of-function stroke studies in the adult have demonstrated that neuroblast migration occurs in association with remodeling of blood vessels [73], via a link between angiogenesis and neurogenesis within the "neurovascular niche," and that blockage of angiogenesis abolishes neurogenesis after adult stroke [73]. Brain vessels with active endothelial proliferation in the ischemic boundaries of the injured regions 14 days after neonatal stroke showed abnormal expression of the endothelial barrier antigen (EBA) [69], a protein necessary for proper BBB function in adolescent and adult rats [74–76] and which expression is maturation-dependent [77, 78]. It is tempting to speculate that the relatively preserved BBB after neonatal stroke may negatively impact angiogenesis and account for a delay in angiogenesis and ultimate endogenous neurogenesis, but the relationships between the processes are still poorly understood.

Consistent with the findings in injured P7 rats, the extent of angiogenesis was also limited 1 and 2 weeks after tMCAO in P10 [46, 48]. Angiogenesis was low despite rapid induction of first neuronal and, then, of astrocytic VEGF [34]. Angiogenesis and neurogenesis were enhanced by delayed administration of rhVEGF and disrupted by VEGFR2 inhibition, demonstrating the role of VEGF signaling for the maintenance of angiogenesis and repair in the injured ischemic core [46, 48].

9 Conclusions and Future Directions

Successful development of therapeutics for neonatal stroke depends on accurate understanding of the neurovascular interface. Recent studies have improved our understanding of the events at the BBB after neonatal ischemic injury by revealing that the developmental step of the BBB at the time of ischemic insult contributes to the differing patterns of brain damage between neonates and adults and that careful consideration should be given about whether the BBB is in fact disrupted, allowing therapies to reach an injured neonatal brain. However, it is important to recognize that there are too many unknowns. We know little about relationships between BBB integrity and white matter maturation after stroke. No studies systematically examined whether these processes are sex-dependent. There have been no studies looking in depth at the effects of hypothermia on neurovascular integrity in injured neonatal brain. Future studies should also shed light on relationships between neurovascular integrity and interaction with neuroprogenitors, endogenous or engrafted, and migration and differentiation of neural progenitors during stroke-induced neurogenesis. Although BCSFB is critically important for functioning of a neonatal brain, there have been no studies on addressing functionality of this barrier in injured neonatal brain.

Sources of Funding NINDS_NS80015, NINDS_NS44025, NINDS_NS76726, The Leducq Foundation DSRR_P34404.

References

1. Engelhardt B, Sorokin L. The blood-brain and the blood-cerebrospinal fluid barriers: function and dysfunction. Semin Immunopathol. 2009;31(4):497–511.
2. Obermeier B, Daneman R, Ransohoff RM. Development, maintenance and disruption of the blood-brain barrier. Nat Med. 2013;19(12):1584–96.
3. Engelhardt B, Liebner S. Novel insights into the development and maintenance of the blood-brain barrier. Cell Tissue Res. 2014;355(3):687–99.
4. Armulik A, et al. Pericytes regulate the blood-brain barrier. Nature. 2010;468(7323):557–61.
5. Daneman R, et al. Pericytes are required for blood-brain barrier integrity during embryogenesis. Nature. 2010;468(7323):562–6.
6. Cahoy JD, et al. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J Neurosci. 2008;28(1):264–78.
7. Zhang Y, Barres BA. Astrocyte heterogeneity: an underappreciated topic in neurobiology. Curr Opin Neurobiol. 2010;20(5):588–94.
8. Fantin A, et al. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. Blood. 2010;116(5):829–40.
9. Shalaby F, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature. 1995;376(6535):62–6.
10. Carmeliet P, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 1996;380(6573):435–9.
11. Arnold TD, et al. Excessive vascular sprouting underlies cerebral hemorrhage in mice lacking alphaVbeta8-TGFbeta signaling in the brain. Development. 2014;141(23):4489–99.
12. Daneman R, et al. Wnt/beta-catenin signaling is required for CNS, but not non-CNS, angiogenesis. Proc Natl Acad Sci U S A. 2009;106(2):641–6.
13. Stenman JM, et al. Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature. Science. 2008;322(5905):1247–50.
14. Liebner S, et al. Wnt/beta-catenin signaling controls development of the blood-brain barrier. J Cell Biol. 2008;183(3):409–17.
15. Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 2008;57(2):178–201.
16. Gould DB, et al. Mutations in Col4a1 cause perinatal cerebral hemorrhage and porencephaly. Science. 2005;308(5725):1167–71.
17. Nguyen HL, et al. TGF-beta signaling in endothelial cells, but not neuroepithelial cells, is essential for cerebral vascular development. Lab Invest. 2011;91(11):1554–63.
18. Sridurongrit S, et al. Signaling via the Tgf-beta type I receptor Alk5 in heart development. Dev Biol. 2008;322(1):208–18.
19. Park SO, et al. Real-time imaging of de novo arteriovenous malformation in a mouse model of hereditary hemorrhagic telangiectasia. J Clin Invest. 2009;119(11):3487–96.
20. Ek CJ, et al. Efflux mechanisms at the developing brain barriers: ABC-transporters in the fetal and postnatal rat. Toxicol Lett. 2010;197(1):51–9.
21. Urayama A, et al. Mannose 6-phosphate receptor-mediated transport of sulfamidase across the blood-brain barrier in the newborn mouse. Mol Ther. 2008;16(7):1261–6.
22. Dziegielewska KM, et al. Fetuin in the developing neocortex of the rat: distribution and origin. J Comp Neurol. 2000;423(3):373–88.
23. Vates GE, et al. Pericyte constriction after stroke: the jury is still out. Nat Med. 2010;16(9):959; author reply 960.
24. Denes A, et al. Inflammation and brain injury: acute cerebral ischaemia, peripheral and central inflammation. Brain Behav Immun. 2010;24(5):708–23.
25. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. Nat Med. 2011;17(7):796–808.
26. Knowland D, et al. Stepwise recruitment of transcellular and paracellular pathways underlies blood-brain barrier breakdown in stroke. Neuron. 2014;82(3):603–17.

27. Enzmann G, et al. The neurovascular unit as a selective barrier to polymorphonuclear granulocyte (PMN) infiltration into the brain after ischemic injury. Acta Neuropathol. 2013;125(3): 395–412.
28. Osborn L, et al. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell. 1989;59(6):1203–11.
29. Stanimirovic DB, et al. Increase in surface expression of ICAM-1, VCAM-1 and E-selectin in human cerebromicrovascular endothelial cells subjected to ischemia-like insults. Acta Neurochir Suppl. 1997;70:12–6.
30. Anthony DC, et al. Age-related effects of interleukin-1 beta on polymorphonuclear neutrophil-dependent increases in blood-brain barrier permeability in rats. Brain. 1997;120(Pt 3):435–44.
31. Stolp HB, et al. Long-term changes in blood-brain barrier permeability and white matter following prolonged systemic inflammation in early development in the rat. Eur J Neurosci. 2005;22(11):2805–16.
32. Renolleau S, et al. A model of transient unilateral focal ischemia with reperfusion in the P7 neonatal rat: morphological changes indicative of apoptosis. Stroke. 1998;29(7):1454–60; discussion 1461.
33. Derugin N, Ferriero DM, Vexler ZS. Neonatal reversible focal cerebral ischemia: a new model. Neurosci Res. 1998;32(4):349–53.
34. Mu D, et al. Regulation of hypoxia-inducible factor 1alpha and induction of vascular endothelial growth factor in a rat neonatal stroke model. Neurobiol Dis. 2003;14(3):524–34.
35. Woo MS, et al. Genetic deletion of CD36 enhances injury after acute neonatal stroke. Ann Neurol. 2012;72:961–70.
36. Derugin N, et al. Evolution of brain injury after transient middle cerebral artery occlusion in neonatal rat. Stroke. 2000;31:1752–61.
37. Rice 3rd JE, Vannucci RC, Brierley JB. The influence of immaturity on hypoxic-ischemic brain damage in the rat. Ann Neurol. 1981;9(2):131–41.
38. Sheldon RA, et al. Manipulation of antioxidant pathways in neonatal murine brain. Pediatr Res. 2004;56(4):656–62.
39. Northington FJ, Chavez-Valdez R, Martin LJ. Neuronal cell death in neonatal hypoxia-ischemia. Ann Neurol. 2011;69(5):743–58.
40. van Velthoven CT, et al. Stem cells for neonatal stroke- the future is here. Front Cell Neurosci. 2014;8:207.
41. Fernandez-Lopez D, et al. Mechanisms of perinatal arterial ischemic stroke. J Cereb Blood Flow Metab. 2014;34(6):921–32.
42. Mallard C, Vexler ZS. Modeling ischemia in the immature brain: how translational are animal models? Stroke. 2015;46(10):3006–11.
43. Hagberg H, et al. The role of inflammation in perinatal brain injury. Nat Rev Neurol. 2015;11(4):192–208.
44. Fernandez-Lopez D, et al. Blood-brain barrier permeability is increased after acute adult stroke but not neonatal stroke in the rat. J Neurosci. 2012;32(28):9588–600.
45. Ek CJ, et al. Brain barrier properties and cerebral blood flow in neonatal mice exposed to cerebral hypoxia-ischemia. J Cereb Blood Flow Metab. 2015;35(5):818–27.
46. Shimotake J, et al. Vascular endothelial growth factor receptor-2 inhibition promotes cell death and limits endothelial cell proliferation in a neonatal rodent model of stroke. Stroke. 2010;41(2):343–9.
47. Dzietko M, et al. VEGF treatment in a rodent model of neonatal stroke. In: PAS meeting. Boston; 2012.
48. Dzietko M, et al. Delayed VEGF treatment enhances angiogenesis and recovery after neonatal focal rodent stroke. Transl Stroke Res. 2013;4(2):189–200.
49. Kniesel U, Risau W, Wolburg H. Development of blood-brain barrier tight junctions in the rat cortex. Brain Res Dev Brain Res. 1996;96(1–2):229–40.
50. Engelhardt B. Development of the blood-brain barrier. Cell Tissue Res. 2003;314(1):119–29.
51. Benjelloun N, et al. Inflammatory responses in the cerebral cortex after ischemia in the P7 neonatal Rat. Stroke. 1999;30(9):1916–23; discussion 1923–4.

52. Wang R, et al. Albumin reduces blood-brain barrier permeability but does not alter infarct size in a rat model of neonatal stroke. Pediatr Res. 2007;62(3):261–6.
53. Dzietko M, et al. MRI as a translational tool for the study of neonatal stroke. J Child Neurol. 2011;26(9):1145–53.
54. Goetz JG, et al. Concerted regulation of focal adhesion dynamics by galectin-3 and tyrosine-phosphorylated caveolin-1. J Cell Biol. 2008;180(6):1261–75.
55. Fukuda S, et al. Focal cerebral ischemia induces active proteases that degrade microvascular matrix. Stroke. 2004;35(4):998–1004.
56. Doverhag C, et al. Galectin-3 contributes to neonatal hypoxic-ischemic brain injury. Neurobiol Dis. 2010;38(1):36–46.
57. Lalancette-Hebert M, et al. Galectin-3 is required for resident microglia activation and proliferation in response to ischemic injury. J Neurosci. 2012;32(30):10383–95.
58. Zador Z, et al. Role of aquaporin-4 in cerebral edema and stroke. Handb Exp Pharmacol. 2009;190:159–70.
59. Papadopoulos MC, et al. Aquaporin-4 facilitates reabsorption of excess fluid in vasogenic brain edema. FASEB J. 2004;18(11):1291–3.
60. Hudome S, et al. The role of neutrophils in the production of hypoxic-ischemic brain injury in the neonatal rat. Pediatr Res. 1997;41(5):607–16.
61. Bona E, et al. Chemokine and inflammatory cell response to hypoxia-ischemia in immature rats. Pediatr Res. 1999;45(4 Pt 1):500–9.
62. Yamasaki Y, et al. New therapeutic possibility of blocking cytokine-induced neutrophil chemoattractant on transient ischemic brain damage in rats. Brain Res. 1997;759(1):103–11.
63. Denker S, et al. Macrophages are comprised of resident brain microglia not infiltrating peripheral monocytes acutely after neonatal stroke. J Neurochem. 2007;100(4):893–904.
64. Gliem M, et al. Macrophages prevent hemorrhagic infarct transformation in murine stroke models. Ann Neurol. 2012;71(6):743–52.
65. Chu HX, et al. Immune cell infiltration in malignant middle cerebral artery infarction: comparison with transient cerebral ischemia. J Cereb Blood Flow Metab. 2014;34(3):450–9.
66. Catania A, Lipton JM. Peptide modulation of fever and inflammation within the brain. Ann N Y Acad Sci. 1998;856:62–8.
67. Ogunshola OO, et al. Neuronal VEGF expression correlates with angiogenesis in postnatal developing rat brain. Brain Res Dev Brain Res. 2000;119(1):139–53.
68. Iwai M, et al. Erythropoietin promotes neuronal replacement through revascularization and neurogenesis after neonatal hypoxia/ischemia in rats. Stroke. 2007;38(10):2795–803.
69. Fernandez-Lopez D, et al. Acute and chronic vascular responses to experimental focal arterial stroke in the neonate rat. Transl Stroke Res. 2013;4(2):179–88.
70. Beck H, Plate KH. Angiogenesis after cerebral ischemia. Acta Neuropathol. 2009;117(5):481–96.
71. Hayashi T, et al. Temporal profile of angiogenesis and expression of related genes in the brain after ischemia. J Cereb Blood Flow Metab. 2003;23(2):166–80.
72. Marti HJ, et al. Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. Am J Pathol. 2000;156(3):965–76.
73. Ohab JJ, et al. A neurovascular niche for neurogenesis after stroke. J Neurosci. 2006;26(50):13007–16.
74. Ghabriel MN, et al. Immunological targeting of the endothelial barrier antigen (EBA) in vivo leads to opening of the blood-brain barrier. Brain Res. 2000;878(1–2):127–35.
75. Lu H, et al. Temporary disruption of the rat blood-brain barrier with a monoclonal antibody: a novel method for dynamic manganese-enhanced MRI. Neuroimage. 2010;50(1):7–14.
76. Saubamea B, et al. Heterogeneity in the rat brain vasculature revealed by quantitative confocal analysis of endothelial barrier antigen and P-glycoprotein expression. J Cereb Blood Flow Metab. 2012;32(1):81–92.
77. Rosenstein JM, et al. Immunocytochemical expression of the endothelial barrier antigen (EBA) during brain angiogenesis. Brain Res Dev Brain Res. 1992;66(1):47–54.
78. Sternberger NH, Sternberger LA. Blood-brain barrier protein recognized by monoclonal antibody. Proc Natl Acad Sci U S A. 1987;84(22):8169–73.

Angiogenesis: A Realistic Therapy for Ischemic Stroke

Ke-Jie Yin and Xinxin Yang

1 Introduction

Stroke is the fourth leading causes of death and the leading cause of adult disability in the Western society. Currently, thrombolytic therapy within a 3–4.5 h of narrow time window is the only acute therapeutic intervention for ischemic stroke, and development of effective therapies is urgently required [1–3]. During the past two and half decades, neuroprotection has been a major focus of ischemic stroke therapy research, yet it has also been most perplexing to investigators. All drugs that were neuroprotective in animal studies failed to show significant effectiveness in stroke patients, implying that solely focusing on neuroprotection is not sufficient and other methods for treating ischemic stroke should be explored. Thus, greater attention has been paid to the local environment of the surviving neuron, such as the cerebral microvasculature and other non-neuronal brain elements [4–8].

Angiogenesis is a normal physiological process in tissue growth and development that may also occur as a natural defense response against neurological diseases, such as stroke. Extensive animal studies have shown that post-ischemic angiogenesis plays an important role in the recovery of blood flow in affected brain tissue [9–14]. It is well known that angiogenic vessels in the ischemic boundary zone (IBZ) may contribute to recovery of tissue-at-risk by restoring metabolism in surviving neurons as well as provide the essential neurotrophic support to newly generated neurons. Robust increase of microvessel density has been observed in the penumbral areas, and the number of new angiogenic vessels is correlated with longer survival in stroke patients [15, 16], suggesting active angiogenesis may be beneficial for neurological functional recovery. These results strongly support inducible

K.-J. Yin, M.D., Ph.D. (✉) • X. Yang
Department of Neurology, University of Pittsburgh School of Medicine,
S514 BST, 200 Lothrop Street, Pittsburgh, PA 15213, USA
e-mail: yink2@upmc.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_5

angiogenesis (drug-based) as a potential therapy for acute ischemic stroke. In addition to endogenous angiogenesis, therapeutic angiogenesis is a novel treatment for ischemic stroke. Through increasing blood flow in or around the ischemic area, in particular the penumbra which likely is salvageable only through quick efforts to restore tissue perfusion after stroke onset, the aim of therapeutic angiogenesis is to promote or improve tissue preservation and regeneration [9, 10, 17–19].

During stroke, ischemic insults rapidly trigger transcription of various genes and proteins that may be associated with the process of angiogenesis [9–14]. Among them, vascular endothelial growth factor (VEGF) acts as a central mediator in post-ischemic angiogenesis. Infusion of VEGF into brain tissue or overexpression of VEGF in transgenic mice has been documented to promote angiogenesis, decrease infarct volume, and reduce neurological deficits after focal cerebral ischemia [20–22]. In brain tissue, VEGF is produced and secreted by many neurovascular cells (neurons, astrocytes, and vascular endothelial cells) [9, 23–25]. VEGF from various cellular sources binds to its receptors on nearby vascular endothelial cells to directly initiate an angiogenic response. The binding of VEGF to its receptors (VEGFR-1 or -2) on the surface of endothelial cells activates intracellular tyrosine kinases and triggers multiple downstream signals (PI3K/Akt and MEK/ERK protein kinase pathways) that promote angiogenesis [10, 26]. Increased levels of VEGF have been identified in human brains and serum after ischemic stroke [27, 28].

Indeed, it has been well established that stimulation of angiogenesis can be therapeutic in ischemic heart or cerebrovascular disease, peripheral arterial disease, and wound healing. In the healthy condition, angiogenesis is strictly controlled by a dynamic balance between pro-angiogenic and anti-angiogenic factors. When this balance is disturbed, it usually causes pathological angiogenesis which results in increased blood-vessel formation in diseases that depend on angiogenesis. Accumulating endogenous pro-angiogenic molecules have been identified, including matrix metalloproteinases (MMPs), cytokines, integrins, and growth factors such as VEGF, fibroblast growth factors (FGF), transforming growth factors (TGF), epidermal growth factor (EGF), and angiogenesis-regulating microRNAs (AngiomiRs) [9, 10, 13, 14, 19].

In this review article, we summarize the research progress describing the role and molecular regulating mechanisms of post-stroke angiogenesis. We also discuss the potential clinical applications of therapeutic angiogenesis as new treatment approaches for ischemic stroke.

2 Overview of Angiogenesis

Angiogenesis is defined as new vessels that are formed in the pathological and physiological process from preexisting vessels in a multistep process in order to deliver nutrients and oxygen to various organs and tissues [9], which is a key restorative mechanism in response to ischemia [29, 30]. Under ischemia condition, hypoxia is a key stimulus for angiogenesis and involved in the angiogenesis through the

activation of hypoxia-inducible factor-1a [31, 32]. Besides, pro-angiogenic molecules such as vascular endothelial growth factor-A (VEGF-A) and VEGF receptor 2 (VEGFR-2), angiopoietins (Ang-1 and -2) and cognate receptor Tie-2, neuropilin-1, and basic fibroblast growth factor (bFGF) are also stimulated [26, 33]. These growth factors activate otherwise quiescent endothelial cells to start the angiogenic cascade. Among these factors, some of them act in a synergic manner but some in a direct-opposed manner. In most cases, angiogenesis exerts preventive effect in organs. For example, angiogenesis could significantly reduce ischemia injury after cerebral and myocardial infarction [34]. However, in other case, angiogenesis could accelerate tissue injury. For example, angiogenesis could exacerbate the injury of tumor tissues [35]. However, therapeutics which reduced angiogenesis is proved to improve tumor prognosis. In the healthy condition, angiogenesis is strictly controlled by a dynamic balance between pro-angiogenic and anti-angiogenic factors. Increased or decreased blood-vessel formation in diseases could be caused when this balance is disrupted.

3 Angiogenesis After Ischemic Stroke

Ischemic stroke occurs immediately after the supply of brain is stopped. Part of the natural recovery process after stroke is angiogenesis [36], which is a biological process involving the growth of new blood vessels from preexisting vessels [13, 14]. The formation of new blood vessels around the infarct may be important for restoration of adequate perfusion for brain tissue. Subsequently, re-establishment of the functional cerebral microvasculature network will promote stroke recovery. In infarction region, neurons in the core of infarction were usually dead. But many apoptotic neurons existed in the peri-infarction, which is called penumbra. Vascular angiogenic remodeling mainly occurred in the penumbra which was beneficial to reduce the apoptosis of neurons and subsequently improved the prognosis of stroke [37]. Thus, it is important for stroke recovery to restore penumbra after stroke.

Although angiogenesis is completely suppressed under normal physiological conditions in adult brains, studies from human and experimental stroke indicate that neovascularization is present in the adult brains after cerebral ischemia [13, 14, 38–40]. Accumulating studies have showed that promotion of angiogenesis was helpful in reducing infarction in experimental animals [41–43]. It has been reported that cerebral vascular endothelial cells start to proliferate in the peri-infarcted region as early as 12–24 h after the onset of stroke [38–40]. This ischemia/hypoxia-induced vascular remodeling leads to increased microvessel density surrounding the infarcted brain area 3 days following ischemic injury. Indeed, many factors such as hemeoxygenase-1 and VEGF have been proved to improve neurological outcome in MCAO animals by enhancing angiogenesis [44].

Consistent with basic research in experimental stroke animal models, it is demonstrated that angiogenesis also occurred in stroke patients. It has been previously reported that young stroke patients had a better prognosis than old stroke patients

[45]. This means angiogenesis is easy to occur in young patients and old patients have worse ability in vessels formation. Other studies showed that stroke patients who had a history of transient ischemic attack (TIA) had a better prognosis than those without TIA history [46]. It is speculated that signals for angiogenesis should be activated when TIA occurred. Mechanistically, stroke patients with greater cerebral blood vessel density appear to make better progress and survive longer than patients with lower vascular density, suggesting that active post-stroke angiogenesis is helpful for long-term neurological functional recovery. Of importance, angiogenesis usually took place after some time when stroke is onset in patients. Then, many neurons were dead especially in those with occlusion in large vessels.

4 Regulation of Angiogenesis After Ischemic Stroke

Since angiogenesis is important for the recovery of stroke, it is important to know how to regulate angiogenesis. Cerebral angiogenesis is strictly controlled by many key angiogenic factors that play an important role in the development of angiogenesis in response to various pathological conditions such as stroke [9–14]. These stroke-associated angiogenic factors include VEGF and its receptors [20, 21, 25, 40, 47], bFGF and its receptors [48, 49], platelet-derived growth factor (PDGF) and its receptors [50, 51], transforming growth factor beta (TGFβ) [52, 53], MMPs [54], thrombospondin-1 (TSP-1) [55], angiopoietins and Tie receptors [56–58], endothelial nitric oxide synthase (eNOS) [59–61], hypoxia-inducible factor 1 [32], and many others [62].

In addition to the classic angiogenic regulators described above, recent studies have revealed important roles for microRNAs (miRs) in regulating angiogenesis [13, 14, 63–65]. MiRs are small endogenous RNA molecules (~21-25 nt) that repress gene translation by hybridizing to 3′-UTRs of one or more mRNAs in a sequence-specific manner. The discovery of miRs has shed light on how non-coding RNAs play critical roles in angiogenesis [13, 14, 63–65]. The initial support for the regulation of angiogenesis by miRs arose from genetic manipulations of the Dicer gene [66, 67]. Dicer knockout mice exhibit embryonic lethality because of abnormal vascular wall structure and arrangement. Mice with vascular-selective Dicer knockout have been reported to show impaired angiogenic ability, such as reduced endothelial tube formation and slowed EC migration, which may result from alterations of key angiogenesis-related genes. Moreover, an increasing number of individual miRs have been shown to regulate angiogenesis signaling pathways, thereby modulating endothelial migration, proliferation, and vascular-forming patterns [13, 14, 63–65]. Angiogenesis-related miRs can be classified into two groups with often opposing effects: pro-angiogenic and anti-angiogenic miRs. Among them, the miR-17-92 cluster, let-7, miR-27b, miR-126, miR-130a, miR-210, miR-296, miR-378, miR-21, and miR-31 exhibit pro-angiogenic effects, whereas miR-15/16, miR-424, miR-221/222, miR-92a, miR-320, miR-200b, miR-217, miR-503, miR-34, and miR-214 are considered anti-angiogenic [13, 14, 63–65].

Accumulating evidence has shown that miRs play a critical role in the pathogenesis of ischemic stroke [13, 14, 68–74]. Altered cerebral miR profiles have been reported in rodent focal and global cerebral ischemia models [75–78]. Ischemic preconditioning was also shown to change miR expression, including miR-132 [79], the miR-200 family, miR-182 family [80], and others [81], which may promote ischemic tolerance via neuroprotective signaling pathways. Altered miR levels were also found in blood samples of rodent stroke models [76, 78] and stroke patients [82–84] and can serve as potential biomarkers. We and others are among the first to identify the function of individual miRs in stroke pathology. Notably, we have defined that miR-497, a miR-15a homolog, is dramatically induced and promotes ischemic neuronal death in vitro and in vivo by inhibiting bcl-2 and bcl-w [85]. Moreover, we provided the first evidence that inhibition of endothelial miR-15a contributes to the PPARδ-mediated cerebral vasoprotective role in stroke [86]. Other miRs including miR-145 [75], miR-320a [87], miR-21 [88], miR-223 [89], miR-23a [90], miR-181 [91, 92], Let7f [93], miR-331 [94], miR-146a [95], miR-199a-5p [96], miR-124a [97], miR-124 [98], miR-133b [99], miR-29c [100], and miR-17-92 cluster [101] also mediate ischemia-induced neuronal death, neurogenesis, and neurological recovery.

Although many angiogenesis-regulating miRs play a role in development, cancer, and cardiovascular diseases, none of them have been systematically studied in the ischemic brain [13, 14]. In animal experiments, our group found that vascular endothelial cell (EC)-enriched miR-15a/16-1 cluster, a kind of stroke-associated miR, could suppress tube formation, cell migration, and cell differentiation in ECs [43]. Moreover, we found that EC-selective miR-15a transgenic overexpression in mice led to reduced blood vessel formation and local blood flow perfusion in the ischemic hindlimbs at 1–3 weeks after hindlimb ischemia [43]. Of note, we found that miR-15a exerts its anti-angiogenetic effect by direct inhibiting FGF2 and VEGF activities [43]. This means miR-15a is an anti-angiogenetic miR. A very recent study showed that miR-107 was strongly expressed in IBZ after permanent middle cerebral artery occlusion in rats and inhibition of miR-107 could reduce capillary density in the IBZ after stroke [102]. Mechanistically, they found that miR-107 could directly downregulate Dicer-1, a gene that encodes an enzyme essential for processing miRNA precursors. This resulted in translational inhibition of VEGF mRNA, thereby increasing the expression of endothelial cell-derived VEGF (VEGF165/VEGF164), leading to angiogenesis after stroke. Using HUVECs and MCAO rat, Li and his collages showed that miR-376b-5p repressed angiogenesis in vivo and in vitro, and miR-376b-5p inhibited angiogenesis in HUVECs by targeting the HIF-1α-mediated VEGFA/Notch1 signaling pathway [103]. Other groups found that miR-210, a hypoxia-induced miR, is significantly upregulated in adult rat ischemic brain cortexes in which the expression of Notch1 signaling was also increased. Gain-of-miR-210 function in cultured HUVE-12 cells caused activation of the Notch1 signaling cascade and induced endothelial cells to migrate and form capillary-like structures. These data may imply that miR-210 is a kind of pro-angiogenic miR [104]. However, this study does not establish a causal link between miR-210 and post-stroke cerebral angiogenesis. Consistent with this finding,

miR-210 has also been shown to regulate angiogenesis in normal brain tissue [105]. Besides brain miRNAs, many circulating miRNA has close relationship with ischemic stroke. For example, previous studies showed that plasma miRNA such as miR-124 and miR-290 were increased from 6 to 24 h in stroke animals [76, 106]. These data showed the potential biomarker of miRNA for onset of ischemic stroke and the potential role of brain-specific miRNAs to serve as biomarkers of tissue injury in MCAO animals.

In human subjects, it is demonstrated that many circulating miRs are associated with vascular endothelial function and angiogenesis and vascular remodeling under different ischemic conditions in stroke patients. In young stroke patients, circulating plasma miRs are associated with vascular endothelial function and angiogenesis (hsa-let-7f, miR-130a, -150, -17, -19a, -19b, -20a, -222, and -378) and vascular remodeling (miR-21, -126, and -150) have been found to be differentially regulated under ischemic conditions. Similarly, miRs that are expressed in hypoxic conditions (miR-23, -24, -26, -103, -107, and -181) and cardiac ischemia/reperfusion (miR-15, -16, -21, -23a, -29, -30a, -150, and -195) have also been detected in blood samples from stroke patients [82]. These miRNA plays an important role in angiogenesis, neuroinflammation, immune activation, leukocyte extravasation, and thrombosis [107]. The expression of certain miRNA may be different in different types of stroke. For example, it has been reported that circulating let-7b decreased in patients with large-vessel atherosclerosis than healthy controls, but it increased in patients with other kinds of ischemic stroke [107]. Moreover, circulating miRNA even could be used to predict the outcome of patients. Decrease of miR-210 was associated with poor outcome [108]. Conversely, reduction of anti-miR such as miR-30 predicts good outcome in stroke patients.

In addition to miRNAs, it has been demonstrated that cerebral ischemia rapidly changes the expression profiles of other classes of ncRNAs, such as lncRNAs and piRNAs, and imply that manipulation of these ncRNAs may regulate post-ischemic cerebrovascular remodeling. Till now, little is known regarding the expression and functional role of cerebral vasculature and/or endothelium lncRNAs and piRNAs after ischemic stroke [13, 14].

5 Functional Roles of Angiogenesis in Stroke Long-Term Remodeling and Functional Recovery

Angiogenesis, neurogenesis, and synaptogenesis could be occurred simultaneously, and function synergistically to improve long-term brain remodeling and promote functional recovery after ischemic stroke. It has been reported that NF-κB could enhance both angiogenesis and synaptogenesis in experimental stroke models [109]. Moreover, Avraham et al. found that following stroke, administration of exogenous leptin significantly induces neural stem cells in the cerebral cortex near the lesion site [110]. An increase in the number of newborn neurons and glia were observed in

leptin-treated animals as well. Leptin treatment also significantly increased the density of brain blood vessels in the penumbral cortex. In addition to neurogenesis and synaptogenesis, angiogenesis could also promote oligodendrogenesis, evidenced by a recent report by Zhang and colleagues that recombinant human erythropoietin could increase differentiation of SVZ neural progenitor cells into oligodendrocytes and effectively enhance neurological outcomes in stroke rats [111].

Accumulative studies have shown that angiogenesis appears to improve long-term functional recovery by promoting brain remodeling, repair, and plasticity. Cerebral injection of AAV-Netrin-1 gene has been shown to effectively improve neurological outcomes in stroke rats during 1–4 weeks [112], with a simultaneous increase in newly generated blood vessels after treatment. Striking findings from another group documented that BSc2118, a proteasome inhibitor, could enhance angiogenesis and improve functional outcomes even at 3 months after ischemic stroke [113]. Consistent with the described-above findings, a recent study also showed that fat-1 transgenic mice that cause overexpression of omega-3 polyunsaturated fatty acids (*n*-3 PUFAs) exhibited long-term histological and behavioral protection against ischemic stroke [114]. Mechanistically, *n*-3 PUFAs induced upregulation and release of a pro-angiogenic factor, angiopoietin 2 (Ang 2), in astrocytes after ischemic stimuli both in vitro and in vivo. Ang 2 facilitated endothelial proliferation and barrier formation in vitro by potentiating the effects of VEGF on phospholipase Cγ1 and Src signaling. Blockade of Src activity with a Src inhibitor AZD0530 impaired *n*-3 PUFA-induced angiogenesis and exacerbated long-term neurological outcomes in fat-1 Tg mice following ischemic stroke. These findings provide strong evidence that post-stroke cerebral angiogenesis contributes to long-term functional recovery in experimental stroke models.

6 Therapeutic Angiogenesis in Ischemic Stroke

Therapeutic angiogenesis is to employ clinical methods to enhance or improve blood vessels growth or development of collateral blood vessels within the ischemic tissue. Currently, there are three major ways to promote angiogenesis, namely, pharmacological/genetic approaches, physical activity, and cell therapy (Fig. 1).

Since cerebral neovascularization is involved in the pathogenesis of stroke, it could be helpful in the recovery of cerebral injury by modulating angiogenesis. Indeed, cumulative studies showed that angiogenesis is a therapeutic target of ischemic stroke [115]. Many pharmacological/genetic approaches could be used to serve as modulating angiogenic factors for angiogenesis in ischemic stroke. These factors reduced ischemic injury and improved neurologic outcomes via promoting angiogenesis under ischemic conditions. There are two main kinds of delivery way, namely direct delivery and virus-gene-associated delivery. Accumulating studies showed that many angiogenic factors such as VEGF [116], angiopoietin 1 [117], fibroblast growth factor (FGF-2), tumor necrosis factor α [118], hepatocyte growth factor (HGF) [119], bFGF [120], heparin-binding epidermal growth factor-like

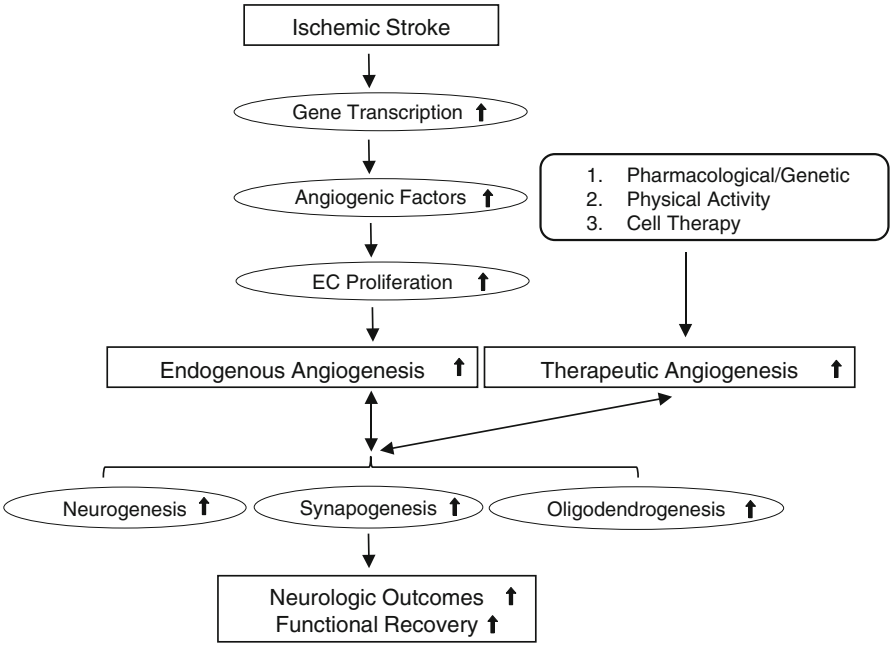

Fig. 1 Schematic representation of the post-stroke angiogenesis and therapeutic angiogenesis after ischemic stroke. Ischemic stimuli in brains trigger gene transcriptions and translations of pro-angiogenic factors, which subsequently induce endothelial cell proliferation and promote endogenous angiogenesis. Newly generated brain microvessels function synergistically with cerebral neurogenesis, synaptogenesis, and oligodendrogenesis to perform long-term neural/vascular remodeling and improve functional recovery after ischemic stroke. Therapeutic angiogenesis via pharmacological/genetic approaches, physical activity, and cell therapy further enhance post-stroke angiogenesis, thereby resulting in therapy-promoted functional recovery after ischemic brain injury

growth factor (HB-EGF), granulocyte-colony stimulating factor (G-CSF) could also decrease ischemic brain damage in animal models [9, 10]. Besides the direct effect of angiogenic factors, some proteins could enhance the expression of angiogenic factors and consequently improve ischemic stroke. For example, Zan and his colleague found that phosphorylation of Src at Y418 was increased as early as 3 h and peaked at 6 h following ischemic injury, after decreasing, it peaked again at 3–7 days [121]. Increases in Src mRNA and phosphorylation correlated positively with levels of VEGF and Ang-2, and negatively with levels of angiopoietin-1 (Ang-1) and zonula occludens-1. More importantly, increase in angiogenic factors was helpful in reducing vascular permeability, cerebral infarct size, and neurologic dysfunction. Moreover, many pharmacological approaches, including phosphodiesterase type-5 (PDE-5) inhibitors, statins, adrenomedullin, erythropoietin, kallikreins, nitric oxide donors, angiotensin II type 1 receptor blockade, have also shown to increase cerebral angiogenesis around the infarcted area and improved functional recovery in rodent stroke models [10].

Additionally, it has been reported that exercise could induce angiogenesis and improve motor deficit in animal models of stroke. For instance, Ma et al. reported that exercise enhanced matrix metalloproteinase 2 (MMP2) and VEGF-related genes and proteins expression while improving regional cerebral blood flow and neurobehavioral score [122]. Another group found that pre-ischemic exercise reduces brain injury in rats subjected to 2 h MCA occlusion followed by 48 h reperfusion via upregulating angiogenic factors such as VEGF [123]. Also Gertz et al. [124] demonstrated that continuous voluntary running in stroke mice led to long-term upregulation of eNOS in the vasculature and of EPCs, which was associated with higher numbers of circulating EPCs and an enhanced cerebral neovascularization, and resulted in an improved functional outcome and increased density of perfused microvessels.

Cell therapy is a novel method in which many angiogenic factors-producing cells are used to promote angiogenesis in ischemic tissue [18, 125]. A variety of cell types have been used for the purpose of therapeutic angiogenesis in experimental stroke models such as monocytes, endothelial progenitor cells, marrow stromal cells, mesenchymal stem cells, umbilical cord blood, and neural stem cells [10, 18]. Mechanistically, these cells are able to stimulate angiogenesis at the ischemic boundary region through direct expression and release of pro-angiogenic factors induced by local paracrine stimuli from ischemia. These cells can also act as a shuttle to transfer an angiogenic gene toward ischemic brain tissue [10, 18].

In summary, recent therapeutic angiogenesis through pharmacological/genetic delivery of various angiogenic factors, physical activity, and cell therapy has shown us several promising results in promoting cerebral neovascularization, blood flow recovery, and neurological function recovery in ischemic stroke [10, 18]. Although selective regulation of post-stroke cerebral angiogenesis appears to be a promising restorative therapy for ischemic stroke, there are several potential challenges or limitations for application of therapeutic angiogenesis in stroke. These obstacles include incomplete understanding of the function and underlying mechanisms of cerebral neovascularization in the stroke pathogenesis, brain edema, ineffective delivery system to achieve cell-specific delivery in vivo, limited target specificity, inflammatory responses, vascular malformation, hypotension, and off-target tumorigenesis [17, 18]. Therefore, a better understanding of the cellular and molecular signaling cascades of post-stroke cerebral neovascularization is needed for successful application of angiogenesis-based neurorestorative therapies after ischemic stroke.

Acknowledgments This work was supported by the National Institutes of Health Grants: NS094930, NS091175, and NS086820 (K.J. Yin).

References

1. Schellinger PD, Kaste M, Hacke W. An update on thrombolytic therapy for acute stroke. Curr Opin Neurol. 2004;17(1):69–77.
2. Schellinger PD, Warach S. Therapeutic time window of thrombolytic therapy following stroke. Curr Atheroscler Rep. 2004;6(4):288–94.
3. Stapf C, Mohr JP. Ischemic stroke therapy. Annu Rev Med. 2002;53:453–75.
4. Barone FC. Ischemic stroke intervention requires mixed cellular protection of the penumbra. Curr Opin Investig Drugs. 2009;10(3):220–3.
5. Del Zoppo GJ, Milner R, Mabuchi T, Hung S, Wang X, Koziol JA. Vascular matrix adhesion and the blood-brain barrier. Biochem Soc Trans. 2006;34(Pt 6):1261–6.
6. Ginsberg MD. Current status of neuroprotection for cerebral ischemia: synoptic overview. Stroke. 2009;40(3 Suppl):S111–4.
7. Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci. 2003;4(5):399–415.
8. Yuan J. Neuroprotective strategies targeting apoptotic and necrotic cell death for stroke. Apoptosis. 2009;14(4):469–77.
9. Hayashi T, Deguchi K, Nagotani S, Zhang H, Sehara Y, Tsuchiya A, et al. Cerebral ischemia and angiogenesis. Curr Neurovasc Res. 2006;3(2):119–29.
10. Beck H, Plate KH. Angiogenesis after cerebral ischemia. Acta Neuropathol. 2009;117(5):481–96.
11. Arai K, Jin G, Navaratna D, Lo EH. Brain angiogenesis in developmental and pathological processes: neurovascular injury and angiogenic recovery after stroke. FEBS J. 2009;276(17):4644–52.
12. Zhang ZG, Chopp M. Neurorestorative therapies for stroke: underlying mechanisms and translation to the clinic. Lancet Neurol. 2009;8(5):491–500.
13. Yin KJ, Hamblin M, Chen YE. Non-coding RNAs in cerebral endothelial pathophysiology: emerging roles in stroke. Neurochem Int. 2014;77:9–16.
14. Yin KJ, Hamblin M, Chen YE. Angiogenesis-regulating microRNAs and ischemic stroke. Curr Vasc Pharmacol. 2015;13(3):352–65.
15. Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM. Some remarks on the growth-rate and angiogenesis of microvessels in ischemic stroke. Morphometric and immunocytochemical studies. Patol Pol. 1993;44(4):203–9.
16. Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM. Role of angiogenesis in patients with cerebral ischemic stroke. Stroke. 1994;25(9):1794–8.
17. Chen YC, Wu JS, Yang ST, Huang CY, Chang C, Sun GY, et al. Stroke, angiogenesis and phytochemicals. Front Biosci. 2012;4:599–610.
18. Al Sabti H. Therapeutic angiogenesis in cardiovascular disease. J Cardiothorac Surg. 2007;2:49.
19. Manoonkitiwongsa PS. Critical questions for preclinical trials on safety and efficacy of vascular endothelial growth factor-based therapeutic angiogenesis for ischemic stroke. CNS Neurol Disord Drug Targets. 2011;10(2):215–34.
20. Zhang ZG, Zhang L, Jiang Q, Zhang R, Davies K, Powers C, et al. VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. J Clin Invest. 2000;106(7):829–38.
21. Sun Y, Jin K, Xie L, Childs J, Mao XO, Logvinova A, et al. VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. J Clin Invest. 2003;111(12):1843–51.
22. Wang Y, Kilic E, Kilic U, Weber B, Bassetti CL, Marti HH, et al. VEGF overexpression induces post-ischaemic neuroprotection, but facilitates haemodynamic steal phenomena. Brain. 2005;128(Pt 1):52–63.

23. Lennmyr F, Ata KA, Funa K, Olsson Y, Terent A. Expression of vascular endothelial growth factor (VEGF) and its receptors (Flt-1 and Flk-1) following permanent and transient occlusion of the middle cerebral artery in the rat. J Neuropathol Exp Neurol. 1998;57(9):874–82.
24. Issa R, Krupinski J, Bujny T, Kumar S, Kaluza J, Kumar P. Vascular endothelial growth factor and its receptor, KDR, in human brain tissue after ischemic stroke. Lab Invest. 1999;79(4):417–25.
25. Kovacs Z, Ikezaki K, Samoto K, Inamura T, Fukui M. VEGF and flt. Expression time kinetics in rat brain infarct. Stroke. 1996;27(10):1865–72; discussion 72–3.
26. Greenberg DA, Jin K. From angiogenesis to neuropathology. Nature. 2005;438(7070):954–9.
27. Gunsilius E, Petzer AL, Stockhammer G, Kahler CM, Gastl G. Serial measurement of vascular endothelial growth factor and transforming growth factor-beta1 in serum of patients with acute ischemic stroke. Stroke. 2001;32(1):275–8.
28. Slevin M, Krupinski J, Slowik A, Kumar P, Szczudlik A, Gaffney J. Serial measurement of vascular endothelial growth factor and transforming growth factor-beta1 in serum of patients with acute ischemic stroke. Stroke. 2000;31(8):1863–70.
29. Stapor P, Wang X, Goveia J, Moens S, Carmeliet P. Angiogenesis revisited—role and therapeutic potential of targeting endothelial metabolism. J Cell Sci. 2014;127(Pt 20):4331–41.
30. Stapor PC, Sweat RS, Dashti DC, Betancourt AM, Murfee WL. Pericyte dynamics during angiogenesis: new insights from new identities. J Vasc Res. 2014;51(3):163–74.
31. Kyzas PA, Stefanou D, Batistatou A, Agnantis NJ. Hypoxia-induced tumor angiogenic pathway in head and neck cancer: an in vivo study. Cancer Lett. 2005;225(2):297–304.
32. Shi H. Hypoxia inducible factor 1 as a therapeutic target in ischemic stroke. Curr Med Chem. 2009;16(34):4593–600.
33. Hayashi T, Abe K, Suzuki H, Itoyama Y. Rapid induction of vascular endothelial growth factor gene expression after transient middle cerebral artery occlusion in rats. Stroke. 1997;28(10):2039–44.
34. Bai YY, Wang L, Chang D, Zhao Z, Lu CQ, Wang G, et al. Synergistic effects of transplanted endothelial progenitor cells and RWJ 67657 in diabetic ischemic stroke models. Stroke. 2015;46(7):1938–46.
35. Gopinathan G, Milagre C, Pearce OM, Reynolds LE, Hodivala-Dilke K, Leinster DA, et al. Interleukin-6 stimulates defective angiogenesis. Cancer Res. 2015;75(15):3098–107.
36. Zhang ZG, Chopp M. Promoting brain remodeling to aid in stroke recovery. Trends Mol Med. 2015;21(9):543–8.
37. Zhao X, Wang H, Sun G, Zhang J, Edwards NJ, Aronowski J. Neuronal interleukin-4 as a modulator of microglial pathways and ischemic brain damage. J Neurosci. 2015;35(32):11281–91.
38. Beck H, Acker T, Wiessner C, Allegrini PR, Plate KH. Expression of angiopoietin-1, angiopoietin-2, and tie receptors after middle cerebral artery occlusion in the rat. Am J Pathol. 2000;157(5):1473–83.
39. Hayashi T, Noshita N, Sugawara T, Chan PH. Temporal profile of angiogenesis and expression of related genes in the brain after ischemia. J Cereb Blood Flow Metab. 2003;23(2):166–80.
40. Marti HJ, Bernaudin M, Bellail A, Schoch H, Euler M, Petit E, et al. Hypoxia-induced vascular endothelial growth factor expression precedes neovascularization after cerebral ischemia. Am J Pathol. 2000;156(3):965–76.
41. Yang Y, Salayandia VM, Thompson JF, Yang LY, Estrada EY, Yang Y. Attenuation of acute stroke injury in rat brain by minocycline promotes blood-brain barrier remodeling and alternative microglia/macrophage activation during recovery. J Neuroinflammation. 2015;12:26.
42. Zents K, Copray S. The therapeutic potential of induced pluripotent stem cells after stroke: evidence from rodent models. Curr Stem Cell Res Ther. 2015;11(2):166–74.

43. Yin KJ, Olsen K, Hamblin M, Zhang J, Schwendeman SP, Chen YE. Vascular endothelial cell-specific microRNA-15a inhibits angiogenesis in hindlimb ischemia. J Biol Chem. 2012;287(32):27055–64.
44. Shanab AY, Elshaer SL, El-Azab MF, Soliman S, Sabbineni H, Matragoon S, et al. Candesartan stimulates reparative angiogenesis in ischemic retinopathy model: role of heme-oxygenase-1 (HO-1). Angiogenesis. 2015;18(2):137–50.
45. Szpak GM, Lechowicz W, Lewandowska E, Bertrand E, Wierzba-Bobrowicz T, Dymecki J. Border zone neovascularization in cerebral ischemic infarct. Folia Neuropathol. 1999;37(4):264–8.
46. Wegener S, Gottschalk B, Jovanovic V, Knab R, Fiebach JB, Schellinger PD, et al. Transient ischemic attacks before ischemic stroke: preconditioning the human brain? A multicenter magnetic resonance imaging study. Stroke. 2004;35(3):616–21.
47. Cobbs CS, Chen J, Greenberg DA, Graham SH. Vascular endothelial growth factor expression in transient focal cerebral ischemia in the rat. Neurosci Lett. 1998;249(2–3):79–82.
48. Chen HH, Chien CH, Liu HM. Correlation between angiogenesis and basic fibroblast growth factor expression in experimental brain infarct. Stroke. 1994;25(8):1651–7.
49. Issa R, AlQteishat A, Mitsios N, Saka M, Krupinski J, Tarkowski E, et al. Expression of basic fibroblast growth factor mRNA and protein in the human brain following ischaemic stroke. Angiogenesis. 2005;8(1):53–62.
50. Iihara K, Sasahara M, Hashimoto N, Hazama F. Induction of platelet-derived growth factor beta-receptor in focal ischemia of rat brain. J Cereb Blood Flow Metab. 1996;16(5):941–9.
51. Iihara K, Sasahara M, Hashimoto N, Uemura Y, Kikuchi H, Hazama F. Ischemia induces the expression of the platelet-derived growth factor-B chain in neurons and brain macrophages in vivo. J Cereb Blood Flow Metab. 1994;14(5):818–24.
52. Krupinski J, Kumar P, Kumar S, Kaluza J. Increased expression of TGF-beta 1 in brain tissue after ischemic stroke in humans. Stroke. 1996;27(5):852–7.
53. Yamashita K, Gerken U, Vogel P, Hossmann K, Wiessner C. Biphasic expression of TGF-beta1 mRNA in the rat brain following permanent occlusion of the middle cerebral artery. Brain Res. 1999;836(1–2):139–45.
54. Zhao BQ, Wang S, Kim HY, Storrie H, Rosen BR, Mooney DJ, et al. Role of matrix metalloproteinases in delayed cortical responses after stroke. Nat Med. 2006;12(4):441–5.
55. Lin TN, Kim GM, Chen JJ, Cheung WM, He YY, Hsu CY. Differential regulation of thrombospondin-1 and thrombospondin-2 after focal cerebral ischemia/reperfusion. Stroke. 2003;34(1):177–86.
56. Lin TN, Wang CK, Cheung WM, Hsu CY. Induction of angiopoietin and Tie receptor mRNA expression after cerebral ischemia-reperfusion. J Cereb Blood Flow Metab. 2000;20(2):387–95.
57. Zhang Z, Chopp M. Vascular endothelial growth factor and angiopoietins in focal cerebral ischemia. Trends Cardiovasc Med. 2002;12(2):62–6.
58. Zhang ZG, Chopp M, Lu D, Wayne T, Zhang RL, Morris D. Receptor tyrosine kinase tie 1 mRNA is upregulated on cerebral microvessels after embolic middle cerebral artery occlusion in rat. Brain Res. 1999;847(2):338–42.
59. Chen J, Zacharek A, Zhang C, Jiang H, Li Y, Roberts C, et al. Endothelial nitric oxide synthase regulates brain-derived neurotrophic factor expression and neurogenesis after stroke in mice. J Neurosci. 2005;25(9):2366–75.
60. Huang Z, Huang PL, Ma J, Meng W, Ayata C, Fishman MC, et al. Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. J Cereb Blood Flow Metab. 1996;16(5):981–7.
61. Chen J, Cui X, Zacharek A, Roberts C, Chopp M. eNOS mediates TO90317 treatment-induced angiogenesis and functional outcome after stroke in mice. Stroke. 2009;40(7):2532–8.

62. Zhang ZG, Tsang W, Zhang L, Powers C, Chopp M. Up-regulation of neuropilin-1 in neovasculature after focal cerebral ischemia in the adult rat. J Cereb Blood Flow Metab. 2001;21(5):541–9.
63. Wang S, Olson EN. AngiomiRs—key regulators of angiogenesis. Curr Opin Genet Dev. 2009;19(3):205–11.
64. Kuehbacher A, Urbich C, Dimmeler S. Targeting microRNA expression to regulate angiogenesis. Trends Pharmacol Sci. 2008;29(1):12–5.
65. Suarez Y, Sessa WC. MicroRNAs as novel regulators of angiogenesis. Circ Res. 2009;104(4):442–54.
66. Kuehbacher A, Urbich C, Zeiher AM, Dimmeler S. Role of dicer and drosha for endothelial microRNA expression and angiogenesis. Circ Res. 2007;101(1):59–68.
67. Suarez Y, Fernandez-Hernando C, Yu J, Gerber SA, Harrison KD, Pober JS, et al. Dicer-dependent endothelial microRNAs are necessary for postnatal angiogenesis. Proc Natl Acad Sci U S A. 2008;105(37):14082–7.
68. Rink C, Khanna S. MicroRNA in ischemic stroke etiology and pathology. Physiol Genomics. 2011;43(10):521–8.
69. Saugstad JA. MicroRNAs as effectors of brain function with roles in ischemia and injury, neuroprotection, and neurodegeneration. J Cereb Blood Flow Metab. 2010;30(9):1564–76.
70. Tan JR, Koo YX, Kaur P, Liu F, Armugam A, Wong PT, et al. microRNAs in stroke pathogenesis. Curr Mol Med. 2011;11(2):76–92.
71. Vemuganti R. The microRNAs and stroke: no need to be coded to be counted. Transl Stroke Res. 2010;1(3):158–60.
72. Ouyang YB, Stary CM, Yang GY, Giffard R. microRNAs: innovative targets for cerebral ischemia and stroke. Curr Drug Targets. 2013;14(1):90–101.
73. Eacker SM, Dawson TM, Dawson VL. The interplay of microRNA and neuronal activity in health and disease. Front Cell Neurosci. 2013;7:136.
74. Liu XS, Chopp M, Zhang RL, Zhang ZG. MicroRNAs in cerebral ischemia-induced neurogenesis. J Neuropathol Exp Neurol. 2013;72(8):718–22.
75. Dharap A, Bowen K, Place R, Li LC, Vemuganti R. Transient focal ischemia induces extensive temporal changes in rat cerebral microRNAome. J Cereb Blood Flow Metab. 2009;29(4):675–87.
76. Jeyaseelan K, Lim KY, Armugam A. MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. Stroke. 2008;39(3):959–66.
77. Yuan Y, Wang JY, Xu LY, Cai R, Chen Z, Luo BY. MicroRNA expression changes in the hippocampi of rats subjected to global ischemia. J Clin Neurosci. 2010;17(6):774–8.
78. Liu DZ, Tian Y, Ander BP, Xu H, Stamova BS, Zhan X, et al. Brain and blood microRNA expression profiling of ischemic stroke, intracerebral hemorrhage, and kainate seizures. J Cereb Blood Flow Metab. 2010;30(1):92–101.
79. Lusardi TA, Farr CD, Faulkner CL, Pignataro G, Yang T, Lan J, et al. Ischemic preconditioning regulates expression of microRNAs and a predicted target, MeCP2, in mouse cortex. J Cereb Blood Flow Metab. 2010;30(4):744–56.
80. Lee ST, Chu K, Jung KH, Yoon HJ, Jeon D, Kang KM, et al. MicroRNAs induced during ischemic preconditioning. Stroke. 2010;41(8):1646–51.
81. Dharap A, Vemuganti R. Ischemic pre-conditioning alters cerebral microRNAs that are upstream to neuroprotective signaling pathways. J Neurochem. 2010;113(6):1685–91.
82. Tan KS, Armugam A, Sepramaniam S, Lim KY, Setyowati KD, Wang CW, et al. Expression profile of MicroRNAs in young stroke patients. PLoS One. 2009;4(11):e7689.
83. Gan CS, Wang CW, Tan KS. Circulatory microRNA-145 expression is increased in cerebral ischemia. Genet Mol Res. 2012;11(1):147–52.
84. Tsai PC, Liao YC, Wang YS, Lin HF, Lin RT, Juo SH. Serum microRNA-21 and microRNA-221 as potential biomarkers for cerebrovascular disease. J Vasc Res. 2013;50(4):346–54.

85. Yin KJ, Deng Z, Huang H, Hamblin M, Xie C, Zhang J, et al. miR-497 regulates neuronal death in mouse brain after transient focal cerebral ischemia. Neurobiol Dis. 2010;38(1):17–26.
86. Yin KJ, Deng Z, Hamblin M, Xiang Y, Huang H, Zhang J, et al. Peroxisome proliferator-activated receptor delta regulation of miR-15a in ischemia-induced cerebral vascular endothelial injury. J Neurosci. 2010;30(18):6398–408.
87. Sepramaniam S, Armugam A, Lim KY, Karolina DS, Swaminathan P, Tan JR, et al. MicroRNA 320a functions as a novel endogenous modulator of aquaporins 1 and 4 as well as a potential therapeutic target in cerebral ischemia. J Biol Chem. 2010;285(38):29223–30.
88. Buller B, Liu X, Wang X, Zhang RL, Zhang L, Hozeska-Solgot A, et al. MicroRNA-21 protects neurons from ischemic death. FEBS J. 2010;277(20):4299–307.
89. Harraz MM, Eacker SM, Wang X, Dawson TM, Dawson VL. MicroRNA-223 is neuroprotective by targeting glutamate receptors. Proc Natl Acad Sci U S A. 2012;109(46):18962–7.
90. Siegel C, Li J, Liu F, Benashski SE, McCullough LD. miR-23a regulation of X-linked inhibitor of apoptosis (XIAP) contributes to sex differences in the response to cerebral ischemia. Proc Natl Acad Sci U S A. 2011;108(28):11662–7.
91. Ouyang YB, Lu Y, Yue S, Xu LJ, Xiong XX, White RE, et al. miR-181 regulates GRP78 and influences outcome from cerebral ischemia in vitro and in vivo. Neurobiol Dis. 2012;45(1):555–63.
92. Moon JM, Xu L, Giffard RG. Inhibition of microRNA-181 reduces forebrain ischemia-induced neuronal loss. J Cereb Blood Flow Metab. 2013;33(12):1976–82.
93. Selvamani A, Sathyan P, Miranda RC, Sohrabji F. An antagomir to microRNA Let7f promotes neuroprotection in an ischemic stroke model. PLoS One. 2012;7(2):e32662.
94. Hunsberger JG, Fessler EB, Wang Z, Elkahloun AG, Chuang DM. Post-insult valproic acid-regulated microRNAs: potential targets for cerebral ischemia. Am J Transl Res. 2012;4(3):316–32.
95. Zhang L, Chopp M, Liu X, Teng H, Tang T, Kassis H, et al. Combination therapy with VELCADE and tissue plasminogen activator is neuroprotective in aged rats after stroke and targets MicroRNA-146a and the toll-like receptor signaling pathway. Arterioscler Thromb Vasc Biol. 2012;32(8):1856–64.
96. Park HA, Kubicki N, Gnyawali S, Chan YC, Roy S, Khanna S, et al. Natural vitamin E alpha-tocotrienol protects against ischemic stroke by induction of multidrug resistance-associated protein 1. Stroke. 2011;42(8):2308–14.
97. Liu XS, Chopp M, Zhang RL, Tao T, Wang XL, Kassis H, et al. MicroRNA profiling in subventricular zone after stroke: MiR-124a regulates proliferation of neural progenitor cells through Notch signaling pathway. PLoS One. 2011;6(8):e23461.
98. Doeppner TR, Doehring M, Bretschneider E, Zechariah A, Kaltwasser B, Muller B, et al. MicroRNA-124 protects against focal cerebral ischemia via mechanisms involving Usp14-dependent REST degradation. Acta Neuropathol. 2013;126(2):251–65.
99. Xin H, Li Y, Liu Z, Wang X, Shang X, Cui Y, et al. Mir-133b promotes neural plasticity and functional recovery after treatment of stroke with multipotent mesenchymal stromal cells in rats via transfer of exosome-enriched extracellular particles. Stem Cells. 2013;31(12):2737–46.
100. Pandi G, Nakka VP, Dharap A, Roopra A, Vemuganti R. MicroRNA miR-29c down-regulation leading to de-repression of its target DNA methyltransferase 3a promotes ischemic brain damage. PLoS One. 2013;8(3):e58039.
101. Liu XS, Chopp M, Wang XL, Zhang L, Hozeska-Solgot A, Tang T, et al. MicroRNA-17-92 cluster mediates the proliferation and survival of neural progenitor cells after stroke. J Biol Chem. 2013;288(18):12478–88.
102. Li Y, Mao L, Gao Y, Baral S, Zhou Y, Hu B. MicroRNA-107 contributes to post-stroke angiogenesis by targeting Dicer-1. Sci Rep. 2015;5:13316.
103. Li LJ, Huang Q, Zhang N, Wang GB, Liu YH. miR-376b-5p regulates angiogenesis in cerebral ischemia. Mol Med Rep. 2014;10(1):527–35.

104. Lou YL, Guo F, Liu F, Gao FL, Zhang PQ, Niu X, et al. miR-210 activates notch signaling pathway in angiogenesis induced by cerebral ischemia. Mol Cell Biochem. 2012;370(1–2):45–51.
105. Zeng L, He X, Wang Y, Tang Y, Zheng C, Cai H, et al. MicroRNA-210 overexpression induces angiogenesis and neurogenesis in the normal adult mouse brain. Gene Ther. 2013;21(1):37–43.
106. Laterza OF, Lim L, Garrett-Engele PW, Vlasakova K, Muniappa N, Tanaka WK, et al. Plasma microRNAs as sensitive and specific biomarkers of tissue injury. Clin Chem. 2009;55(11):1977–83.
107. Li M, Zhang J. Circulating microRNAs: potential and emerging biomarkers for diagnosis of cardiovascular and cerebrovascular diseases. Biomed Res Int. 2015;2015:730535.
108. Zeng L, Liu J, Wang Y, Wang L, Weng S, Tang Y, et al. MicroRNA-210 as a novel blood biomarker in acute cerebral ischemia. Front Biosci (Elite Ed). 2011;3:1265–72.
109. Cui L, Duchamp NS, Boston DJ, Ren X, Zhang X, Hu H, et al. NF-kappaB is involved in brain repair by stem cell factor and granulocyte-colony stimulating factor in chronic stroke. Exp Neurol. 2015;263:17–27.
110. Avraham Y, Davidi N, Lassri V, Vorobiev L, Kabesa M, Dayan M, et al. Leptin induces neuroprotection neurogenesis and angiogenesis after stroke. Curr Neurovasc Res. 2011;8(4):313–22.
111. Zhang L, Chopp M, Zhang RL, Wang L, Zhang J, Wang Y, et al. Erythropoietin amplifies stroke-induced oligodendrogenesis in the rat. PLoS One. 2010;5(6):e11016.
112. Lu H, Wang Y, He X, Yuan F, Lin X, Xie B, et al. Netrin-1 hyperexpression in mouse brain promotes angiogenesis and long-term neurological recovery after transient focal ischemia. Stroke. 2012;43(3):838–43.
113. Doeppner TR, Mlynarczuk-Bialy I, Kuckelkorn U, Kaltwasser B, Herz J, Hasan MR, et al. The novel proteasome inhibitor BSc2118 protects against cerebral ischaemia through HIF1A accumulation and enhanced angioneurogenesis. Brain. 2012;135(Pt 11):3282–97.
114. Wang J, Shi Y, Zhang L, Zhang F, Hu X, Zhang W, et al. Omega-3 polyunsaturated fatty acids enhance cerebral angiogenesis and provide long-term protection after stroke. Neurobiol Dis. 2014;68:91–103.
115. Zechariah A, ElAli A, Doeppner TR, Jin F, Hasan MR, Helfrich I, et al. Vascular endothelial growth factor promotes pericyte coverage of brain capillaries, improves cerebral blood flow during subsequent focal cerebral ischemia, and preserves the metabolic penumbra. Stroke. 2013;44(6):1690–7.
116. Soliman S, Ishrat T, Pillai A, Somanath PR, Ergul A, El-Remessy AB, et al. Candesartan induces a prolonged proangiogenic effect and augments endothelium-mediated neuroprotection after oxygen and glucose deprivation: role of vascular endothelial growth factors A and B. J Pharmacol Exp Ther. 2014;349(3):444–57.
117. Camos S, Gubern C, Sobrado M, Rodriguez R, Romera VG, Moro MA, et al. The high-mobility group I-Y transcription factor is involved in cerebral ischemia and modulates the expression of angiogenic proteins. Neuroscience. 2014;269:112–30.
118. Wang L, Chopp M, Teng H, Bolz M, Francisco MA, Aluigi DM, et al. Tumor necrosis factor alpha primes cerebral endothelial cells for erythropoietin-induced angiogenesis. J Cereb Blood Flow Metab. 2011;31(2):640–7.
119. Shimamura M, Sato N, Oshima K, Aoki M, Kurinami H, Waguri S, et al. Novel therapeutic strategy to treat brain ischemia: overexpression of hepatocyte growth factor gene reduced ischemic injury without cerebral edema in rat model. Circulation. 2004;109(3):424–31.
120. Jiang N, Finklestein SP, Do T, Caday CG, Charette M, Chopp M. Delayed intravenous administration of basic fibroblast growth factor (bFGF) reduces infarct volume in a model of focal cerebral ischemia/reperfusion in the rat. J Neurol Sci. 1996;139(2):173–9.
121. Zan L, Zhang X, Xi Y, Wu H, Song Y, Teng G, et al. Src regulates angiogenic factors and vascular permeability after focal cerebral ischemia-reperfusion. Neuroscience. 2014;262:118–28.

122. Ma Y, Qiang L, He M. Exercise therapy augments the ischemia-induced proangiogenic state and results in sustained improvement after stroke. Int J Mol Sci. 2013;14(4):8570–84.
123. Ding YH, Luan XD, Li J, Rafols JA, Guthinkonda M, Diaz FG, et al. Exercise-induced overexpression of angiogenic factors and reduction of ischemia/reperfusion injury in stroke. Curr Neurovasc Res. 2004;1(5):411–20.
124. Gertz K, Priller J, Kronenberg G, Fink KB, Winter B, Schrock H, et al. Physical activity improves long-term stroke outcome via endothelial nitric oxide synthase-dependent augmentation of neovascularization and cerebral blood flow. Circ Res. 2006;99(10):1132–40.
125. Suzuki K, Murtuza B, Smolenski RT, Sammut IA, Suzuki N, Kaneda Y, et al. Cell transplantation for the treatment of acute myocardial infarction using vascular endothelial growth factor-expressing skeletal myoblasts. Circulation. 2001;104(12 Suppl 1):I207–12.

Part II
Glial Cells in Stroke

Astrocytes as a Target for Ischemic Stroke

Shinghua Ding

1 A Brief Overview of Astrocytes

Astrocytes are star-like cells and the most diverse glial cell type in the central nervous system (CNS). The classical definition of astrocytes is based on morphology and specific protein markers. Although there are many types of astrocyte in a normal brain, our discussion in this chapter will focus on two major types of astrocytes in the adult brain: Fibrous astrocytes present in white matter tracts such as the corpus callosum and protoplasmic astrocytes present in gray matter such as the cortex. Kimelberg proposed the following eight criteria to define mature astrocytes [1]:

1. Electrically non-excitable.
2. A very negative membrane potential determined by the transmembrane K^+ gradient.
3. Expression of functional transporters for glutamate and GABA uptake.
4. A large number of intermediate filament bundles, which are the sites of the astrocyte-specific protein glial fibrillary acidic protein (GFAP).
5. Glycogen granules.
6. Processes from each cell surrounding blood vessels.
7. Many more processes from each cell surrounding synapses.
8. Linkage to other astrocytes by gap junctions consisting of connexins 43 and 30.

Astrocytes are traditionally considered as the housekeeping cells needed to maintain homeostasis in CNS. Growing evidence suggests that astrocytes also play an active role in modulating neuronal function. Protoplasmic astrocytes exhibit the following functions under normal conditions [1, 2]:

S. Ding, Ph.D. (✉)
Department of Bioengineering, Dalton Cardiovascular Research Center,
University of Missouri, 134 Research Park Drive, Columbia, MO 65211, USA
e-mail: dings@missouri.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_6

1. Extracellular K^+ buffering.
2. Control of extracellular H^+ and brain pH.
3. Uptake of glutamate and GABA with their transporters.
4. Mobilizing intracellular Ca^{2+} stores by activation of G-protein coupled receptors (GPCRs) such as mGLuR5 and P2Y.
5. Control of cerebral blood flow.
6. Control of water transport by aquaporins.
7. Astrocyte-neuron lactate shuttle.
8. Modulation and control of synaptic activity.

Many astrocytes conform to the aforementioned criteria and functional roles, but astrocytes are heterogeneous in morphology, molecular expression, and physiological function [3, 4]. Morphologically, protoplasmic astrocytes are highly branched. A protoplasmic astrocyte in gray matter has several primary processes each with elaborated sub-branched fine process arborizations to form a bush-like astrocytic territory. Protoplasmic astrocytes exhibit distinct territorial domain with little overlap between neighboring astrocytes [5, 6]. Fibrous astrocytes have thicker and less branched processes. They exhibit a high degree of overlap. Intermediate filament protein GFAP is primarily expressed in the thick main processes in astrocytes and has been considered as a "pan-astrocyte" marker; however, its expression levels are quite different between fibrous and protoplasmic astrocytes. GFAP is expressed in almost every individual fibrous astrocyte, but protoplasmic astrocytes in the cortex express much lower levels of GFAP than do protoplasmic astrocytes in the hippocampus [7]. Recent transcriptome study revealed that the Aldh1L1 gene is most widely and homogenously expressed in astrocytes, and immunostaining revealed that the Aldh1L1 protein is highly expressed in the cell body and extensive processes of an astrocyte [8]. Therefore, Aldh1L1 is now considered as a new "pan-astrocyte" marker.

Astrocytes exhibit different electrophysiological properties. Astrocytes can be classified as outward rectifying astrocytes and variably rectifying astrocytes based on current–voltage relationship [9]. Two-photon (2-P) microscopy revealed that astrocytes also exhibit different properties of Ca^{2+} signaling in vivo*;* for example, astrocytes in the cortical layer 1 (L1) nearly double the Ca^{2+} activity compared to the astrocytes in L2/3 in anaesthetized rats; moreover, Ca^{2+} signals in the processes in the same astrocyte are asynchronous in L1 while those in L2/3 are more synchronous [10]. The morphological, molecular, and functional heterogeneity of astrocytes indicates that astrocytes play diverse and complex physiological and pathological roles in the CNS.

Astrocytes are electrically non-excitable, but their excitability is manifested by Ca^{2+} signaling through the activation of various GPCRs. Astrocytes express GPCRs for glutamate, γ-aminobutyric acid (GABA), ATP, serotonin, norepinephrine, and dopamine. Astrocytes can mediate Ca^{2+} signaling and intercellular waves in vivo by the activation of metabotropic glutamate receptors (mGluRs) [11–13], P2Y receptors [13–17], $GABA_B$ receptors ($GABA_B$Rs) [14, 18], noradrenergic receptors [19], and dopamine receptors [20, 21]. GPCR stimulation activates phospholipase-C (PLC) with subsequent IP_3R-mediated Ca^{2+} release from internal stores in endoplas-

mic reticulum (ER) [22–24]. Among the three types of IP_3R (IP_3R1-3), IP_3R2 seems to be the predominant type in astrocytes in the rodent brain [25–27]. IP_3R2 knockout (IP_3R2 KO) mice do not exhibit GPCR agonists-evoked Ca^{2+} release in astrocytes in brain slice and in vivo, demonstrating that IP_3R2 is a key mediator of intracellular Ca^{2+} release in astrocytes [24, 28, 29]. Ca^{2+} signaling is now considered as a primary form of cellular excitability in astrocytes that can be measured by fluorescent imaging.

2 Astrocytes in Ischemic Stroke

Focal ischemic stroke (FIS) is initiated by the mechanical occlusion of cerebral vessels by thrombus or embolus thereby reducing or blocking blood flow; it is also called focal cerebral ischemia. FIS accounts for approximately 80 % of all human strokes and causes brain infarction and human disability. It is the leading cause of human death and disability and has a major impact on public health. The degree of blood flow reduction largely determines the brain infarction. Due to the lack of glucose and oxygen in the ischemic region, synthesis of ATP through glycolysis and oxidative phosphorylation is impaired. Thus, ischemia results in energy depletion and subsequent loss of ionic gradients and membrane potential depolarization in neurons and astrocytes. This further induces the release of glutamate and other neurotransmitters from presynaptic terminals to the extracellular space. This energy depletion also contributes to the malfunction of astrocytic and neuronal glutamate transporters, which are responsible for the clearance of the glutamate released into the synaptic cleft.

Cells in the ischemic core region die rapidly from the onset of ischemia due to severely impaired energy production and the ensuing breakdown of ionic homeostasis, but in the penumbra, the region surrounding the core of a focal ischemic locus, the brain tissue is hypoperfused with collateral blood flow with partial preservation of energy metabolism. This region can completely or partially recover after reperfusion, but can progress to infarction without treatment due to the ongoing excitotoxicity [30], thus the primary goal for stroke therapy is to salvage the penumbra in the acute phase of ischemic stroke. Clinically, current strategies to treat ischemic stroke are aimed at restoring blood supply by administration of thrombolytic drugs, which is only effective within a narrow window of about 3–4 h following the onset of ischemia [31, 32]. Thus, identifying novel therapeutic targets and elucidating cellular and molecular mechanisms by which ischemia induces neuronal death and brain damage are of great importance to provide effective therapeutic avenues.

Astrocytes respond to almost all neural diseases including FIS; however, compared with neurons, the role of astrocytes in ischemia is less understood. After the onset of ischemia, astrocytes undergo numerous pathological processes [33–35]. Astrocytes rapidly swell after ischemia, which is contributed to by a number of factors [36–38]. Swelling spreads from the ischemic core to the penumbral tissue. Many of swollen astrocytes in the ischemic core eventually lyse, while astrocytes in the penumbral region exhibit reversible swelling [37]. Astrocytes are generally

more resistant to ischemic insults than neurons [39] primarily due to utilization of glycogen stores as an alternative energy source [30, 40–42]. Astrocytes can supply energy to neurons through lactate shuttle [43]. Astrocytes can also convert glycogen to glucose-1P and produce ATP by glycolysis [41]. Astrocytes become reactive over time after ischemia, a process called reactive astrogliosis [33, 44–47]. Reactive astrocytes eventually form a glial scar around the ischemic core. Some reviews have provided various aspects of astrocytes in stroke and therapeutic potential [35, 48–51]. This chapter focuses on discussing the Ca^{2+} signaling in astrocytes in the acute phase of ischemia, dynamic changes of morphology and proliferation in the subacute phase, signaling pathways of reactive astrogliosis, stem cell properties of reactive astrocytes, and the potential of astrocytes as a target in stroke therapy.

2.1 Astrocyte and Glutamate Uptake in Ischemic Stroke

Glutamate excitotoxicity is considered the primary mediator of acute neuronal death [52]. The excessive accumulation of extracellular glutamate overstimulates ionotropic and metabotropic glutamate receptors, especially ionotropic *N*-methyl-D-aspartate (NMDA) receptors (NMDARs), and results in pathological overloading of intracellular Ca^{2+} in neurons [53, 54], which is linked to the triggering of activations of downstream phospholipases and proteases that cause degradation of membranes and proteins and eventually neuronal death [39, 55]. Thus, glutamate excitotoxicity not only causes acute neuronal death, but it also initiates molecular events that lead to delayed neuronal death and brain damage [55]. It has been known that glutamate transporters in astrocytes are impeded due to energy depletion. Glutamate can even be released from astrocytes by the reversed operation of transporter in ischemia [56, 57], leading to elevation of extracellular glutamate and aggravating excitotoxic neuronal death. From study using cortical cultures, neuronal vulnerability to glutamate is 100-fold greater in astrocyte-poor cultures than in astrocyte-rich cultures. Knockdown of glial glutamate transporter (GLT-1) significantly increases in neuronal death in a global ischemia mouse model [58]. These studies demonstrate that astrocytes play an important role in reducing glutamate excitotoxicity in ischemia through uptaking glutamate by their glutamate transporters. Thus, enhancing the glutamate transporter function is a promising therapeutic strategy to reduce acute neuronal death and brain damage after ischemic stroke.

2.2 Ca^{2+} Signaling in Astrocytes After Ischemic Stroke

Neuronal glutamate and Ca^{2+} excitotoxicity is widely acknowledged [52, 59]; however, whether and how astrocytic Ca^{2+} is altered after ischemia has been less understood. Since astrocytic Ca^{2+} signaling is the signature feature of astrocytic excitability and due to the tripartite nature of synapse, Ca^{2+}-dependent gliotransmitter release is

expected to affect neuronal excitotoxicity during a stroke. Several studies using in vitro brain slice and in vivo animal models reported astrocytic Ca^{2+} dysregulation after ischemia.

Using acute brain slices which can mimic tissue environment, Duffy and MacVicar [60] found that a short episode (5 min) of simultaneous hypoxia and hypoglycemia can induce intracellular Ca^{2+} increase in astrocytes in acute brain slices within an average of 7.5 min. Their report also recorded 2.5 min as the time needed to reach a peak. Ca^{2+} levels in astrocytes remained elevated for a variable period of time ranging from several minutes to 1 h after reoxygenation. Astrocytic Ca^{2+} elevation could be still detected in the absence of extracellular Ca^{2+} albeit with a relative consistent duration. They further showed using electrophysiological recordings that hypoxia and hypoglycemia can also depolarize astrocytes. Their study suggests that astrocytes can mediate Ca^{2+} increase from the internal store release and influx of a voltage-dependent Ca^{2+} channel.

Dong et al. showed that OGD can induce slow inward currents (SICs) mediated by extrasynaptic NMDA receptors in rat CA1 pyramidal neurons in brain slices [61]. SICs can be inhibited by dialysis of the Ca^{2+} chelator BAPTA into astrocytic network, indicating that the activation of extrasynaptic NMDA receptors depended on astrocytic Ca^{2+} activity. Using 2-P microscopy, it was found that frequent astrocytic Ca^{2+} elevations were observed during OGD, with over 60 % of astrocytes displayed detectable Ca^{2+} elevations within the 10 min of OGD. In addition, most astrocytes displayed more than two transients. They further demonstrated that astrocytic Ca^{2+} elevations and the frequency of SICs during OGD were largely reduced in IP_3R2 KO mice as compared with wild type (WT) mice.

Ding et al. for the first time determined whether astrocytes exhibit altered Ca^{2+} signaling in vivo after ischemia [14]. Using 2-P microscopy, they imaged astrocytic Ca^{2+} signals after photothrombosis (PT)-induced focal ischemia in urethane-anesthetized adult mice. They found that astrocytes exhibit enhanced Ca^{2+} signaling characterized as intercellular Ca^{2+} waves, which starts ~20 min after PT, and Ca^{2+} signals reach the plateau 60 min after PT. Both amplitude and frequency of PT-induced astrocytic Ca^{2+} signals were dramatically increased compared to relative quiescent Ca^{2+} signaling prior to PT. Most Ca^{2+} signals were initiated and returned to the basal level at the same time among astrocytes in the imaging field, i.e., they were highly synchronized transient signals. To further determine the nature of astrocytic Ca^{2+} signals in ischemia, antagonists for GPCRs including mGluR5, $GABA_BR$, and P2Y receptors were administered after emergence of Ca^{2+} signals. Both 2-methyl 6-(phenylethynyl)pyridine hydrochloride (MPEP), an antagonist of mGluR5, and CGP54626, the antagonist of the $GABA_B$ receptor, significantly reduced Ca^{2+} signals (~55 %). But suramin, a non-specific inhibitor for P2Y receptors, did not reduce the PT-induced Ca^{2+} signals. A general P2 receptor antagonist, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), did not attenuate the astrocytic Ca^{2+} signal either. Similarly, inhibition of adenosine receptor A1 did not inhibit PT-induced astrocytic Ca^{2+} signals. Thus, the pharmacological study suggests that glutamate and GABA are likely to be released to the extracellular space following PT to stimulate intercellular Ca^{2+} waves in the astrocytic network through

mGluR5 and $GABA_BR$. It is surprising that neither P2Y nor A1 receptors contribute to the enhanced Ca^{2+} signaling after PT, presumably due to the rapid degradation of ATP and adenosine by enzymes such as ectonucleotidases after ischemia [62]. Furthermore, application of BAPTA-AM significantly reduced brain infarct 24 h after PT. The results represent the first in vivo study of astrocytic Ca^{2+} responses after ischemia and suggest that a blockade of Ca^{2+} increase in astrocytes can reduce the release of glutamate from astrocytes and cause neuronal death and brain damage. The emergence of Ca^{2+} increase in the acute phase might represent the initial response of astrocytes to ischemic insults. In future study, it is important to determine the time course of neuronal and astrocytic Ca^{2+} signaling and overloading after ischemia as neuronal Ca^{2+} overloading is one of the major events that cause excitotoxicity.

Astrocytes also exhibit altered Ca^{2+} signaling in the chronic phase of ischemia. Winship et al. studied the neuronal and astrocytic Ca^{2+} signaling and functional rewiring in somatosensory neurons in the ipsilateral hemisphere after PT [63]. They examined astrocytic Ca^{2+} responses in anesthetized mice to study whether the prevalence of these responses changed in the peri-infarct cortex half, 1 and 2 months after PT. A significant increase was found in the prevalence of responses to preferred limb stimulation. The selectivity of astrocyte responses in regions with overlap between contralateral hindlimb- and contralateral forelimb-evoked intrinsic optical signal and regions without intrinsic optical signal overlap were also significantly different. These results suggest that mechanisms for rapid neuron–astrocyte communication are preserved, or even enhanced, after stroke in the penumbra.

Astrocytes also play a role in the functional recovery within the contralateral hemisphere. Takatsura et al. performed in vivo Ca^{2+} imaging to examine the neuronal and astrocytic Ca^{2+} responses in the region contralateral to the stroke site at different times following PT [64]. Their results showed that the number of astrocytes with a Ca^{2+} response to limb stimulation significantly increased in the contralateral somatosensory cortex responding to ipsilateral limb stimulation during the first and second week after infarction as compared with the sham group. A significantly larger number of astrocytes responded only to the single-limb stimulation in the sham group as compared with stroke groups, but a smaller number of astrocytes responded to multiple-limb stimulation in the sham group as compared to the stroke groups. Interestingly, unlike neurons, astrocytes showed no preference in response to contralateral and ipsilateral limb stimulation. The amplitude of Ca^{2+} response was also increased in stroke groups as compared with sham groups. A large increase in glutamate concentration were observed 2 weeks after the stroke compared with the sham and 1-week group based on a microdialysis study; however, glutamine concentration was much higher in the contralateral side in the 1-week group than in the sham and 2-week group. Astrocytic plasma membrane glutamate transporter 1 (GLT-1) may contribute to the uptake of glutamate in the 1-week groups. These findings demonstrate that activated astrocytes increased the uptake of glutamate by glutamate transporters during the first week, and indicate that astrocytes play an important role in functional recovery and cortical remodeling in the area contralateral to ischemic lesion in the post-ischemic period.

IP_3R2 knockout mice exhibit smaller infarct volume than WT littermates in acute ischemia [61, 65] as well as in chronic phases of ischemia [65], indicating that IR_3R2-mediated Ca^{2+} signaling contributes to neuronal excitotoxicity and acute brain damage after ischemia. IP_3R2 KO mice also exhibited less neuronal apoptosis, reactive astrogliosis, and tissue loss than WT mice [65]. The study from Li et al. further shows that IP_3R2 KO mice exhibited reduced functional deficits after PT using behavioral tests, including cylinder, hanging wire, pole and adhesive tests [65]. These studies demonstrate that disruption of astrocytic Ca^{2+} signaling has beneficial effects on neuronal and brain protection and functional deficits after stroke by reducing Ca^{2+} dependent glutamate release and revealed a novel non-cell-autonomous neuronal and brain protective function of astrocytes in ischemic stroke, whereby suggest that the astrocytic IP_3R2-mediated Ca^{2+} signaling pathway might be a promising target for stroke therapy.

Astrocytic Ca^{2+} signaling may play different roles in neuronal death and brain damage in different brain injury models. In a tiny ischemic lesion model generated by a single vessel laser irradiation, an increase in astrocytic Ca^{2+} can stimulate energy metabolism and ATP production in astrocytic mitochondria and, thus, reduce brain damage in WT mice as compared with IP_3R2 KO mice [66]. In the stab wound injury (SWI) model which also produces a tiny lesion, IP_3R2 KO mice had increased neuronal loss as compared with WT mice [67]. In MCAo and PT models, which cause large infarction, WT mice exhibit larger infarction than IP_3R2 KO mice [61, 65]. Thus, astrocytic Ca^{2+} signals have different effects on neuronal death and brain damage in mild vs. severe brain injury models.

2.3 Morphology of Reactive Astrocytes After FIS

Since the clinical aim of stroke therapy is to salvage the penumbral cells, understanding the spatial and temporal changes of reactive astrocytes at molecular and cellular levels will provide therapeutic insights for brain repair after stroke. The hallmark of reactive astrogliosis after FIS is the morphological changes and the increased expression levels of GFAP [33, 44, 46, 50, 68, 69]. These changes include the eventual formation of a glial scar, which establishes both a physical and biochemical barrier that separates the ischemic core from the vital tissues. Although there is little expression of GFAP in the astrocytes within the cortex under normal condition [45, 68, 70], cortical astrocytes undergo dramatic increases in GFAP levels over time after FIS. Thus, GFAP expression has been used to study the morphological changes of reactive astrocytes following ischemia. Using endotelin-1 collagenase type IV-S-induced ischemia model, Mesttriner et al. [71] conducted a detailed study on morphology of reactive astrocytes in the penumbra in the chronical stage, i.e., 30 days after ischemic stroke in rats. Their study showed that not only GFAP+ astrocyte density significantly increased after stroke but the ramification and length of primary processes also increased compared with the astrocytes in a sham control group. Wagner et al. studied the morphology of reactive astrocytes in

rats at early stage, i.e., day 4 after middle cerebral artery occlusion (MCAo) and showed that the process volume, diameter, length, and branching levels in reactive astrocytes in the penumbra were increased compared with the astrocytes in contralateral hemisphere and in the distant regions from the ischemic core [72]. Moreover, the process volume and diameter in the penumbra were larger than those in the distant regions. The mean process length of astrocytes in the contralateral hemisphere was longer than reactive astrocytes in the penumbra and the remote region, while the mean process length of reactive astrocytes in the remote region was slightly but significantly longer than those in the penumbra. On the other hand, the mean process branching was similar in the penumbra and in the remote region. The data shows that reactive astrocytes become hypertrophic rather than increase the length of their process at a relatively acute stage of ischemia.

Reactive astrogliosis after ischemia is highly dependent on time and location [45, 46, 73]. Li et al. examined the dynamic changes of reactive astrocytes in the cortex using a photothrombosis-induced mouse ischemia model with a high temporal resolution [68]. GFAP expression was examined in mouse brains after 2, 4, 6, 8, 10, 12, and 14 days following PT using immunostaining and confocal microscopy. A significant increase of GFAP expression was observed 2 days after PT, suggesting astrocytes had been activated. Reactive astrocytes exhibited a stellate morphology and more hypertrophy and expressed higher GFAP up to 4 days after PT. After day 6 post PT, reactive astrocytes became densely packed and exhibited a stream-like structure with their elongated processes pointing towards the ischemic core—a feature of reactive astrocyte polarization and astroglial scar formation, but reactive astrocytes became less hypertrophic after day 6 post PT. Ten days after PT, reactive astrocytes in the penumbra remained similar but with longer processes, indicating the maturation of a glial scar. A significant increase in GFAP expression was also observed in the regions further away from the scar border but with similar morphology to the astrocytes under normal conditions. Thus, the morphology of GFAP+ astrocytes in the penumbra underwent dynamic modifications over time following PT.

Astrocytes exhibit non-overlapping domains in healthy mouse brains [6]. In an epileptic brain, reactive astrocytes interdigitate, i.e., exhibit overlapping domains between the neighboring astrocytes [74]. In an electrically induced mouse lesion model, reactive astrocytes in the cortex exhibit minimal interdigitation, which is similar to those in the cortex of control animals [5]. Thus, it is likely that the severity of brain injury determines the degree of overlapping between reactive astrocytes. It will be interesting to investigate whether reactive astrocytes interdigitate after FIS and whether the degree of interdigitation is temporal and spatial dependent.

2.4 Proliferation of Reactive Astrocytes After FIS

Reactive astrocyte proliferation after FIS has been well documented using PT-induced focal ischemia and MCAo models [45, 46, 73, 75–78]. Glial scar formation is associated not only with morphological changes of reactive astrocytes but

also with substantial tissue shrinking [45]. Spontaneous recovery from brain injury in the chronic phase of ischemia may involve reactive astrogliosis [79]. While it is well known that the common feature of reactive astrocytes is increased GFAP expression levels, whether reactive astrogliosis is merely through the upregulation of GFAP or through cell proliferation is less understood. Bromodeoxyuridine (Brdu) labeling and immunostaining are the general approaches used to assess the spatial and temporal changes of reactive astrocyte proliferation [44–46, 73, 75, 80]. Li et al. [68] studied the dynamic change of proliferation of astrocytes and microglia after FIS. They designed a "time-block" protocol to label proliferating cells at different times after PT. Mice were administered with Brdu at days 1, 3, 4, 5, 9, 11, and 13 post-PT for 2 consecutive days and transcardially perfused after 1 day from the last injection. The proliferation of reactive astrocytes was studied using GFAP and Brdu double staining. Brdu+ cells and GFAP+ astrocytes dramatically increased from day 2 post PT and reached their peak at day 4; however, overall, the GFAP+Brdu+ proliferating astrocytes only accounted for a small percentage of total Brdu+ cells, which reached a peak value of about 6 % from days 3 to 4 post PT and then declined sharply. Based on the labeling protocol, the GFAP+Brdu+/Brdu+ ratio at each time point represents the relative rate of the generation of reactive astrocytes. On the other hand, the GFAP+Brdu+/GFAP+ ratio also reached the highest level within days 3–4 post PT. These data demonstrated that FIS increases the population of proliferating reactive astrocytes in a highly temporal dependent manner. The results indicate that glial scar is formed largely from the existing astrocytes through the upregulation of GFAP, rather than from newly generated astrocytes through proliferation. Although proliferation rate reduces dramatically after 8 days following PT, the morphology of reactive astrocytes maintains straight processes pointing to ischemic core for a prolonged time based on the GFAP expression [68, 73, 75]. This phenomenon suggests that the expression of certain genes is irreversible in the penumbra during recovery from focal ischemic injury, and glial scar formation causes substantial tissue remodeling and permanent and persistent structural changes. Although the approach might underestimate the total number of proliferating cells since the injection protocol may not label all the proliferating cells [81], the GFAP+Brdu+/Brdu+ ratio should represent the ratio of newly generated reactive astrocytes out of the total proliferating cells.

2.5 Signaling Pathways of Reactive Astrogliosis After Ischemia

Advancements in genetics and molecular biology have helped to extensively characterize reactive astrocytes after brain injury. A plethora of molecular markers and signaling pathways associated with reactive astrogliosis have been identified [80, 82–85]. The following is a brief summary of the major signaling pathways that have been identified to regulate reactive astrogliosis in ischemic stroke.

Intermediate filaments. The intermediate filament GFAP is expressed both in protoplasmic and fibrous astrocytes. Following ischemic stroke, the peri-lesional

astrocytes become reactivated and dramatically upregulate their GFAP in a temporally and spatially dependent manner. Genetic deletion of GFAP is neuroprotective against metabolic insult and excitotoxicity which is attributed to increased production of the glial-derived neurotrophic factor (GDNF) by GFAP knockout astrocytes [86]. GFAP$^{-/-}$ mice have exhibited a more significant decrease in cortical cerebral blood flow, and a relatively larger infarct volume than WT mice after MCAo, indicating that GFAP-null mice have a high susceptibility to cerebral ischemia, which indicates that astrocytes and GFAP play an important role in the progression of brain damage after ischemia [87]. However, studies using GFAP$^{-/-}$/Vimentin$^{-/-}$ double knockout mice show that in acute ischemia, double deletion of GFAP and vimentin results in decreased glutamate uptake, increased susceptibility to oxidative stress, and significantly increased infarct volumes (two to three times compared with that of WT mice). Hence, the deletion of GFAP and vimentin in the acute stage disrupts glial scar formation and promotes neuronal regeneration after ischemia [88]. Whereas in chronic stages of stroke, attenuation of astroglial reactivity by GFAP and vimentin deletion impaired axonal remodeling and functional recovery [89]. Together, these studies suggest that reactive astrocytes have a protective role in brain ischemia. GFAP and vimentin are essential to retaining the beneficial function of reactive astrocytes in ischemic as well as recovering nervous tissues. Thus, manipulation of astrocytic reactivity may represent a therapeutic target for neurorestorative strategies in ischemic stroke.

p38 MAPK pathway. Mitogen-activated protein kinases (MAPK) are a family of enzymes transducing a wide range of extracellular signals such as inflammation, growth factors, and toxic stimuli as well as integrating corresponding cellular responses [90]. Among p38, MAPKs is of particular importance since it transduces cellular inflammation [91]. p38 MAPK can become activated in neurons, microglia, and astrocytes after ischemic stroke [92–94]. A recent study using an astrocyte-specific p38 MAPK knockout mouse model demonstrated that delayed activation of p38 MAPK following ischemic injury corresponds with upregulation of GFAP in the penumbra region [95], suggesting an important role of p38 MAPK in signaling reactive astrogliosis following ischemic stroke. Furthermore, conditional deletion of using GFAP-Cre/LoxP p38 MAPK mice resulted in a significant reduction of reactive astrogliosis in the penumbra following ischemia compared to WT animals, but had no significant effect on motor functional recovery after ischemia. p38 MAPK signaling may be a critical transducing pathway to modulate reactive astrogliosis in ischemic stroke.

Notch signaling pathway. Notch signaling is a highly conserved pathway critical for the maintenance and self-renewal of progenitor cells and to inhibit precocious neurogenesis [96]. In ischemic stroke, Notch 1 signaling was activated in astrocytes in the peri-infarct region by 24 h [97], and conditional deletion of Notch 1 from GFAP positive cells resulted in a decreased number of proliferating astrocytes and an increased number of invading CD45+ immune cells following ischemic injury [98]. Further study showed that Notch1–STAT3–ETBR axis connects a signaling network that promotes reactive astrocyte proliferation after a stroke [99]. Genetic fate mapping analysis revealed that a subpopulation of reactive astrocyte activated

Notch1 signaling in response to injury and became proliferative [97, 100]. A recent study also showed that Notch1 signaling is important in reactive astrocyte differentiation to neurons in an ischemic brain [101]. These studies suggest that Notch 1 plays different roles including neurogenesis, reactive astrocyte proliferation, and transdifferentiation after brain injury. Some outstanding questions which will be mandatory in future studies are: Why does only a subset of reactive astrocytes express Notch 1 signaling and become proliferative? What is the spatiotemporal pattern of Notch 1 signaling after ischemia? Does inhibiting Notch 1-induced astrocyte proliferation affect functional recovery? Answering these questions will better define the role of Notch 1 in reactive astrogliosis and provide novel therapeutic insights for stroke therapy.

STAT3 signaling. Signal transducer and activator of transcription 3 (STAT3) is a member of the Jak-STAT family and activated by hormones, growth factors, or cytokines [102]. The role of STAT3 in reactive astrogliosis has been extensively studied in spinal cord injury models [81, 103–105]. In ischemic stroke, reperfusion-induced increases in ROS and inflammatory cytokines are known to be powerful stimulants of STAT3 signaling [106, 107], but it remains a matter of extensive debate regarding cell type specific activation of STAT3 in the post ischemic brain. Some reports suggest neurons are the predominant source of activated STAT3 [108, 109], while others have shown that STAT3 is also activated in astrocytes along with neurons [110, 111]. More recently, it suggests that STAT3 can regulate reactive astrogliosis induced by neurotoxic insults where activation of STAT3 in astrocytes and subsequent increases in GFAP expression can be induced by direct neuronal death even in the absence of astrocyte damage [112, 113]. It appears that its role in driving reactive astrogliosis in ischemic stroke remains to be explored.

Transforming growth factor-Beta (TGF-β) signaling. TGF-β is an injury-related peptide and has been shown to increase immunoreactivity in both the infarct and penumbra in stroke patients [114] as well as animal models of FIS [115–117]. TGF-β has been mostly regarded as neuroprotective particularly against the *N*-methyl-D-aspartate receptor (NMDA)-induced excitotoxicity [116, 118]. It is suggested that the protective action of TGF-β is mostly mediated by astrocytes [119, 120]. Most recent data suggests that disrupting astrocytic FGF receptors results in reduced scar size, increased astrocyte activation, and inflammation, but did not affect infarct volume [121, 122]. These studies suggest that TGF-β signaling in astrocytes can limit neuroinflammation, reactive astrogliosis in the peri-infarct cortex and preserve brain function during the subacute period after stroke.

Despite the extensive characterization of reactive gliosis, high heterogeneity and the context-dependent nature of reactive astrogliosis in the injured brain (e.g., acute phase vs. chronic phase, white matter vs. gray matter, penumbral region vs. distant region) complicate our understanding of signaling pathways for regulating this process [3, 85, 123]. Recent studies on the genome-wide transcriptome profiles of pure glial cells using microarray and RNA-Seq technologies provide an unprecedented opportunity to study glia phenotypes and functions in health and disease [8, 80, 124, 125]. Data from these studies open a window for a comprehensive view of complex mechanisms by which astrocytes contribute to brain protection and repair in ischemia.

Especially when using RNA-Seq, a large number of signaling pathways that trigger reactive astrogliosis and molecular changes of astrocytes can be identified. Changes in transcriptome profiles at different times after ischemic stroke will help to identify novel molecular pathways by which astrocytes respond to ischemic insults at different stages [80]. These data will shed new light on the roles of astrocytes in brain protection and repair and provide information for astrocyte-targeting therapeutic strategies for ischemic therapy.

2.6 Stem-Cell-like Properties of Reactive Astrocytes and Endogenous Neuronal Differentiation of Reactive Astrocytes After Ischemic Stroke

Growing evidence has shown that ischemic stroke dramatically increases neurogenesis in the subventricular zone (SVZ) and subgranular layers in dentate gyrus [126]. Endogenous progenitor cells proliferate and differentiate into neurons in response to ischemic insult; the newly generated neurons migrate to the damaged region thereby integrating into the neural network [127–129]. These studies suggest that endogenous neurogenesis contributes to brain repair and spontaneous recovery after ischemia. As we already discussed, normally quiescent astrocytes become reactive and resume proliferation after ischemia. This implies that reactive astrocytes might be in an immature state due to a certain degree of dedifferentiation induced by ischemia. Studies have demonstrated that proliferating astrocytes in specific brain regions act as adult neural stem cells under normal conditions [130, 131]. In addition to many changes in gene profile, reactive astrocytes also exhibit stem cell-like properties [132–136]. These studies suggest that reactive astrocytes may attempt to reconstitute neurons after ischemia. Indeed, they express neural stem-cell-related proteins such as nestin and Sox2 [136], doublecortin (DCX), an immature neural stem cell marker [101, 137], and oligo 2, a transcription factor that regulates neuroglia fate decision [138]. It shows that a sonic hedgehog (SHH) signal can directly act on the astrocytes and is necessary and sufficient to elicit the stem cell response both in vitro and in vivo [133]. Stroke also elicits a latent neurogenic program in striatal astrocytes in a mouse model [101, 139]. It is reported that Notch 1 signaling is reduced in reactive astrocytes after stroke and attenuated Notch 1 signaling is necessary for neurogenesis by striatal astrocytes [101]; furthermore, blocking Notch 1 signaling triggers astrocytes in the striatum and the medial cortex to enter a neurogenic program even in the absence of a stroke, indicating that attenuated Notch 1 signaling is necessary for neurogenesis by striatal astrocytes. After a prolonged time (e.g., 13–16 weeks) following ischemia, reactive astrocyte-derived neurons in striatum not only express mature neuronal markers but also are able to fire the action potential and exhibit synaptic activity, suggesting the integration into their local neural network [139]. These studies provide a molecular base for how reactive astrocytes acquire stem cell lineage and become neurons after a stroke.

Reactive astrocytes also affect the migration of neural progenitor cells after ischemia. Young et al. [140] reported for the first time that ischemic stroke causes substantial reactive astrogliosis in SVZ and the hypertrophic reactive astrocytes and their tortuous processes disrupt the neuroblast migratory scaffold and cause SVZ reorganization after a stroke [140]; thus, reactive astrocytes in SVZ might also play a modulating role in neurogenesis thereby affecting stroke recovery.

2.7 Direct Astrocyte-to-Neuron Conversion After Ischemic Stroke

The fact that reactive astrocytes express neural stem markers and can be transformed into mature neurons after a prolonged time following ischemia indicate that reactive astrocytes have the intrinsic potential to generate neurons and reactive astrocytes-to-neuron conversion can be facilitated under permissive conditions such as overexpression of neurogenic factors. It has been recently reported that astrocytes indeed can be converted into neuroblasts and neurons in vitro and in vivo by forced expression of a single transcriptional factor such as Sox2 [141, 142], Neurog-2 [143–145], NeuroD1 [146], Ascl1 [147], or a combination of multiple transcriptional factors such as Ascl1, Lmx1B, Nurr1, Oct4, Sox2, or Nanog [148, 149]. Astrocyte-converted neurons express mature neuronal markers such as Map2 and NeuN [141, 146]. Electrophysiological recording showed that cultured mouse astrocyte-converted neurons by NeuroD1 are glutamatergic; furthermore, astrocyte-converted neurons by NeuroD1 in mouse brain can exhibit synaptic currents and integrate into neural circuit in vivo [146]. In vitro direct reprogramming study of postnatal astrocytes showed that astrocyte-to-neuron conversion is swift, but different proneural factors can elicit distinct transcriptional programs. Both Neurog-2 and Ascl1 rapidly elicited neurogenic programs to induce glutamatergic and GABAergic neuronal conversion but followed distinct paths with a few common genes in the neurogenic cascades [145]. The study of shared target genes will allow identifying a particularly important subset of downstream targets capable of directly converting astrocytes into functional neurons.

Although, reactive astrocyte-to-neuron conversion by forced expression of transcription factors has not been reported in the context of strokes, these studies suggest that recruiting reactive astrocytes and directly converting them into neurons might be a promising strategy for brain repair after ischemic stroke. Since reactive astrocytes exhibit different proliferating rates at different times after FIS [68], there might be an optimal timing to convert them into neurons using genetic manipulation techniques such as astrocyte-specific inducible transgenic mice and viral transduction. Because stroke causes a large damage in the cortex, it is also interesting to study whether reactive astrocytes in the cortex have the similar capacity of neuronal transformation to striatal astrocytes. Future study is also needed to explore whether any small molecular drugs targeting the signaling pathway of endogenous neuronal

differentiation will facilitate astrocyte-to-neuron conversion [150, 151] and to determine whether astrocyte-derived neurons contribute to brain recovery and improvement of stroke outcomes.

3 Concluding Remarks

Despite tremendous investments in translational research, there are still limited treatment options for stroke therapy. Tissue plasminogen activator (tPA) is the only available FDA-approved drug for acute stroke treatment, but it is effective within a narrow therapeutic window of a few hours after the onset of a stroke. Treatment of a stroke is primarily dependent on supportive care, secondary prevention, and rehabilitation after the acute phase. tPA treatment is also associated with intracranial bleeding. Although various mechanisms showing how ischemia leads to neuronal death and brain damage have been identified using animal models, current neuron-centric strategies have not resulted in major breakthroughs in stroke therapy; therefore, alternative strategies to target astrocytes could be an important direction for stroke therapy. Since astrocytes have intimate contact with neurons and vasculature and undergo dynamic changes in Ca^{2+} signaling, morphology, proliferation, and gene expression after ischemia, targeting reactive astrocytes is a promising strategy for brain damage and repair after stroke. Knowledge regarding the dynamic changes of function and signaling pathways that trigger reactive astrogliosis and facilitate neuronal differentiation should provide information for effective strategies for ischemic brain therapy. Studies on genome-wide transcriptome profiles further provide information for astrocyte-targeted therapeutic strategies for brain protection and repair. However, as astrocytes exhibit heterogeneity in different regions, a comprehensive understanding of reactive astrogliosis is extremely vital to the designing strategies to modulate reactive astrogliosis for brain repair. On the other hand, using the stem-cell-like properties of reactive astrocytes, it is feasible to directly convert them into neurons by expressing transcriptional factors. Thus, targeting astrocytes for stroke therapy is an emerging and promising area.

Acknowledgments This work was supported by the National Institutes of Health [R01NS069726, R01NS094539] and the American Heart Association [13GRNT17020004] to S.D.

References

1. Kimelberg HK. Functions of mature mammalian astrocytes: a current view. Neuroscientist. 2010;16:79–106.
2. Kimelberg H, Nedergaard M. Functions of astrocytes and their potential as therapeutic targets. Neurotherapeutics. 2010;7:338–53.
3. Zhang Y, Barres BA. Astrocyte heterogeneity: an underappreciated topic in neurobiology. Curr Opin Neurobiol. 2010;20:588–94.

4. Matyash V, Kettenmann H. Heterogeneity in astrocyte morphology and physiology. Brain Res Rev. 2010;63:2–10.
5. Wilhelmsson U, Bushong EA, Price DL, Smarr BL, Phung V, Terada M, et al. Redefining the concept of reactive astrocytes as cells that remain within their unique domains upon reaction to injury. Proc Natl Acad Sci U S A. 2006;103:17513–8.
6. Bushong EA, Martone ME, Jones YZ, Ellisman MH. Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. J Neurosci. 2002;22:183–92.
7. Ding S. In vivo astrocytic Ca^{2+} signaling in health and brain disorders. Future Neurol. 2013;8:529–54.
8. Cahoy JD, Emery B, Kaushal A, Foo LC, Zamanian JL, Christopherson KS, et al. A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J Neurosci. 2008;28:264–78.
9. Zhou M, Kimelberg HK. Freshly isolated astrocytes from rat hippocampus show two distinct current patterns and different $[K^+]o$ uptake capabilities. J Neurophysiol. 2000;84:2746–57.
10. Takata N, Hirase H. Cortical layer 1 and layer 2/3 astrocytes exhibit distinct calcium dynamics in vivo. PLoS One. 2008;3:e2525.
11. Ding S, Fellin T, Zhu Y, Lee SY, Auberson YP, Meaney DF, et al. Enhanced astrocytic Ca^{2+} signals contribute to neuronal excitotoxicity after status epilepticus. J Neurosci. 2007;27:10674–84.
12. Fellin T, Pascual O, Gobbo S, Pozzan T, Haydon PG, Carmignoto G. Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. Neuron. 2004;43:729–43.
13. Sun W, McConnell E, Pare JF, Xu Q, Chen M, Peng W, et al. Glutamate-dependent neuroglial calcium signaling differs between young and adult brain. Science. 2013;339:197–200.
14. Ding S, Wang T, Cui W, Haydon PG. Photothrombosis ischemia stimulates a sustained astrocytic Ca^{2+} signaling in vivo. Glia. 2009;57:767–76.
15. Thrane AS, Rangroo Thrane V, Zeppenfeld D, Lou N, Xu Q, Nagelhus EA, et al. General anesthesia selectively disrupts astrocyte calcium signaling in the awake mouse cortex. Proc Natl Acad Sci U S A. 2012;109:18974–9.
16. Wang X, Lou N, Xu Q, Tian GF, Peng WG, Han X, et al. Astrocytic Ca^{2+} signaling evoked by sensory stimulation in vivo. Nat Neurosci. 2006;9:816–23.
17. Nizar K, Uhlirova H, Tian P, Saisan PA, Cheng Q, Reznichenko L, et al. In vivo stimulus-induced vasodilation occurs without IP_3 receptor activation and may precede astrocytic calcium increase. J Neurosci. 2013;33:8411–22.
18. Meier SD, Kafitz KW, Rose CR, Meier SD, Kafitz KW, Rose CR. Developmental profile and mechanisms of GABA-induced calcium signaling in hippocampal astrocytes. Glia. 2008;56:1127–37.
19. Bekar LK, He W, Nedergaard M. Locus coeruleus α-adrenergic-mediated activation of cortical astrocytes in vivo. Cereb Cortex. 2008;18:2789–95.
20. Haydon PG, Carmignoto G. Astrocyte control of synaptic transmission and neurovascular coupling. Physiol Rev. 2006;86:1009–31.
21. Ni Y, Malarkey EB, Parpura V, Ni Y, Malarkey EB, Parpura V. Vesicular release of glutamate mediates bidirectional signaling between astrocytes and neurons. [Review] [150 refs]. J Neurochem. 2007;103:1273–84.
22. Agulhon C, Petravicz J, McMullen AB, Sweger EJ, Minton SK, Taves SR, et al. What is the role of astrocyte calcium in neurophysiology? Neuron. 2008;59:932–46.
23. Haydon PG. GLIA: listening and talking to the synapse. Nat Rev Neurosci. 2001;2:185–93.
24. Petravicz J, Fiacco TA, McCarthy KD. Loss of IP_3 receptor-dependent Ca^{2+} increases in hippocampal astrocytes does not affect baseline CA1 pyramidal neuron synaptic activity. J Neurosci. 2008;28:4967–73.
25. Hertle DN, Yeckel MF, Hertle DN, Yeckel MF. Distribution of inositol-1,4,5-trisphosphate receptor isotypes and ryanodine receptor isotypes during maturation of the rat hippocampus. Neuroscience. 2007;150:625–38.

26. Holtzclaw LA, Pandhit S, Bare DJ, Mignery GA, Russell JT, Holtzclaw LA, et al. Astrocytes in adult rat brain express type 2 inositol 1,4,5-trisphosphate receptors. Glia. 2002;39:69–84.
27. Sharp AH, Nucifora Jr FC, Blondel O, Sheppard CA, Zhang C, Snyder SH, et al. Differential cellular expression of isoforms of inositol 1,4,5-triphosphate receptors in neurons and glia in brain. J Comp Neurol. 1999;406:207–20.
28. Petravicz J, Boyt KM, McCarthy KD. Astrocyte IP_3R2-dependent Ca^{2+} signaling is not a major modulator of neuronal pathways governing behavior. Front Behav Neurosci. 2014;8:e384.
29. Srinivasan R, Huang BS, Venugopal S, Johnston AD, Chai H, Zeng H, et al. Ca^{2+} signaling in astrocytes from Ip3r2-/- mice in brain slices and during startle responses in vivo. Nat Neurosci. 2015;18:708–17.
30. Rossi DJ, Brady JD, Mohr C. Astrocyte metabolism and signaling during brain ischemia. Nat Neurosci. 2007;10:1377–86.
31. Stapf C, Mohr JP. Ischemic stroke therapy. Annu Rev Med. 2002;53:453–75.
32. Huang Y, McNamara JO. Ischemic stroke: “acidotoxicity” is a perpetrator.[comment]. [Review] [7 refs]. Cell. 2004;118(6):665–6.
33. Nedergaard M, Dirnagl U. Role of glial cells in cerebral ischemia. Glia. 2005;50:281–6.
34. Barber PA, Demchuk AMH. Biochemistry ischemic stroke. Adv Neurol. 2003;92:151–64.
35. Swanson RA, Ying W, Kauppinen TM. Astrocyte influences on ischemic neuronal death. Curr Mol Med. 2004;4:193–205.
36. Kimelberg HK. Astrocytic swelling in cerebral ischemia as a possible cause of injury and target for therapy. Glia. 2005;50:389–97.
37. Zheng W, Watts LT, Holstein DM, Prajapati SI, Keller C, Grass EH, et al. Purinergic receptor stimulation reduces cytotoxic edema and brain infarcts in mouse induced by photothrombosis by energizing glial mitochondria. PLoS One. 2010;5:e14401.
38. Risher WC, Croom D, Kirov SA. Persistent astroglial swelling accompanies rapid reversible dendritic injury during stroke-induced spreading depolarizations. Glia. 2012;60:1709–20.
39. Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci. 2003;4:399–415.
40. Kasischke KA, Vishwasrao HD, Fisher PJ, Zipfel WR, Webb WW. Neural activity triggers neuronal oxidative metabolism followed by astrocytic glycolysis. Science. 2004;305:99–103.
41. Brown AM. Brain glycogen re-awakened. J Neurochem. 2004;89:537–52.
42. Gurer G, Gursoy-Ozdemir Y, Erdemli E, Can A, Dalkara T. Astrocytes are more resistant to focal cerebral ischemia than neurons and die by a delayed necrosis. Brain Pathol. 2009;19:630–41.
43. Brown AM, Ransom BR. Astrocyte glycogen and brain energy metabolism. Glia. 2007;55:1263–71.
44. Panickar KS, Norenberg MD. Astrocytes in cerebral ischemic injury: morphological and general considerations. Glia. 2005;50:287–98.
45. Li H, Zhang N, Sun G, Ding S. Inhibition of the group I mGluRs reduces acute brain damage and improves long-term histological outcomes after photothrombosis-induced ischaemia. ASN Neuro. 2013;5:e00117.
46. Barreto GE, Sun X, Xu L, Giffard RG. Astrocyte proliferation following stroke in the mouse depends on distance from the infarct. PLoS One. 2011;6:e27881.
47. Pekny M, Nilsson M. Astrocyte activation and reactive gliosis. Glia. 2005;50:427–34.
48. Zhao Y, Rempe D. Targeting astrocytes for stroke therapy. Neurotherapeutics. 2010;7:439–51.
49. Gleichman AJ, Carmichael ST. Astrocytic therapies for neuronal repair in stroke. Neurosci Lett. 2014;565:47–52.
50. Choudhury GR, Ding S. Reactive astrocytes and therapeutic potential in focal ischemic stroke. Neurobiol Dis. 2016;85:234–44.
51. Ding S. Ca^{2+} signaling in astrocytes and its role in ischemic stroke. In: Parpura V, Schousboe A, Verkhratsky A, editors. Glutamate and ATP at the interface of metabolism and signaling in the brain. 11th ed. Cham: Springer; 2014. p. 189–211.

52. Choi D. Glutamate neurotoxicity and diseases of the nervous system. Neuron. 1988;1:623–34.
53. Medvedeva YV, Lin B, Shuttleworth CW, Weiss JH. Intracellular Zn^{2+} accumulation contributes to synaptic failure, mitochondrial depolarization, and cell death in an acute slice oxygen-glucose deprivation model of ischemia. J Neurosci. 2009;29:1105–14.
54. Tanaka E, Yamamoto S, Kudo Y, Mihara S, Higashi H. Mechanisms underlying the rapid depolarization produced by deprivation of oxygen and glucose in rat hippocampal CA1 neurons in vitro. J Neurophysiol. 1997;78:891–902.
55. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci. 1999;22:391–7.
56. Rossi DJ, Oshima T, Attwell D. Glutamate release in severe brain ischaemia is mainly by reversed uptake. Nature. 2000;403:316–21.
57. Phillis JW, Ren J, ÖRegan MH. Transporter reversal as a mechanism of glutamate release from the ischemic rat cerebral cortex: studies with dl-threo-β-benzyloxyaspartate. Brain Res. 2000;868:105–12.
58. Mitani A, Tanaka K. Functional changes of glial glutamate transporter GLT-1 during ischemia: an in vivo study in the hippocampal CA1 of normal mice and mutant mice lacking GLT-1. J Neurosci. 2003;23:7176–82.
59. Szydlowska K, Tymianski M. Calcium, ischemia and excitotoxicity. Cell Calcium. 2010;47:122–9.
60. Duffy S, MacVicar BA. In vitro ischemia promotes calcium influx and intracellular calcium release in hippocampal astrocytes. J Neurosci. 1996;16:71–81.
61. Dong Q, He J, Chai Z. Astrocytic Ca^{2+} waves mediate activation of extrasynaptic NMDA receptors in hippocampal neurons to aggravate brain damage during ischemia. Neurobiol Dis. 2013;58:68–75.
62. Dunwiddie TV, Masino SA. The role and regulation of adenosine in the central nervous system. Annu Rev Neurosci. 2001;24:31–55.
63. Winship IR, Murphy TH. In vivo calcium imaging reveals functional rewiring of single somatosensory neurons after stroke. J Neurosci. 2008;28:6592–606.
64. Takatsuru Y, Eto K, Kaneko R, Masuda H, Shimokawa N, Koibuchi N, et al. Critical role of the astrocyte for functional remodeling in contralateral hemisphere of somatosensory cortex after stroke. J Neurosci. 2013;33:4683–92.
65. Li H, Xie Y, Zhang N, Yu Y, Zhang Q, Ding S. Disruption of IP_3R2-mediated Ca^{2+} signaling pathway in astrocytes ameliorates neuronal death and brain damage while reducing behavioral deficits after focal ischemic stroke. Cell Calcium. 2015;58(6):565–76.
66. Zheng W, Talley Watts L, Holstein DM, Wewer J, Lechleiter JD. P2Y1R-initiated, IP_3R-dependent stimulation of astrocyte mitochondrial metabolism reduces and partially reverses ischemic neuronal damage in mouse. J Cereb Blood Flow Metab. 2013;33:600–11.
67. Kanemaru K, Kubota J, Sekiya H, Hirose K, Okubo Y, Iino M. Calcium-dependent N-cadherin up-regulation mediates reactive astrogliosis and neuroprotection after brain injury. Proc Natl Acad Sci U S A. 2013;110:11612–7.
68. Li H, Zhang N, Lin H, Yu Y, Cai QM, et al. Histological, cellular and behavioral assessments of stroke outcomes after photothrombosis-induced ischemia in adult mice. BMC Neurosci. 2014;15:58.
69. Ding S. Dynamic reactive astrocytes after focal ischemia. Neural Regen Res. 2014;9:2048–52.
70. Zhang W, Xie Y, Wang T, Bi J, Li H, Zhang LQ, et al. Neuronal protective role of PBEF in a mouse model of cerebral ischemia. J Cereb Blood Flow Metab. 2010;30:1962–71.
71. Mestriner RG, Saur L, Bagatini PB, Baptista PPA, Vaz SP, Ferreira K, et al. Astrocyte morphology after ischemic and hemorrhagic experimental stroke has no influence on the different recovery patterns. Behav Brain Res. 2015;278:257–61.
72. Wagner DC, Scheibe J, Glocke I, Weise G, Deten A, Boltze J, Kranz A. Object-based analysis of astroglial reaction and astrocyte subtype morphology after ischemic brain injury. Acta Neurobiol Exp (Wars). 2013;73:79–87.

73. Haupt C, Witte OW, Frahm C. Up-regulation of Connexin 43 in the glial scar following photothrombotic ischemic injury. Mol Cell Neurosci. 2007;35:89–99.
74. Oberheim NA, Tian GF, Han X, Peng W, Takano T, Ransom B, et al. Loss of astrocytic domain organization in the epileptic brain. J Neurosci. 2008;28:3264–76.
75. Nowicka D. Spatiotemporal dynamics of astroglial and microglial responses after photothrombotic stroke in the rat brain. Acta Neurobiol Exp. 2008;68(2):155–68.
76. Shen J, Ishii Y, Xu G, Dang TC, Hamashima T, Matsushima T, et al. PDGFR-[beta] as a positive regulator of tissue repair in a mouse model of focal cerebral ischemia. J Cereb Blood Flow Metab. 2012;32:353–67.
77. Stoll G, Jander S, Schroeter M. Inflammation and glial responses in ischemic brain lesions. Prog Neurobiol. 1998;56:149–71.
78. Schroeter M, Jander S, Stoll G. Non-invasive induction of focal cerebral ischemia in mice by photothrombosis of cortical microvessels: characterization of inflammatory responses. J Neurosci Methods. 2002;117:43–9.
79. Cramer SC. Repairing the human brain after stroke: I. Mechanisms of spontaneous recovery. Ann Neurol. 2008;63:272–87.
80. Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, et al. Genomic analysis of reactive astrogliosis. J Neurosci. 2012;32:6391–410.
81. Wanner IB, Anderson MA, Song B, Levine J, Fernandez A, Gray-Thompson Z, et al. Glial scar borders are formed by newly proliferated, elongated astrocytes that interact to corral inflammatory and fibrotic cells via STAT3-dependent mechanisms after spinal cord injury. J Neurosci. 2013;33:12870–86.
82. Ridet JL, Privat A, Malhotra SK, Gage FH. Reactive astrocytes: cellular and molecular cues to biological function. Trends Neurosci. 1997;20:570–7.
83. Colangelo AM, Alberghina L, Papa M. Astrogliosis as a therapeutic target for neurodegenerative diseases. Neurosci Lett. 2014;565:59–64.
84. Sofroniew M, Vinters H. Astrocytes: biology and pathology. Acta Neuropathol. 2010;119:7–35.
85. Kang W, Hebert J. Signaling pathways in reactive astrocytes, a genetic perspective. Mol Neurobiol. 2011;43(3):147–54.
86. Hanbury R, Ling ZD, Wuu J, Kordower JH. GFAP knockout mice have increased levels of GDNF that protect striatal neurons from metabolic and excitotoxic insults. J Comp Neurol. 2003;461:307–16.
87. Nawashiro H, Brenner M, Fukui S, Shima K, Hallenbeck JM. High susceptibility to cerebral ischemia in GFAP-null mice. J Cereb Blood Flow Metab. 2000;20:1040–4.
88. Li L, Lundkvist A, Andersson D, Wilhelmsson U, Nagai N, Pardo AC, et al. Protective role of reactive astrocytes in brain ischemia. J Cereb Blood Flow Metab. 2007;28:468–81.
89. Liu Z, Li Y, Cui Y, Roberts C, Lu M, Wilhelmsson U, et al. Beneficial effects of gfap/vimentin reactive astrocytes for axonal remodeling and motor behavioral recovery in mice after stroke. Glia. 2014;62:2022–33.
90. Kaminska B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy-from molecular mechanisms to therapeutic benefits. Biochim Biophys Acta. 2005;1754:253–62.
91. Cuadrado A, Nebreda AR. Mechanisms and functions of p38 MAPK signalling. Biochem J. 2010;429:403–17.
92. Nozaki K, Nishimura M, Hashimoto N. Mitogen-activated protein kinases and cerebral ischemia. Mol Neurobiol. 2001;23:1–19.
93. Ferrer I, Friguls B, Dalfo E, Planas AM. Early modifications in the expression of mitogen-activated protein kinase (MAPK/ERK), stress-activated kinases SAPK/JNK and p38, and their phosphorylated substrates following focal cerebral ischemia. Acta Neuropathol. 2003;105:425–37.
94. Krupinski J, Slevin M, Marti E, Catena E, Rubio F, Gaffney J. Time-course phosphorylation of the mitogen activated protein (MAP) kinase group of signalling proteins and related molecules following middle cerebral artery occlusion (MCAO) in rats. Neuropathol Appl Neurobiol. 2003;29:144–58.

95. Roy Choudhury G, Ryou MG, Poteet E, Wen Y, He R, Sun F, et al. Involvement of p38 MAPK in reactive astrogliosis induced by ischemic stroke. Brain Res. 2014;1551:45–58.
96. Yoon K, Gaiano N. Notch signaling in the mammalian central nervous system: insights from mouse mutants. Nat Neurosci. 2005;8:709–15.
97. Marumo T, Takagi Y, Muraki K, Hashimoto N, Miyamoto S, Tanigaki K. Notch signaling regulates nucleocytoplasmic Olig2 translocation in reactive astrocytes differentiation after ischemic stroke. Neurosci Res. 2013;75:204–9.
98. Shimada IS, Borders A, Aronshtam A, Spees JL. Proliferating reactive astrocytes are regulated by Notch-1 in the peri-infarct area after stroke. Stroke. 2011;42:3231–7.
99. LeComte MD, Shimada IS, Sherwin C, Spees JL. Notch1-STAT3-ETBR signaling axis controls reactive astrocyte proliferation after brain injury. Proc Natl Acad Sci U S A. 2015;112:8726–31.
100. Komitova M, Serwanski DR, Lu QR, Nishiyama A. NG2 cells are not a major source of reactive astrocytes after neocortical stab wound injury. Glia. 2011;59:800–9.
101. Magnusson JP, Goritz C, Tatarishvili J, Dias DO, Smith EMK, Lindvall O, et al. A latent neurogenic program in astrocytes regulated by Notch signaling in the mouse. Science. 2014;346:237–41.
102. Nicolas CS, Amici M, Bortolotto ZA, Doherty A, Csaba Z, Fafouri A, et al. The role of JAK-STAT signaling within the CNS. JAKSTAT. 2013;2:e22925.
103. Herrmann JE, Imura T, Song B, Qi J, Ao Y, Nguyen TK, et al. STAT3 is a critical regulator of astrogliosis and scar formation after spinal cord injury. J Neurosci. 2008;28:7231–43.
104. Sofroniew MV. Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci. 2009;32:638–47.
105. Karimi-Abdolrezaee S, Billakanti R. Reactive astrogliosis after spinal cord injury-beneficial and detrimental effects. Mol Neurobiol. 2012;46:251–64.
106. Lei C, Deng J, Wang B, Cheng D, Yang Q, Dong H, et al. Reactive oxygen species scavenger inhibits STAT3 activation after transient focal cerebral ischemia-reperfusion injury in rats. Anesth Analg. 2011;113:153–9.
107. Zhu H, Zou L, Tian J, Du G, Gao Y. SMND-309, a novel derivative of salvianolic acid B, protects rat brains ischemia and reperfusion injury by targeting the JAK2/STAT3 pathway. Eur J Pharmacol. 2013;714:23–31.
108. Suzuki S, Tanaka K, Nogawa S, Dembo T, Kosakai A, Fukuuchi Y. Phosphorylation of signal transducer and activator of transcription-3 (Stat3) after focal cerebral ischemia in rats. Exp Neurol. 2001;170:63–71.
109. Wen TC, Peng H, Hata R, Desaki J, Sakanaka M. Induction of phosphorylated-Stat3 following focal cerebral ischemia in mice. Neurosci Lett. 2001;303:153–6.
110. Justicia C, Gabriel C, Planas AM. Activation of the JAK/STAT pathway following transient focal cerebral ischemia: signaling through Jak1 and Stat3 in astrocytes. Glia. 2000;30:253–70.
111. Choi JS, Kim SY, Cha JH, Choi YS, Sung KW, Oh ST, et al. Upregulation of gp130 and STAT3 activation in the rat hippocampus following transient forebrain ischemia. Glia. 2003;41:237–46.
112. Sriram K, Benkovic SA, Hebert MA, Miller DB, O'Callaghan JP. Induction of gp130-related cytokines and activation of JAK2/STAT3 pathway in astrocytes precedes up-regulation of glial fibrillary acidic protein in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of neurodegeneration: key signaling pathway for astrogliosis in vivo? J Biol Chem. 2004;279:19936–47.
113. O'Callaghan JP, Kelly KA, VanGilder RL, Sofroniew MV, Miller DB. Early activation of STAT3 regulates reactive astrogliosis induced by diverse forms of neurotoxicity. PLos One. 2014;9:e102003.
114. Krupinski J, Kumar P, Kumar S, Kaluza J. Increased expression of TGF-beta 1 in brain tissue after ischemic stroke in humans. Stroke. 1996;27:852–7.
115. Wang X, Yue TL, White RF, Barone FC, Feuerstein GZ. Transforming growth factor-beta 1 exhibits delayed gene expression following focal cerebral ischemia. Brain Res Bull. 1995;36:607–9.

116. Ruocco A, Nicole O, Docagne F, Ali C, Chazalviel L, Komesli S, et al. A transforming growth factor-beta antagonist unmasks the neuroprotective role of this endogenous cytokine in excitotoxic and ischemic brain injury. J Cereb Blood Flow Metab. 1999;19:1345–53.
117. Ali C, Docagne F, Nicole O, Lesne S, Toutain J, Young A, et al. Increased expression of transforming growth factor-beta after cerebral ischemia in the baboon: an endogenous marker of neuronal stress? J Cereb Blood Flow Metab. 2001;21:820–7.
118. Prehn JH, Backhauss C, Krieglstein J. Transforming growth factor-beta 1 prevents glutamate neurotoxicity in rat neocortical cultures and protects mouse neocortex from ischemic injury in vivo. J Cereb Blood Flow Metab. 1993;13:521–5.
119. Buisson A, Nicole O, Docagne F, Sartelet H, MacKenzie ET, Vivien D. Up-regulation of a serine protease inhibitor in astrocytes mediates the neuroprotective activity of transforming growth factor beta1. FASEB J. 1998;12:1683–91.
120. Docagne F, Nicole O, Marti HH, MacKenzie ET, Buisson A, Vivien D. Transforming growth factor-beta1 as a regulator of the serpins/t-PA axis in cerebral ischemia. FASEB J. 1999;13:1315–24.
121. Kang W, Balordi F, Su N, Chen L, Fishell G, Hebert JM. Astrocyte activation is suppressed in both normal and injured brain by FGF signaling. Proc Natl Acad Sci U S A. 2014;111:E2987–95.
122. Cekanaviciute E, Fathali N, Doyle KP, Williams AM, Han J, Buckwalter MS. Astrocytic transforming growth factor-beta signaling reduces subacute neuroinflammation after stroke in mice. Glia. 2014;62:1227–40.
123. Anderson MA, Ao Y, Sofroniew MV. Heterogeneity of reactive astrocytes. Neurosci Lett. 2014;565:23–9.
124. Zhang Y, Chen K, Sloan SA, Bennett ML, Scholze AR, O'Keeffe S, et al. An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. J Neurosci. 2014;34:11929–47.
125. Doyle JP, Dougherty JD, Heiman M, Schmidt EF, Stevens TR, Ma G, et al. Application of a translational profiling approach for the comparative analysis of CNS cell types. Cell. 2008;135:749–62.
126. Tobin MK, Bonds JA, Minshall RD, Pelligrino DA, Testai FD, Lazarov O. Neurogenesis and inflammation after ischemic stroke: what is known and where we go from here. J Cereb Blood Flow Metab. 2014;34:1573–84.
127. Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, et al. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell. 2002;110:429–41.
128. Jin K, Minami M, Lan JQ, Mao XO, Batteur S, Simon RP, et al. Neurogenesis in dentate subgranular zone and rostral subventricular zone after focal cerebral ischemia in the rat. Proc Natl Acad Sci U S A. 2001;98:4710–5.
129. Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med. 2002;8:963–70.
130. Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell. 1999;97:703–16.
131. Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A. Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci. 2001;21:7153–60.
132. Buffo A, Rite I, Tripathi P, Lepier A, Colak D, Horn AP, et al. Origin and progeny of reactive gliosis: a source of multipotent cells in the injured brain. Proc Natl Acad Sci U S A. 2008;105:3581–6.
133. Sirko S, Behrendt G, Johansson P, Tripathi P, Costa MR, Bek S, et al. Reactive glia in the injured brain acquire stem cell properties in response to sonic hedgehog. Cell Stem Cell. 2013;12:426–39.
134. Robel S, Berninger B, Gotz M. The stem cell potential of glia: lessons from reactive gliosis. Nat Rev Neurosci. 2011;12:88–104.

135. Dimou LG, Götz M. Glial cells as progenitors and stem cells: new roles in the healthy and diseased brain. Physiol Rev. 2014;94:709–37.
136. Shimada IS, LeComte MD, Granger JC, Quinlan NJ, Spees JL. Self-renewal and differentiation of reactive astrocyte-derived neural stem/progenitor cells isolated from the cortical peri-infarct area after stroke. J Neurosci. 2012;32:7926–40.
137. Ohab JJ, Fleming S, Blesch A, Carmichael ST. A neurovascular niche for neurogenesis after stroke. J Neurosci. 2006;26:13007–16.
138. Buffo A, Vosko MR, Ertork D, Hamann GF, Jucker M, Rowitch D, et al. Expression pattern of the transcription factor Olig2 in response to brain injuries: implications for neuronal repair. Proc Natl Acad Sci U S A. 2005;102:18183–8.
139. Duan CL, Liu CW, Shen SW, Yu Z, Mo JL, Chen XH, et al. Striatal astrocytes transdifferentiate into functional mature neurons following ischemic brain injury. Glia. 2015;63:1660–70.
140. Young CC, van der Harg JM, Lewis NJ, Brooks KJ, Buchan AM, Szele FG. Ependymal ciliary dysfunction and reactive astrocytosis in a reorganized subventricular zone after stroke. Cereb Cortex. 2013;23:647–59.
141. Su Z, Niu W, Liu ML, Zou Y, Zhang CL. In vivo conversion of astrocytes to neurons in the injured adult spinal cord. Nat Commun. 2014;5:e3338.
142. Niu W, Zang T, Zou Y, Fang S, Smith DK, Bachoo R, et al. In vivo reprogramming of astrocytes to neuroblasts in the adult brain. Nat Cell Biol. 2013;15:1164–75.
143. Berninger B, Costa MR, Koch U, Schroeder T, Sutor B, Grothe B, et al. Functional properties of neurons derived from in vitro reprogrammed postnatal astroglia. J Neurosci. 2007;27:8654–64.
144. Heinrich C, Blum R, Gascón S, Masserdotti G, Tripathi P, Sánchez R, et al. Directing astroglia from the cerebral cortex into subtype specific functional neurons. PLoS Biol. 2010;8:e1000373.
145. Masserdotti G, Gillotin S, Sutor B, Drechsel D, Irmler M, Jorgensen H, et al. Transcriptional mechanisms of proneural factors and REST in regulating neuronal reprogramming of astrocytes. Cell Stem Cell. 2015;17:74–88.
146. Guo Z, Zhang L, Wu Z, Chen Y, Wang F, Chen G. In vivo direct reprogramming of reactive glial cells into functional neurons after brain injury and in an Alzheimer's disease model. Cell Stem Cell. 2013;14:188–202.
147. Heinrich C, Gascon S, Masserdotti G, Lepier A, Sanchez R, Simon-Ebert T, et al. Generation of subtype-specific neurons from postnatal astroglia of the mouse cerebral cortex. Nat Protoc. 2011;6:214–28.
148. Addis RC, Hsu FC, Wright RL, Dichter MA, Coulter DA, Gearhart JD. Efficient conversion of astrocytes to functional midbrain dopaminergic neurons using a single polycistronic vector. PLoS One. 2011;6:e28719.
149. Corti S, Nizzardo M, Simone C, Falcone M, Donadoni C, Salani S, et al. Direct reprogramming of human astrocytes into neural stem cells and neurons. Exp Cell Res. 2012;318:1528–41.
150. Li X, Zuo X, Jing J, Ma Y, Wang J, Liu D, et al. Small-molecule-driven direct reprogramming of mouse fibroblasts into functional neurons. Cell Stem Cell. 2015;17:195–203.
151. Hu W, Qiu B, Guan W, Wang Q, Wang M, Li W, et al. Direct conversion of normal and alzheimer's disease human fibroblasts into neuronal cells by small molecules. Cell Stem Cell. 2015;17:204–12.

Microglia: A Double-Sided Sword in Stroke

Hong Shi, Mingyue Xu, Yejie Shi, Yanqin Gao, Jun Chen, and Xiaoming Hu

Abbreviations

CD200R	CD200 receptor
CNS	Central nervous system
CX3CR1	CX3C chemokine receptor 1

H. Shi
State Key Laboratory of Medical Neurobiology and Institute of Brain Sciences, Fudan University, Shanghai, China

Department of Anesthesiology of Shanghai Pulmonary Hospital, Tongji University, Shanghai, China
e-mail: 13651958255@139.com

M. Xu • Y. Gao
State Key Laboratory of Medical Neurobiology and Institute of Brain Sciences, Fudan University, Shanghai, China

Center of Cerebrovascular Disease Research, University of Pittsburgh School of Medicine, 200 Lothrop Street, Pittsburgh, PA 15213, USA
e-mail: elune2009@126.com; yqgao@shmu.edu.cn

Y. Shi
Center of Cerebrovascular Disease Research, University of Pittsburgh School of Medicine, 200 Lothrop Street, Pittsburgh, PA 15213, USA
e-mail: shiy3@upmc.edu

J. Chen • X. Hu (✉)
Department of Neurology, University of Pittsburgh, Pittsburgh, PA, USA

Pittsburgh Institute of Brain Disorders and Recovery, University of Pittsburgh, Pittsburgh, PA, USA

State Key laboratory of Medical Neurobiology, Fudan University, Shanghai, China
e-mail: chenj2@upmc.edu; hux2@upmc.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_7

DCs	Dendritic cells
Gals	Galectins
HMGB1	High-mobility group box 1
HSP	Heat shock protein
IFN-γ	Interferon-gamma
IgSF	Immunoglobulin superfamily
iNOS	Inducible nitric oxide synthase
KO	Knockout
NO	Nitric oxide
Nox	NADPH oxidase
NSC	Neural stem cells
OPC	Oligodendrocyte progenitor cell
pro-MMP-9	pro-matrix metalloproteinase-9
RAGE	Receptor for advanced glycation endproducts
ROS	Reactive oxygen species
SGZ	Subgranular zone
SVZ	Subventricular zone
TGF-β	Transforming growth factor-β
TIMP-1	Tissue inhibitor of metalloproteinases-1
TNF-α	Tumor necrosis factor-α
TREM	Triggering receptors expressed on myeloid cells
VEGF	Vascular endothelial growth factor

1 Introduction

Microglia were first described by Pio del Rio Hortega as early as in 1919 [1]. Although the precise origin of microglia is still in debate up to date, it is commonly accepted that the microglial progenitors are derived from the yolk sac. They migrate into the central nervous system (CNS) during early embryogenesis and give rise to microglia throughout the brain parenchyma [2, 3].

Microglia have multiple vital functions in the CNS. Under physiological state, microglia display a ramified phenotype, characterized by a small cell body and numerous branched processes. These so-called “resting microglia” actively survey the surrounding environment and respond promptly to even subtle changes in the CNS. When a microvascular or an isolated neuron is impaired, the surveying microglia may offer structural and trophic support to them so that the normal functions could be preserved. Microglia also remove the sick individual neurons or other CNS cells. These daily functions of microglia are called housekeeping activity, which contributes to the maintenance of CNS homeostasis [1, 4–6]. Upon the noxious cues such as brain infection or injury, microglia expand their cell bodies and retract their processes, becoming reactive microglia. These reactive microglia are the first line of defense in the compromised brain. Due to the importance of microglia

in brain homeostasis and brain injuries, their activities are well-regulated by a large group of surface receptors [7].

Activation of microglia occurs within 1 h after stroke, much earlier than the infiltration of other immune cells from the periphery [8]. Interestingly, the activated microglia appear to be double-edged swords in the battle of brain injuries. The beneficial effects of microglia include cell debris clearance and trophic factor production [4, 9–11]. In contrast, overactivated microglia can expand tissue damage and hinder CNS repair by releasing large amount of inflammatory mediators or free radicals [11–13]. These seemingly contradictory functions of microglia might be at least partially due to their diverse phenotypes in response to different microenvironmental cues. Actually, microglia exhibit a spectrum of different but overlapping functional phenotypes upon activation. In a conceptual framework, M1 (classically activated) and M2 (alternatively activated) dichotomy have been used to represent two extreme activation states of microglia. Although being oversimplified, the M1 and M2 classification is a useful concept to understand microglia functional status in the progress of injury/repair and to explore therapeutic strategies targeting microglia responses (Fig. 1) [14].

2 Surface Receptors That Modulate Microglial Responses in the Ischemic Brain

Microglia make contact with other cells via recognizing the signals released after CNS insults. Two types of signals, the so-called "Off" and "On" signals are identified by different microglial surface receptors (Table 1). The "Off" signals occur constantly under normal physiological conditions, maintaining microglia at the quiescent surveillant state. In contrast, the "On" signals are released under various pathological conditions, leading to microglial activation and mobilization. Several receptors have been identified to recognize the "On" or "Off" signals released in the ischemic brain and play an important role in stroke pathology. In this section, we will discuss some of these microglial receptors, including the "Off" receptors TREM2, CD200, CX3CR1, as well as the "On" receptors RAGE and galectins.

2.1 Triggering Receptors Expressed on Myeloid Cells

Triggering receptors expressed on myeloid cells (TREM) belong to the immunoglobulin superfamily (IgSF), which plays an important role in regulating immunity responses and medicating cell surface recognition. It is expressed in a variety of immune cells, including microglia, dendritic cells (DCs), monocytes, and neutrophils [15]. Among these cells, TREM2 is the most relevant to microglia due to its constitutive expression.

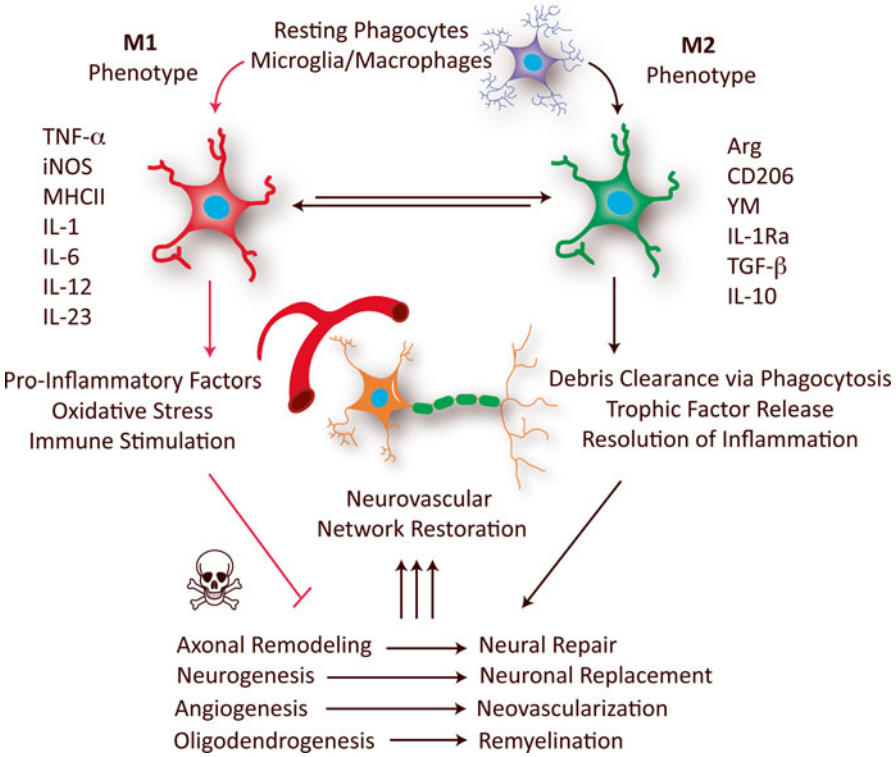

Fig. 1 Microglia response to different stimuli with either M1 or M2 phenotype. M1 microglia are characterized by production and secretion of proinflammation factors such as TNFα, iNOS, MHCII, IL-1, IL-6, IL-2, IL-23, etc., leading to exacerbated tissue damage. In contrast, M2 microglia polarization are characterized with the expression of Arg, CD206, YM, IL-1Ra, IL-10, and TGF-β, etc. M2 microglia improve brain repair/regeneration by enhancing phagocytosis, releasing tropic factors, and resolving cerebral inflammation. Arg: arginase; IL: interleukin; IL-1Ra: IL-1 receptor antagonist; iNOS: inducible nitric oxide synthase; MHCII: major histocompatibility complex II; TGF-β, transforming growth factor-β; TNFα: tumor necrosis factor-α. (Adapted from Hu et al., Nat Rev Neurol. 2015; 11(1):56–64.)

TREM2 migrates to the cell surface from the intracellular pool upon stimulation by ionomycin or interferon-gamma (IFN-γ) [16, 17], promoting microglial anti-inflammatory responses and decreasing the production of TNF-α or IL-6 [18, 19]. Loss of a single copy of TREM2 alters the morphological phenotype of microglia and induces neurotoxicity [20]. Studies also reveal increased microglial expression of TREM2 after ischemic stroke. In in vitro ischemic model, TREM2 deficiency in microglia ameliorates microglial activation and inhibits the phagocytosis of injured neurons. In a mouse model of stroke, TREM2 knockout (KO) results in reduced phagocytosis and impaired infarct brain tissue resorption. As a result, TREM2 KO mice exhibited worsened neurological recovery and increased brain tissue loss in

Table 1 Microglial surface receptors in stroke

Function	Receptor type	Receptor name	References
"Off"	Immunoglobulin superfamily	TREM2	[21, 22, 105]
	Chemokine receptors	CX3CR1	[34, 106–109]
	Immunoglobulin superfamily	CD200R	[26, 110, 111]
"On"	Immunoglobulin superfamily	RAGE	[44, 112–114]
	Chemokine receptors	CCR2	[115–119]
		CCR5	[120–123]
	Purinergic receptors	A2A receptor	[124–126]
		A3 receptor	[127, 128]
		P2X4	[129, 130]
		P2X7	[131–133]
	Carbohydrate-binding receptor	Galectins	[48, 51]
		Selectins	[134–136]
	Pattern recognition receptors	TLRs	[137]
	Scavenger receptor	CD36	[138–140]
	Phosphatidylserine receptors		[141–143]

the ipsilateral hemisphere [21, 22]. In contrast, TREM2 overexpression promotes microglial migration, lipid catabolism, and the phagocytosis of neuronal cell debris in stroke as well as in Alzheimer's disease (AD) [17, 23].

The ligands of TREM2 have not yet been identified, which prevents the understanding of microglial activation through this receptor. Multiple types of cultured neuronal cells have been shown to bind to TREM2-Fc fusion protein but not TREM1-Fc, suggesting that TREM2 ligand(s) are constitutively expressed on healthy neurons [24]. The identity of TREM2 ligand(s), however, remains elusive. Interestingly enough, a recent study showed that TREM2-Fc fusion protein pulled down nucleic acids from ischemic brain lysate, suggesting that nucleic acids might be a potential ligand for TREM2 in brain ischemia [21]. Another study has shown that heat shock protein (HSP) 60 expresses on neuron and astrocytes surface and might be a TREM2-binding protein [25]. Accordingly, treatment with HSP60 can stimulate microglial phagocytosis effectively in a TREM2-dependent manner.

2.2 CD200 Receptor

CD200 receptor (CD200R) is another member of the immunoglobulin receptor family. CD200R expression is restricted to cells of the myeloid lineage, including CNS microglia [26]. CD200, the sole ligand to CD200R, is expressed by a variety of cells including neurons. The CD200-CD200R signaling plays a critical role in keeping microglia at the physiological "resting" state. Microglia in CD200-deficient

mice display spontaneous activation, characterized by morphological changes and upregulation of CD11b and CD45 [27]. In experimental autoimmune encephalomyelitis (EAE) and experimental Parkinson's disease (PD), disruption of the CD200-CD200R binding results in enhanced microglial activation and worsens the outcome [28]. Shortly after ischemic injury, CD200 mRNA is decreased, correlating with enhanced microglial proinflammatory activation [29]. These evidence strongly support the role of CD200R signaling in the maintenance of immune homeostasis in the CNS.

2.3 CX3CR1

CX3CR1 is a G-protein-coupled chemokine receptor expressed mainly in microglia in the CNS. Its unique ligand CX3CL1 is highly expressed in neurons, CX3CR1 has been shown to play an important role in neuron–microglia crosstalk and the maintenance of CNS homeostasis. CX3CR1 participates in microglia-mediated synaptic pruning [30]. In addition, CX3CR1 regulates a variety of microglial functions, including migration, proliferation, adhesion, phagocytosis, and inflammatory cytokine production [31, 32]. The role of CX3CR1 signaling in CNS disorders is controversial. On the one hand, CX3CR1-deficient microglia produce less TNF-α, NO and superoxide in AD [33]. In models of ischemic brain injuries, inhibition of CX3CR1 signaling has been shown to be neuroprotective by reducing activation of resident microglia and inhibiting recruitment of peripheral macrophages [34, 35]. On the other hand, some studies found that microglia without CX3CR1 are neurotoxic due to the loss of inhibitory signaling [36]. The role of the CX3CR1-CX3CL1 signaling may be context-dependent under different pathological conditions and in different stages of disease progression.

2.4 Receptor for Advanced Glycation Endproducts

Receptor for advanced glycation endproducts (RAGE) belongs to the immunoglobulin superfamily and is highly expressed in immune cells [37]. RAGE exaggerates tissue injury under various pathological conditions by potentiating host immune responses [38]. Microglial RAGE recognizes ligands such as high-mobility group box 1 (HMGB1), S100 [39, 40], Aβ, and AGE [41, 42]. The role of microglial RAGE in brain injuries has been reported. The expression of RAGE in macrophages/microglia increased in active lesions while its ligand, S100β expression was strikingly upregulated in reactive astrocytes in a demyelination model [40]. Another study showed that HMGB1 is released from injured neurons after ischemia and contributes to tissue injury through its interaction with RAGE [43]. HMGB1-RAGE signaling results in functional exhaustion of lymphocytes and mature monocytes

after extensive ischemia, which is a hallmark of immune suppression [43, 44]. These studies suggest that HMGB1-RAGE signaling is critical for microglia/macrophage activation upon neuronal necrosis.

2.5 Galectins

Galectins (Gals) are a family of β-galactoside-binding lectins. They bind to cell surface to form multivalent complexes and induce intracellular signals regulating cell survival and differentiation [45, 46]. Among all the Gals, Galectin-3 (Gal-3) has been shown to be important in stroke pathology. Gal-3 has been shown to be expressed on microglia and acts as an endogenous ligand of TLR4. Depletion of Gal-3 induced an anti-inflammatory and neuroprotective effects after global brain ischemia, suggesting a detrimental role of this receptor in ischemic stroke [47]. However, other studies argued that Gal-3 expression might have beneficial effects in the ischemic brain. Gal-3 gene deletion prevented VEGF upregulation after MCAO, which inhibited increases in endothelial proliferation and angiogenesis in the striatum [48, 49]. Another study proved that Gal-3 mediated angiogenesis and microglia migration [50]. In addition, Gal-3 modulates microglial response by upregulating phagocytic receptor TREM-2b during cuprizone-induced demyelination which can help the myelination process by enhancing oligodendrocyte differentiation [51, 52].

Recent research have identified more and more surface receptors on microglia mediating microglial recognition of "On" or "Off" signals in the ischemic brain. Integrated actions of these receptors result in fine-tuned microglial functions including phagocytosis, cell migration, inflammatory mediation and trophic factor release. These tightly regulated microglia play diverse and dynamic functions in the progress of stroke and in the process of brain repair.

3 Dual Roles of Microglia in the Ischemic Brain

Detrimental effects of activated microglia in the ischemic brain. It is well known that excessive microglial activation may lead to expansion of ischemic brain damage and deterioration of neurological outcomes [53, 54]. The toxicity of microglia, especially the M1 microglia, is mediated by the release of a variety of harmful substances, including nitric oxide (NO), reactive oxygen species (ROS), and proinflammatory cytokines [55]. In addition, overactivated microglia impair post-stroke neurogenesis [12, 13], prevent axon regeneration [56], and limit the efficacy of thrombolytic therapy [57].

Proinflammatory cytokines/chemokine-mediated neurotoxicity. Acute brain injuries lead to rapid damage to CNS cells including endothelial cells, astrocytes

and neurons. Those damaged cells release a set of activation signals such as ATP, glutamate, HMBG1 et al. In response to these alarming signals, microglia may release large amount of proinflammatory cytokines including IL-1, IL-6, IL-12, and TNF-α. The accumulation of these cytokines may exacerbate brain damage. TNF-α is a typical and potent proinflammatory cytokine released from activated microglia. It plays a crucial role in cerebral ischemia and brain inflammation. TNF-α deficiency is associated with reduced microglial population size and Toll-like receptor 2 expression, which can influence the neuronal response to injury [58]. TNF-α has also been demonstrated to induce cell death via caspase-8-mediated apoptosis and RIPK1-mediated necroptosis [59, 60]. Moreover, microglial TNF-α has been reported to block both neurogenesis and oligodendrogenesis [61].

In addition to the secretion of proinflammation cytokines, microglia also release chemokines to recruit peripheral leukocytes. For example, microglia secretes CCL2 when triggered by the proinflammatory stimuli such as IFN-γ, TNF-α, and IL-1β [62]. CCL2 is a potent chemokine for peripheral monocyte recruitment [63]. Once reaching the injured site, monocytes further differentiated into activated macrophages, and contribute to tissue damage. CCL2 or CCR2 knockout mice exhibited reduced number of phagocytic monocytes and smaller infarcts after middle cerebral artery occlusion. Therefore, microglia-released CCL2 may contribute to the recruitment of monocytes and the exacerbation of tissue damage [64].

Free radical-mediated neurotoxicity. Oxidative stress is important in ischemic/reperfusion injury following stroke [65]. Activated microglia, together with intracellular organelles of injured cells and infiltrating immune cells, are main sources of ROS in the ischemic brain [66, 67]. NADPH oxidase (Nox) is the major enzyme that catalyzes the production of ROS. Nox generates the highly reactive free radical, superoxide, via its Nox2 catalytic subunit [68]. The innate immune cells including microglia have been reported to express this enzyme. Apocynin, a known antioxidant and inhibitor of Nox2 NADPH, significantly reduced infarct volume when applied 1 h prior to stroke or 24 or 48 h after stroke [69].

Activated microglia also upregulate inducible nitric oxide synthase (iNOS) and produce NO. ROS can react with NO to form peroxynitrite, a more toxic product that cause cell damage. Iadecola C et al. have demonstrated that inhibiting iNOS was able to ameliorate brain damage after stroke [70, 71], suggesting the importance of NO and derivates in the ischemic brain injury.

Microglial inhibition of axonal regeneration. It is noted that the loss of neurological function after ischemic brain injury might be attributed to the interruption of axonal connectivity rather than the neuronal loss. Activated microglia/macrophages have been shown to hinder axonal regeneration by physical cell–cell interactions with neurons [72]. M1 Microglia activated by LPS inhibited neurite outgrowth and induced growth cone collapse of cortical neurons in vitro, a pattern that was only observed when there was direct contact between microglia and neurons [73]. These studies suggest that microglia/macrophages play a role in the failure of axonal regeneration in the CNS.

3.1 *Beneficial Effects of Activated Microglia*

Resolution of local inflammation While the M1 microglia promote local inflammation, the M2 microglia have been proposed to play an anti-inflammatory role. In the normal CNS, microglia have been documented to produce anti-inflammation cytokines such as IL-4 and TGF-β1 [74]. Zhou et al. also demonstrated that IL-4-induced M2 microglia increase the expression and secretion of TGF-β2 [75], which may subsequently involve in the resolution of local inflammation. In addition to direct secretion of anti-inflammation cytokines, microglial clearance of cell debris through phagocytosis also helps to mitigate successive inflammation. Phagocytosis of apoptotic cells are mediated by the interaction of engulfment receptors on microglia and the "eat me" signal from apoptotic cells [76–78].

Activated microglia in oligodendrogenesis. Oligodendrocytes are myelinating glial cells that ensheath axons, ensuring fast and saltatory impulse propagation. Oligodendrocytes are differentiated from the oligodendrocyte progenitor cells (OPCs), which disperse throughout the adult brain. OPCs continue to proliferate in healthy adult brain and generate oligodendrocytes. Recent study demonstrated that those adult-born OLs could remodel existing myelin and play a role in myelin homeostasis [79]. After stroke, OPCs are activated and migrate toward the infarct core, accounting for the recovery of oligodendrocytes and remyelination around the peri-infarct core [80].

Accumulating studies have highlighted the importance of microglia-released trophic factors in the process of remyelination after brain injure. IGF-1 secreted from activated microglia has been reported to induce hippocampus-derived neural progenitor cells to differentiate into oligodendrocytes in adult rats, and protects immature oligodendrocytes from glutamate-mediated apoptosis [81, 82]. Moreover, IL-1β-difficiency mice devoid of microglial IGF-1 displayed impaired OPC differentiation into mature oligodendrocytes, leading to the remyelination failure [83]. Lalive and colleagues demonstrated that HGF released from microglia is both a chemotactic and differentiation factor for OPCs in injured mature CNS [84]. In addition, microglial CNTF has also been reported to provide a protective role in the remyelination phase [85, 86]. A recent study with specific microglial phenotype depletion further confirmed that M2 microglia promote, while M1 microglia impair OPC differentiation and remyelination.

Microglia in neurogenesis. Microglia play an important role in neurogenesis during brain development. Microglial cells display special amoeboid morphology in early developing brain. These amoeboid microglia possess high phagocytic capacity and actively clear the apoptotic neurons to ensure correct cytoarchitecture [87]. In addition, microglia actively secrete pro-apoptotic factors to induce neuron death during development. For example, NGF secreted from microglia has been reported to promote the death of neurotrophin receptor p75-positive neurons in retina [88], and microglial TNF-α-induced cell death was demonstrated to control the number of hippocampal progenitor cells [89]. Moreover, microglia release high levels of superoxide ions to promote neuronal death in the cerebellum [90].

Ischemic brain injury triggers neurogenesis in the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) lining the lateral ventricles [91]. Microglia has been demonstrated to actively promote post-stroke neurogenesis. In rat, treatment of minocycline immediately after transient cerebral ischemia suppressed microglial activation, leading to significantly decreased neurogenesis [92]. In vitro study by Walton et al. confirmed that coculture with microglia or their conditioned medium greatly enhanced neurogenesis of neural stem cells (NSC) [93].

Microglia secret an array of factors to promote neurogenesis. Liao et al. reported that microglial cells can interact with tenascin-R and secrete NGF, BDNF, and TGFβ to promote NSC proliferation and differentiation into neurons [94]. Battista et al. have also reported that activated microglia secrete TGFβ and promote neurogenesis in the dentate gyrus of the hippocampus in adrenalectomized animals [95]. Moreover, microglial IGF-1 is known to suppress apoptosis and increase proliferation and differentiation of neural stem cells [9]. Taken together, those studies suggested an active role of microglia in the recovery course after stroke by enhancing neurogenesis.

Microglia in angiogenesis. In the developing CNS, microglia progenitors derived from the yolk sac invade brain parenchyma before vascularization takes place, and has been proposed to promote the formation of brain blood vessels [96]. An evidence for the function of microglia in angiogenesis comes from the PU.1-deficient mice. PU.1 is a transcription factor that is indispensable for microglia development. Mice with PU.1 deficiency display impaired microglial development, which is accompanied by reduced vascular branching and complexity [97]. Kubota et al. showed that CSF1 op/op mice with inactive CSF1 displayed reduced retinal microglia and a significant decrease in branching of the primary vascular plexus in developing retina [98]. Similarly, depletion of retinal microglia by intravitreal clodronate liposome application led to reduced number of retinal microglia associated with decreased retinal vascular density [99]. Those studies strongly suggested an angiogenic role of microglia in the developing brain. The function of microglia in angiogenesis in the ischemic brain, however, has not been well-addressed.

To date, the molecular mechanisms underlying microglial promotion of angiogenesis remain elusive. Rymo et al. showed that coculture of microglia with aortic ring stimulated vessel sprouting, and this effect was dependent on soluble factors released from microglia rather than direct cell–cell interaction [100]. Recently, Li et al. demonstrated that microglial TNF-α upregulate the production of ephrin-A3 and ephrin-A4 in brain microvascular endothelium cells, resulting in the formation of capillary-like tube in vitro [101]. M2 microglia/macrophages produce robust pro-angiogenic factors such as vascular endothelial growth factor (VEGF) [102] and IL-8 [103]. M2 macrophages also release pro-matrix metalloproteinase-9 (pro-MMP-9) in a unique, tissue inhibitor of metalloproteinases-1 (TIMP-1)-free form to enhance their angiogenic capacity [104]. Those studies suggested that microglia may promote brain angiogenesis through an array of soluble factors. Further studies to identify those factors are warranted.

4 Conclusions

Microglia play diverse roles in the injured brain. Our current understanding of microglial functions in the ischemic brain is far from complete. Multiple lines of evidence suggest dynamic changes in microglia after stroke, with a trend of phenotype switch from protective M2-like polarity to toxic M1-like polarity [12, 55]. Such transition not only explains the dual-faced nature of microglia, but also provides targets for immunomodulatory therapies after stroke. Further identification of the microglial receptors and/or signaling pathways that are in charge of phenotype switch is essential for the phenotypic research in the stroke field.

Sources of Funding X Hu is supported by a NIH/National Institute of neurological disorders and stroke (NINDS) grant (NS092618) and a grant from the American Heart Association (13SDG14570025); J Chen is supported by the NIH/NINDS grants (NS095671 and NS089534) and a VA research career scientist award; Y Gao is supported by the Chinese Natural Science Foundation grants (81171149 and 81371306).

References

1. Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. Physiol Rev. 2011;91(2):461–553.
2. Chan WY, Kohsaka S, Rezaie P. The origin and cell lineage of microglia: new concepts. Brain Res Rev. 2007;53(2):344–54.
3. Saijo K, Glass CK. Microglial cell origin and phenotypes in health and disease. Nat Rev Immunol. 2011;11(11):775–87.
4. Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. Nat Neurosci. 2007;10(11):1387–94.
5. Trapp BD, Wujek JR, Criste GA, Jalabi W, Yin X, Kidd GJ, et al. Evidence for synaptic stripping by cortical microglia. Glia. 2007;55(4):360–8.
6. Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J. Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. J Neurosci. 2009;29(13):3974–80.
7. Hu X, Liou AK, Leak RK, Xu M, An C, Suenaga J, et al. Neurobiology of microglial action in CNS injuries: receptor-mediated signaling mechanisms and functional roles. Prog Neurobiol. 2014;119–120:60–84.
8. Wang J, Dore S. Heme oxygenase-1 exacerbates early brain injury after intracerebral haemorrhage. Brain. 2007;130(Pt 6):1643–52. Epub 2007/05/26.
9. Thored P, Heldmann U, Gomes-Leal W, Gisler R, Darsalia V, Taneera J, et al. Long-term accumulation of microglia with proneurogenic phenotype concomitant with persistent neurogenesis in adult subventricular zone after stroke. Glia. 2009;57(8):835–49. Epub 2008/12/05.
10. Kwon MJ, Kim J, Shin H, Jeong SR, Kang YM, Choi JY, et al. Contribution of macrophages to enhanced regenerative capacity of dorsal root Ganglia sensory neurons by conditioning injury. J Neurosci. 2013;33(38):15095–108. Epub 2013/09/21.
11. Miron VE, Boyd A, Zhao JW, Yuen TJ, Ruckh JM, Shadrach JL, et al. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. Nat Neurosci. 2013;16(9):1211–8. Epub 2013/07/23.
12. Ekdahl CT, Claasen JH, Bonde S, Kokaia Z, Lindvall O. Inflammation is detrimental for neurogenesis in adult brain. Proc Natl Acad Sci U S A. 2003;100(23):13632–7.

13. Liu Z, Fan Y, Won SJ, Neumann M, Hu D, Zhou L, et al. Chronic treatment with minocycline preserves adult new neurons and reduces functional impairment after focal cerebral ischemia. Stroke. 2007;38(1):146–52.
14. Hu X, Leak RK, Shi Y, Suenaga J, Gao Y, Zheng P, et al. Microglial and macrophage polarization-new prospects for brain repair. Nat Rev Neurol. 2015;11(1):56–64.
15. Ford JW, McVicar DW. TREM and TREM-like receptors in inflammation and disease. Curr Opin Immunol. 2009;21(1):38–46.
16. Hamerman JA, Jarjoura JR, Humphrey MB, Nakamura MC, Seaman WE, Lanier LL. Cutting edge: inhibition of TLR and FcR responses in macrophages by triggering receptor expressed on myeloid cells (TREM)-2 and DAP12. J Immunol. 2006;177(4):2051–5.
17. Takahashi K, Rochford CD, Neumann H. Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. J Exp Med. 2005;201(4):647–57.
18. Sessa G, Podini P, Mariani M, Meroni A, Spreafico R, Sinigaglia F, et al. Distribution and signaling of TREM2/DAP12, the receptor system mutated in human polycystic lipomembraneous osteodysplasia with sclerosing leukoencephalopathy dementia. Eur J Neurosci. 2004;20(10):2617–28.
19. Zhu C, Herrmann US, Li B, Abakumova I, Moos R, Schwarz P, et al. Triggering receptor expressed on myeloid cells-2 is involved in prion-induced microglial activation but does not contribute to prion pathogenesis in mouse brains. Neurobiol Aging. 2015;36(5):1994–2003.
20. Ulrich JD, Finn MB, Wang Y, Shen A, Mahan TE, Jiang H, et al. Altered microglial response to Abeta plaques in APPPS1-21 mice heterozygous for TREM2. Mol Neurodegener. 2014;9:20. Epub 2014/06/05.
21. Kawabori M, Kacimi R, Kauppinen T, Calosing C, Kim JY, Hsieh CL, et al. Triggering receptor expressed on myeloid cells 2 (TREM2) deficiency attenuates phagocytic activities of microglia and exacerbates ischemic damage in experimental stroke. J Neurosci. 2015;35(8):3384–96.
22. Sieber MW, Jaenisch N, Brehm M, Guenther M, Linnartz-Gerlach B, Neumann H, et al. Attenuated inflammatory response in triggering receptor expressed on myeloid cells 2 (TREM2) knock-out mice following stroke. PLoS One. 2013;8(1), e52982.
23. Jay TR, Miller CM, Cheng PJ, Graham LC, Bemiller S, Broihier ML, et al. TREM2 deficiency eliminates TREM2+ inflammatory macrophages and ameliorates pathology in Alzheimer's disease mouse models. J Exp Med. 2015;212(3):287–95.
24. Hsieh CL, Koike M, Spusta SC, Niemi EC, Yenari M, Nakamura MC, et al. A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. J Neurochem. 2009;109(4):1144–56. Epub 2009/03/24.
25. Stefano L, Racchetti G, Bianco F, Passini N, Gupta RS, Panina Bordignon P, et al. The surface-exposed chaperone, Hsp60, is an agonist of the microglial TREM2 receptor. J Neurochem. 2009;110(1):284–94.
26. Matsumoto S, Tanaka J, Yano H, Takahashi H, Sugimoto K, Ohue S, et al. CD200+ and CD200- macrophages accumulated in ischemic lesions of rat brain: the two populations cannot be classified as either M1 or M2 macrophages. J Neuroimmunol. 2015;282:7–20.
27. Hoek RM, Ruuls SR, Murphy CA, Wright GJ, Goddard R, Zurawski SM, et al. Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science. 2000;290(5497):1768–71. Epub 2000/12/02.
28. Zhang S, Wang XJ, Tian LP, Pan J, Lu GQ, Zhang YJ, et al. CD200-CD200R dysfunction exacerbates microglial activation and dopaminergic neurodegeneration in a rat model of Parkinson's disease. J Neuroinflammation. 2011;8:154.
29. Matsumoto H, Kumon Y, Watanabe H, Ohnishi T, Takahashi H, Imai Y, et al. Expression of CD200 by macrophage-like cells in ischemic core of rat brain after transient middle cerebral artery occlusion. Neurosci Lett. 2007;418(1):44–8. Epub 2007/04/04.
30. Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, Giustetto M, Ferreira TA, Guiducci E, Dumas L, Ragozzino D, Gross CT. Synaptic pruning by microglia is necessary for normal brain development. Science. 2011;333:1456–8.

31. Bhaskar K, Konerth M, Kokiko-Cochran ON, Cardona A, Ransohoff RM, Lamb BT. Regulation of tau pathology by the microglial fractalkine receptor. Neuron. 2010;68(1):19–31.
32. Lyons A, Lynch AM, Downer EJ, Hanley R, O'Sullivan JB, Smith A, et al. Fractalkine-induced activation of the phosphatidylinositol-3 kinase pathway attenuates microglial activation in vivo and in vitro. J Neurochem. 2009;110(5):1547–56.
33. Mattison HA, Nie H, Gao H, Zhou H, Hong JS, Zhang J. Suppressed pro-inflammatory response of microglia in CX3CR1 knockout mice. J Neuroimmunol. 2013;257(1-2):110–5.
34. Tang Z, Gan Y, Liu Q, Yin JX, Liu Q, Shi J, Shi FD. CX3CR1 deficiency suppresses activation and neurotoxicity of microglia/macrophage in experimental ischemic stroke. J Neuroinflammation. 2014;11:11–26.
35. Pimentel-Coelho PM, Michaud JP, Rivest S. Evidence for a gender-specific protective role of innate immune receptors in a model of perinatal brain injury. J Neurosci. 2013;33(28):11556–72.
36. Donnelly DJ, Longbrake EE, Shawler TM, Kigerl KA, Lai W, Tovar CA, et al. Deficient CX3CR1 signaling promotes recovery after mouse spinal cord injury by limiting the recruitment and activation of Ly6Clo/iNOS+ macrophages. J Neurosci. 2011;31(27):9910–22.
37. Fang F, Lue LF, Yan S, Xu H, Luddy JS, Chen D, et al. RAGE-dependent signaling in microglia contributes to neuroinflammation, Abeta accumulation, and impaired learning/memory in a mouse model of Alzheimer's disease. FASEB J. 2010;24(4):1043–55.
38. Sparvero LJ, Asafu-Adjei D, Kang R, Tang D, Amin N, Im J, et al. RAGE (Receptor for Advanced Glycation Endproducts), RAGE ligands, and their role in cancer and inflammation. J Transl Med. 2009;7:17.
39. Bianchi R, Kastrisianaki E, Giambanco I, Donato R. S100B protein stimulates microglia migration via RAGE-dependent up-regulation of chemokine expression and release. J Biol Chem. 2011;286(9):7214–26.
40. Barateiro A, Afonso V, Santos G, Cerqueira JJ, Brites D, van Horssen J, et al. S100B as a potential biomarker and therapeutic target in multiple sclerosis. Mol Neurobiol. 2015. Epub 2015/07/18.
41. Lee S, Varvel NH, Konerth ME, Xu G, Cardona AE, Ransohoff RM, et al. CX3CR1 deficiency alters microglial activation and reduces beta-amyloid deposition in two Alzheimer's disease mouse models. Am J Pathol. 2010;177(5):2549–62.
42. Origlia N, Criscuolo C, Arancio O, Yan SS, Domenici L. RAGE inhibition in microglia prevents ischemia-dependent synaptic dysfunction in an amyloid-enriched environment. J Neurosci. 2014;34(26):8749–60.
43. Muhammad S, Barakat W, Stoyanov S, Murikinati S, Yang H, Tracey KJ, et al. The HMGB1 receptor RAGE mediates ischemic brain damage. J Neurosci. 2008;28(46):12023–31.
44. Liesz A, Dalpke A, Mracsko E, Antoine DJ, Roth S, Zhou W, et al. DAMP signaling is a key pathway inducing immune modulation after brain injury. J Neurosci. 2015;35(2):583–98.
45. Rabinovich GA, Toscan MA, Jackson SS, Vasta GR. Functions of cell surface galectin-glycoprotein lattices. Curr Opin Struct Biol. 2007;17(5):513–20.
46. Vasta GR. Galectins as pattern recognition receptors: structure, function, and evolution. Adv Exp Med Biol. 2012;946:21–36.
47. Burguillos MA, Svensson M, Schulte T, Boza-Serrano A, Garcia-Quintanilla A, Kavanagh E, et al. Microglia-secreted Galectin-3 acts as a toll-like receptor 4 ligand and contributes to microglial activation. Cell Rep. 2015. doi:10.1016/j.celrep.2015.02.012.
48. Young CC, Al-Dalahmah O, Lewis NJ, Brooks KJ, Jenkins MM, Poirier F, et al. Blocked angiogenesis in Galectin-3 null mice does not alter cellular and behavioral recovery after middle cerebral artery occlusion stroke. Neurobiol Dis. 2014;63:155–64.
49. Rabinovich GA, Croci DO. Regulatory circuits mediated by lectin-glycan interactions in autoimmunity and cancer. Immunity. 2012;36(3):322–35.
50. Wesley UV, Vemuganti R, Ayvaci ER, Dempsey RJ. Galectin-3 enhances angiogenic and migratory potential of microglial cells via modulation of integrin linked kinase signaling. Brain Res. 2013;1496:1–9.

51. Lalancette-Hebert M, Swarup V, Beaulieu JM, Bohacek I, Abdelhamid E, Weng YC, et al. Galectin-3 is required for resident microglia activation and proliferation in response to ischemic injury. J Neurosci. 2012;32(30):10383–95.
52. Pasquini LA, Millet V, Hoyos HC, Giannoni JP, Croci DO, Marder M, et al. Galectin-3 drives oligodendrocyte differentiation to control myelin integrity and function. Cell Death Differ. 2011;18(11):1746–56.
53. Barone FC, Feuerstein GZ. Inflammatory mediators and stroke: new opportunities for novel therapeutics. J Cereb Blood Flow Metab. 1999;19(8):819–34.
54. Dirnagl U, Iadecola C, Moskowitz MA. Pathobiology of ischaemic stroke: an integrated view. Trends Neurosci. 1999;22(9):391–7.
55. An C, Shi Y, Li P, Hu X, Gan Y, Stetler RA, et al. Molecular dialogs between the ischemic brain and the peripheral immune system: dualistic roles in injury and repair. Prog Neurobiol. 2014;115:6–24.
56. Schwab ME, Bartholdi D. Degeneration and regeneration of axons in the lesioned spinal cord. Physiol Rev. 1996;76(2):319–70.
57. Jean WC, Spellman SR, Nussbaum ES, Low WC. Reperfusion injury after focal cerebral ischemia: the role of inflammation and the therapeutic horizon. Neurosurgery. 1998;43(6):1382–96; discussion 96–7.
58. Lambertsen KL, Clausen BH, Babcock AA, Gregersen R, Fenger C, Nielsen HH, et al. Microglia protect neurons against ischemia by synthesis of tumor necrosis factor. J Neurosci. 2009;29(5):1319–30.
59. Degterev A, Huang Z, Boyce M, Li Y, Jagtap P, Mizushima N, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol. 2005;1(2):112–9.
60. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol. 2010;11(10):700–14.
61. Butovsky O, Ziv Y, Schwartz A, Landa G, Talpalar AE, Pluchino S, et al. Microglia activated by IL-4 or IFN-gamma differentially induce neurogenesis and oligodendrogenesis from adult stem/progenitor cells. Mol Cell Neurosci. 2006;31(1):149–60.
62. Semple BD, Kossmann T, Morganti-Kossmann MC. Role of chemokines in CNS health and pathology: a focus on the CCL2/CCR2 and CXCL8/CXCR2 networks. J Cereb Blood Flow Metab. 2010;30(3):459–73.
63. Gliem M, Mausberg AK, Lee JI, Simiantonakis I, van Rooijen N, Hartung HP, et al. Macrophages prevent hemorrhagic infarct transformation in murine stroke models. Ann Neurol. 2012;71(6):743–52.
64. Hughes PM, Allegrini PR, Rudin M, Perry VH, Mir AK, Wiessner C. Monocyte chemoattractant protein-1 deficiency is protective in a murine stroke model. J Cereb Blood Flow Metab. 2002;22(3):308–17.
65. Schaller B. Prospects for the future: the role of free radicals in the treatment of stroke. Free Radic Biol Med. 2005;38(4):411–25.
66. Miller AA, Dusting GJ, Roulston CL, Sobey CG. NADPH-oxidase activity is elevated in penumbral and non-ischemic cerebral arteries following stroke. Brain Res. 2006;1111(1):111–6.
67. McCann SK, Dusting GJ, Roulston CL. Early increase of Nox4 NADPH oxidase and superoxide generation following endothelin-1-induced stroke in conscious rats. J Neurosci Res. 2008;86(11):2524–34.
68. Chan EC, Jiang F, Peshavariya HM, Dusting GJ. Regulation of cell proliferation by NADPH oxidase-mediated signaling: potential roles in tissue repair, regenerative medicine and tissue engineering. Pharmacol Ther. 2009;122(2):97–108.
69. Weston RM, Lin B, Dusting GJ, Roulston CL. Targeting oxidative stress injury after ischemic stroke in conscious rats: limited benefits with apocynin highlight the need to incorporate long term recovery. Stroke Res Treat. 2013;2013:648061.

70. Iadecola C, Zhang F, Xu X. Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. Am J Phys. 1995;268(1 Pt 2):R286–92.
71. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Phys. 1996;271(5 Pt 1):C1424–37.
72. Horn KP, Busch SA, Hawthorne AL, van Rooijen N, Silver J. Another barrier to regeneration in the CNS: activated macrophages induce extensive retraction of dystrophic axons through direct physical interactions. J Neurosci. 2008;28(38):9330–41.
73. Kitayama M, Ueno M, Itakura T, Yamashita T. Activated microglia inhibit axonal growth through RGMa. PLoS One. 2011;6(9), e25234.
74. Ponomarev ED, Veremeyko T, Weiner HL. MicroRNAs are universal regulators of differentiation, activation, and polarization of microglia and macrophages in normal and diseased CNS. Glia. 2013;61(1):91–103.
75. Zhou X, Spittau B, Krieglstein K. TGFbeta signalling plays an important role in IL4-induced alternative activation of microglia. J Neuroinflammation. 2012;9:210.
76. Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death Differ. 1998;5(7):551–62.
77. Fu R, Shen Q, Xu P, Luo JJ, Tang Y. Phagocytosis of microglia in the central nervous system diseases. Mol Neurobiol. 2014;49(3):1422–34.
78. Katayama T, Kobayashi H, Okamura T, Yamasaki-Katayama Y, Kibayashi T, Kimura H, et al. Accumulating microglia phagocytose injured neurons in hippocampal slice cultures: involvement of p38 MAP kinase. PLoS One. 2012;7(7), e40813.
79. Young KM, Psachoulia K, Tripathi RB, Dunn SJ, Cossell L, Attwell D, et al. Oligodendrocyte dynamics in the healthy adult CNS: evidence for myelin remodeling. Neuron. 2013;77(5):873–85.
80. Tanaka K, Nogawa S, Suzuki S, Dembo T, Kosakai A. Upregulation of oligodendrocyte progenitor cells associated with restoration of mature oligodendrocytes and myelination in peri-infarct area in the rat brain. Brain Res. 2003;989(2):172–9.
81. Hsieh J, Aimone JB, Kaspar BK, Kuwabara T, Nakashima K, Gage FH. IGF-I instructs multipotent adult neural progenitor cells to become oligodendrocytes. J Cell Biol. 2004;164(1):111–22.
82. Ness JK, Wood TL. Insulin-like growth factor I, but not neurotrophin-3, sustains Akt activation and provides long-term protection of immature oligodendrocytes from glutamate-mediated apoptosis. Mol Cell Neurosci. 2002;20(3):476–88.
83. Mason JL, Ye P, Suzuki K, D'Ercole AJ, Matsushima GK. Insulin-like growth factor-1 inhibits mature oligodendrocyte apoptosis during primary demyelination. J Neurosci. 2000;20(15):5703–8.
84. Lalive PH, Paglinawan R, Biollaz G, Kappos EA, Leone DP, Malipiero U, et al. TGF-beta-treated microglia induce oligodendrocyte precursor cell chemotaxis through the HGF-c-Met pathway. Eur J Immunol. 2005;35(3):727–37.
85. Filipovic R, Zecevic N. Interaction between microglia and oligodendrocyte cell progenitors involves Golli proteins. Ann N Y Acad Sci. 2005;1048:166–74.
86. Tanaka T, Murakami K, Bando Y, Yoshida S. Minocycline reduces remyelination by suppressing ciliary neurotrophic factor expression after cuprizone-induced demyelination. J Neurochem. 2013;127(2):259–70.
87. Vilhardt F. Microglia: phagocyte and glia cell. Int J Biochem Cell Biol. 2005;37(1):17–21.
88. Frade JM, Barde YA. Microglia-derived nerve growth factor causes cell death in the developing retina. Neuron. 1998;20(1):35–41.
89. Cacci E, Claasen JH, Kokaia Z. Microglia-derived tumor necrosis factor-alpha exaggerates death of newborn hippocampal progenitor cells in vitro. J Neurosci Res. 2005;80(6):789–97.
90. Marin-Teva JL, Dusart I, Colin C, Gervais A, van Rooijen N, Mallat M. Microglia promote the death of developing Purkinje cells. Neuron. 2004;41(4):535–47.

91. Bengzon J, Kokaia Z, Elmer E, Nanobashvili A, Kokaia M, Lindvall O. Apoptosis and proliferation of dentate gyrus neurons after single and intermittent limbic seizures. Proc Natl Acad Sci U S A. 1997;94(19):10432–7.
92. Kim BJ, Kim MJ, Park JM, Lee SH, Kim YJ, Ryu S, et al. Reduced neurogenesis after suppressed inflammation by minocycline in transient cerebral ischemia in rat. J Neurol Sci. 2009;279(1–2):70–5.
93. Walton NM, Sutter BM, Laywell ED, Levkoff LH, Kearns SM, Marshall 2nd GP, et al. Microglia instruct subventricular zone neurogenesis. Glia. 2006;54(8):815–25.
94. Liao H, Huang W, Niu R, Sun L, Zhang L. Cross-talk between the epidermal growth factor-like repeats/fibronectin 6-8 repeats domains of Tenascin-R and microglia modulates neural stem/progenitor cell proliferation and differentiation. J Neurosci Res. 2008;86(1):27–34.
95. Battista D, Ferrari CC, Gage FH, Pitossi FJ. Neurogenic niche modulation by activated microglia: transforming growth factor beta increases neurogenesis in the adult dentate gyrus. Eur J Neurosci. 2006;23(1):83–93.
96. Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, et al. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science. 2010;330(6005):841–5.
97. Cecchini MG, Dominguez MG, Mocci S, Wetterwald A, Felix R, Fleisch H, et al. Role of colony stimulating factor-1 in the establishment and regulation of tissue macrophages during postnatal development of the mouse. Development. 1994;120(6):1357–72.
98. Kubota Y, Takubo K, Shimizu T, Ohno H, Kishi K, Shibuya M, et al. M-CSF inhibition selectively targets pathological angiogenesis and lymphangiogenesis. J Exp Med. 2009;206(5):1089–102.
99. Checchin D, Sennlaub F, Levavasseur E, Leduc M, Chemtob S. Potential role of microglia in retinal blood vessel formation. Invest Ophthalmol Vis Sci. 2006;47(8):3595–602.
100. Rymo SF, Gerhardt H, Wolfhagen Sand F, Lang R, Uv A, Betsholtz C. A two-way communication between microglial cells and angiogenic sprouts regulates angiogenesis in aortic ring cultures. PLoS One. 2011;6(1), e15846.
101. Li Y, Liu DX, Li MY, Qin XX, Fang WG, Zhao WD, et al. Ephrin-A3 and ephrin-A4 contribute to microglia-induced angiogenesis in brain endothelial cells. Anat Rec. 2014;297(10):1908–18.
102. Willenborg S, Lucas T, van Loo G, Knipper JA, Krieg T, Haase I, et al. CCR2 recruits an inflammatory macrophage subpopulation critical for angiogenesis in tissue repair. Blood. 2012;120(3):613–25. Epub 2012/05/12.
103. Medina RJ, O'Neill CL, O'Doherty TM, Knott H, Guduric-Fuchs J, Gardiner TA, et al. Myeloid angiogenic cells act as alternative M2 macrophages and modulate angiogenesis through interleukin-8. Mol Med. 2011;17(9–10):1045–55. Epub 2011/06/15.
104. Zajac E, Schweighofer B, Kupriyanova TA, Juncker-Jensen A, Minder P, Quigley JP, et al. Angiogenic capacity of M1- and M2-polarized macrophages is determined by the levels of TIMP-1 complexed with their secreted proMMP-9. Blood. 2013;122(25):4054–67. Epub 2013/11/01.
105. Sugimoto K, Nishioka R, Ikeda A, Mise A, Takahashi H, Yano H, et al. Activated microglia in a rat stroke model express NG2 proteoglycan in peri-infarct tissue through the involvement of TGF-beta1. Glia. 2014;62(2):185–98.
106. Febinger HY, Thomasy HE, Pavlova MN, Ringgold KM, Barf PR, George AM, et al. Time-dependent effects of CX3CR1 in a mouse model of mild traumatic brain injury. J Neuroinflammation. 2015;12:154.
107. Liu Y, Wu XM, Luo QQ, Huang S, Yang QW, Wang FX, et al. CX3CL1/CX3CR1-mediated microglia activation plays a detrimental role in ischemic mice brain via p38MAPK/PKC pathway. J Cereb Blood Flow Metab. 2015;35(10):1623–31.
108. Wu XM, Liu Y, Qian ZM, Luo QQ, Ke Y. CX3CL1/CX3CR1 Axis plays a key role in ischemia-induced oligodendrocyte injury via p38MAPK signaling pathway. Mol Neurobiol. 2015.

109. Walter HL, van der Maten G, Antunes AR, Wieloch T, Ruscher K. Treatment with AMD3100 attenuates the microglial response and improves outcome after experimental stroke. J Neuroinflammation. 2015;12:24.
110. McGuckin CP, Jurga M, Miller AM, Sarnowska A, Wiedner M, Boyle NT, et al. Ischemic brain injury: a consortium analysis of key factors involved in mesenchymal stem cell-mediated inflammatory reduction. Arch Biochem Biophys. 2013;534(1–2):88–97.
111. Dentesano G, Serratosa J, Tusell JM, Ramon P, Valente T, Saura J, et al. CD200R1 and CD200 expression are regulated by PPAR-gamma in activated glial cells. Glia. 2014;62(6):982–98. Epub 2014/03/19.
112. Lee JC, Cho JH, Cho GS, Ahn JH, Park JH, Kim IH, et al. Effect of transient cerebral ischemia on the expression of receptor for advanced glycation end products (RAGE) in the gerbil hippocampus proper. Neurochem Res. 2014;39(8):1553–63.
113. Lok KZ, Basta M, Manzanero S, Arumugam TV. Intravenous immunoglobulin (IVIg) dampens neuronal toll-like receptor-mediated responses in ischemia. J Neuroinflammation. 2015;12:73.
114. Park H, Ku SH, Park H, Hong J, Kim D, Choi BR, et al. RAGE siRNA-mediated gene silencing provides cardioprotection against ventricular arrhythmias in acute ischemia and reperfusion. J Control Release. 2015;217:315–26.
115. Pimentel-Coelho PM, Michaud JP, Rivest S. C-C chemokine receptor type 2 (CCR2) signaling protects neonatal male mice with hypoxic-ischemic hippocampal damage from developing spatial learning deficits. Behav Brain Res. 2015;286:146–51.
116. Arakelyan A, Zakharyan R, Hambardzumyan M, Petrkova J, Olsson MC, Petrek M, et al. Functional genetic polymorphisms of monocyte chemoattractant protein 1 and C-C chemokine receptor type 2 in ischemic stroke. J Interf Cytokine Res. 2014;34(2):100–5. Epub 2013/10/03.
117. Chu HX, Broughton BR, Kim HA, Lee S, Drummond GR, Sobey CG. Evidence that Ly6C(hi) monocytes are protective in acute ischemic stroke by promoting M2 macrophage polarization. Stroke. 2015;46(7):1929–37. Epub 2015/05/23.
118. Hammond MD, Taylor RA, Mullen MT, Ai Y, Aguila HL, Mack M, et al. CCR2+ Ly6C(hi) inflammatory monocyte recruitment exacerbates acute disability following intracerebral hemorrhage. J Neurosci. 2014;34(11):3901–9. Epub 2014/03/14.
119. Wacker BK, Perfater JL, Gidday JM. Hypoxic preconditioning induces stroke tolerance in mice via a cascading HIF, sphingosine kinase, and CCL2 signaling pathway. J Neurochem. 2012;123(6):954–62. Epub 2012/10/10.
120. Tokami H, Ago T, Sugimori H, Kuroda J, Awano H, Suzuki K, Kiyohara Y, Kamouchi M, Kitazono T, REBIOS Investigators. RANTES has a potential to play a neuroprotective role in an autocrine/paracrine manner after ischemic stroke. Brain Res. 2013;1517:122–32.
121. Dziennis S, Mader S, Akiyoshi K, Ren X, Ayala P, Burrows GG, et al. Therapy with recombinant T-cell receptor ligand reduces infarct size and infiltrating inflammatory cells in brain after middle cerebral artery occlusion in mice. Metab Brain Dis. 2011;26(2):123–33. Epub 2011/04/08.
122. Sorce S, Bonnefont J, Julien S, Marq-Lin N, Rodriguez I, Dubois-Dauphin M, et al. Increased brain damage after ischaemic stroke in mice lacking the chemokine receptor CCR5. Br J Pharmacol. 2010;160(2):311–21. Epub 2010/04/29.
123. Tokami H, Ago T, Sugimori H, Kuroda J, Awano H, Suzuki K, et al. RANTES has a potential to play a neuroprotective role in an autocrine/paracrine manner after ischemic stroke. Brain Res. 2013;1517:122–32. Epub 2013/04/23.
124. Fronz U, Deten A, Baumann F, Kranz A, Weidlich S, Hartig W, et al. Continuous adenosine A2A receptor antagonism after focal cerebral ischemia in spontaneously hypertensive rats. Naunyn Schmiedeberg's Arch Pharmacol. 2014;387(2):165–73. Epub 2013/10/31.
125. Melani A, Corti F, Cellai L, Vannucchi MG, Pedata F. Low doses of the selective adenosine A2A receptor agonist CGS21680 are protective in a rat model of transient cerebral ischemia. Brain Res. 2014;1551:59–72. Epub 2014/01/25.

126. Melani A, Dettori I, Corti F, Cellai L, Pedata F. Time-course of protection by the selective A2A receptor antagonist SCH58261 after transient focal cerebral ischemia. Neurol Sci. 2015;36(8):1441–8. Epub 2015/03/26.
127. Choi IY, Lee JC, Ju C, Hwang S, Cho GS, Lee HW, Choi WJ, Jeong LS, Kim WK. A3 adenosine receptor agonist reduces brain ischemic injury and inhibits inflammatory cell migration in rats. Am J Pathol. 2011;179:2042–52.
128. Ryu S, Kwon J, Park H, Choi IY, Hwang S, Gajulapati V, et al. Amelioration of cerebral ischemic injury by a synthetic seco-nucleoside LMT497. Exp Neurol. 2015;24(1):31–40. Epub 2015/03/21.
129. Li F, Wang L, Li JW, Gong M, He L, Feng R, Dai Z, Li SQ. Hypoxia induced amoeboid microglial cell activation in postnatal rat brain is mediated by ATP receptor P2X4. BMC Neurosci. 2011;12:111.
130. Cheng RD, Ren JJ, Zhang YY, Ye XM. P2X4 receptors expressed on microglial cells in post-ischemic inflammation of brain ischemic injury. Neurochem Int. 2014;67:9–13. Epub 2014/02/04.
131. Mahi N, Kumar A, Jaggi AS, Singh N, Dhawan R. Possible role of pannexin 1/P2x7 purinoceptor in neuroprotective mechanism of ischemic postconditioning in mice. J Surg Res. 2015;196:190–9.
132. Bindra CS, Jaggi AS, Singh N. Role of P2X7 purinoceptors in neuroprotective mechanism of ischemic postconditioning in mice. Mol Cell Biochem. 2014;390(1–2):161–73. Epub 2014/02/05.
133. Cisneros-Mejorado A, Gottlieb M, Cavaliere F, Magnus T, Koch-Nolte F, Scemes E, et al. Blockade of P2X7 receptors or pannexin-1 channels similarly attenuates postischemic damage. J Cereb Blood Flow Metab. 2015;35(5):843–50. Epub 2015/01/22.
134. Ma XJ, Cheng JW, Zhang J, Liu AJ, Liu W, Guo W, Shen FM, Lu GC. E-selectin deficiency attenuates brain ischemia in mice. CNS Neurosci Ther. 2012;13:903–8.
135. Das S, Roy S, Kaul S, Jyothy A, Munshi A. E-selectin gene (S128R) polymorphism in hemorrhagic stroke: Comparison with ischemic stroke. Neurosci Lett. 2014;581:125–8.
136. Anuncibay-Soto B, Perez-Rodriguez D, Llorente IL, Regueiro-Purrinos M, Gonzalo-Orden JM, Fernandez-Lopez A. Age-dependent modifications in vascular adhesion molecules and apoptosis after 48-h reperfusion in a rat global cerebral ischemia model. Age (Dordr). 2014;36(5):9703. Epub 2014/09/04.
137. Bell MT, Puskas F, Agoston VA, Cleveland Jr JC, Freeman KA, Gamboni F, et al. Toll-like receptor 4-dependent microglial activation mediates spinal cord ischemia-reperfusion injury. Circulation. 2013;128(11 Suppl 1):S152–6.
138. Woo MS, Wang X, Faustino JV, Derugin N, Wendland MF, Zhou P, et al. Genetic deletion of CD36 enhances injury after acute neonatal stroke. Ann Neurol. 2012;72(6):961–70.
139. Li F, Faustino J, Woo MS, Derugin N, Vexler ZS. Lack of the scavenger receptor CD36 alters microglial phenotypes after neonatal stroke. J Neurochem. 2015;135(3):445–52.
140. Kim E, Febbraio M, Bao Y, Tolhurst AT, Epstein JM, Cho S. CD36 in the periphery and brain synergizes in stroke injury in hyperlipidemia. Ann Neurol. 2012;71(6):753–64. Epub 2012/06/22.
141. Cheyuo C, Aziz M, Yang WL, Jacob A, Zhou M, Wang P. Milk fat globule-EGF factor VIII attenuates CNS injury by promoting neural stem cell proliferation and migration after cerebral ischemia. PLoS One. 2015;10(4), e0122833. Epub 2015/04/14.
142. Cheyuo C, Jacob A, Wu R, Zhou M, Qi L, Dong W, et al. Recombinant human MFG-E8 attenuates cerebral ischemic injury: its role in anti-inflammation and anti-apoptosis. Neuropharmacology. 2012;62(2):890–900. Epub 2011/10/04.
143. Neher JJ, Emmrich JV, Fricker M, Mander PK, Thery C, Brown GC. Phagocytosis executes delayed neuronal death after focal brain ischemia. Proc Natl Acad Sci U S A. 2013;110(43):E4098–107. Epub 2013/10/09.

Crosstalk Between Cerebral Endothelium and Oligodendrocyte After Stroke

Akihiro Shindo, Takakuni Maki, Kanako Itoh, Nobukazu Miyamoto, Naohiro Egawa, Anna C. Liang, Takayuki Noro, Josephine Lok, Eng H. Lo, and Ken Arai

1 Introduction

The concept of the neurovascular unit is now well-accepted in the understanding of brain physiology and pathophysiology. The "neurovascular unit" provides a conceptual framework that emphasizes cell–cell interactions between neuronal, glial, and vascular elements [1–7]. This concept primarily guides research in neuron-related cell–cell interaction mechanisms in gray matter. But cell–cell signaling between non-neuronal cells is also critical for white matter and gray matter function. As shown in the diagram in Fig. 1, cells in cerebral white matter are closely related to each other. For example, oligodendrocytes enwrap axons for efficient conduction of electrical impulses, and the oligodendrocyte–axon interaction is one of the most well-documented aspects of neurovascular unit in white matter. In fact, oligodendrocyte-derived trophic factors such as insulin-like growth factor-1 and glial cell line-derived neurotrophic factor increase axonal length and support neuron survival [9]. Another example of the importance of cell–cell interactions in white matter may be found in the roles of oligodendrocyte precursor cells (OPCs). OPCs

A. Shindo • T. Maki • K. Itoh • N. Miyamoto • N. Egawa • A.C. Liang
T. Noro • E.H. Lo • K. Arai (✉)
Neuroprotection Research Laboratory, Departments of Radiology and Neurology,
Massachusetts General Hospital and Harvard Medical School,
149 13th Street, MGH-CNY149 Room 2401, Charlestown, MA 02129, USA
e-mail: karai@partners.org

J. Lok
Neuroprotection Research Laboratory, Departments of Radiology and Neurology,
Massachusetts General Hospital and Harvard Medical School,
149 13th Street, MGH-CNY149 Room 2401, Charlestown, MA 02129, USA

Department of Pediatrics, Massachusetts General Hospital
and Harvard Medical School, Charlestown, MA, USA

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_8

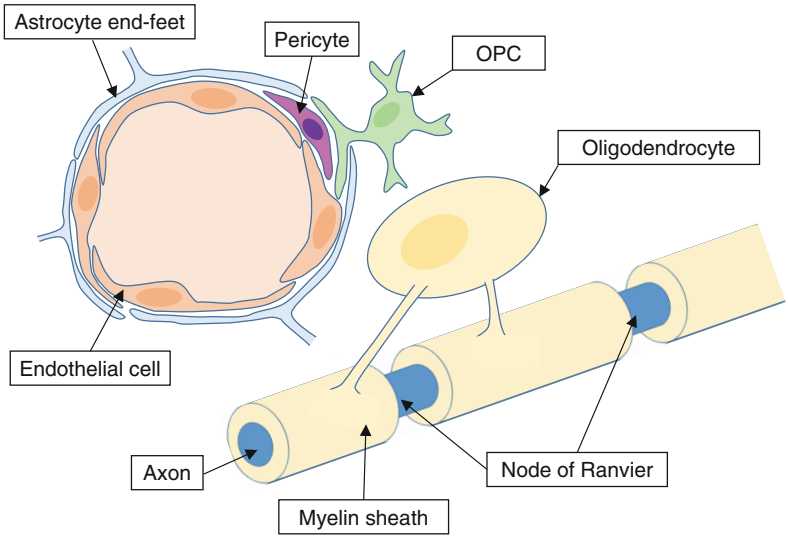

Fig. 1 Schematic of the neurovascular unit in white matter. Neuronal axons, astrocytes, cerebral endothelial cells, pericytes, and oligodendrocytes/OPCs comprise the neurovascular unit in white matter. The concept of the neurovascular unit emphasizes the functional aspects of cell–cell signaling, and in fact, the components are closely located and presumably exchange signals to support their function with each other. This figure was drawn from the authors' previous review article [8]

are an important component in the neurovascular unit in cerebral white matter, and are situated directly next to astrocytes. Recent reports suggest that OPCs are also closely located to cerebral endothelial cells [10, 11]. Although OPCs are most active during development, when they maturate into oligodendrocytes to form myelin sheath, a number of OPCs resides in adult white matter to maintain white matter homeostasis. Importantly, OPCs receive support from their neighboring cells to proliferate and differentiate into mature oligodendrocytes in brain.

Another important aspect within the neurovascular unit is the distinct contribution of cerebral endothelium-to-brain function. The cerebral vascular system is one of the major constituents of the brain, traditionally viewed as a passive conduit for blood. However, recent research has shown that this system plays more active roles in maintaining the central nervous system (CNS) homeostasis. For example, cerebral endothelial cells, along with astrocytes and pericytes, form the blood–brain barrier (BBB). The BBB constitutes an anatomical, physiochemical, and biochemical barrier that controls the exchange of materials between blood, brain, and cerebrospinal fluid. BBB breakdown due to endothelial dysfunction is frequently associated with a myriad of neurological pathologies, including chronic CNS diseases [12–14]. Another important function of the cerebral vascular system is that of providing trophic support to neighboring neurons. By releasing trophic factors, cerebral endothelial cells guide developing axons [15], protect neurons against stress [16, 17], and provide a niche for supporting neural stem/progenitor cells (NSPCs) [18]. NSPCs were shown to have direct coupling with cerebral endothelial

cells [19], and in this so-called "neurovascular niche," cell–cell signaling between cerebral endothelial cells and neuronal precursor cells help mediate and sustain pockets of ongoing neurogenesis and angiogenesis in adult brain [18, 20]. Even under the remodeling phase after brain injury, these close relationships are maintained, and both neurogenesis and angiogenesis occur in the neurovascular niche to promote repair of the brain. Indeed, angiogenic stimulation enhances neurogenesis after stroke [18, 21]. In turn, neuroblasts migrate along perivascular routes and the promotion of neurogenesis enhances vascular re-growth [22].

Much of the research into mechanisms of trophic coupling in the neurovascular unit has focused on endothelium–neuron and endothelium–astrocyte interactions. However, cell–cell interactions between endothelial cells and oligodendrocyte lineage cells are also important in maintaining brain function, especially in white matter. In cerebral white matter, OPCs play essential roles in white matter remodeling/repairing after stroke. Under pathological conditions associated with demyelination, OPCs are activated, recruited to the site of injury, and differentiated into mature oligodendrocytes. These OPC activities proceed in a coordinated fashion in response to complex signaling events involving numerous mediators [23–25]. In the first step, OPCs are activated by phenotypically switching from a mitotically dormant inactive state to a proliferative active state [26, 27]. The next step is OPC recruitment, which is a process involving proliferation and migration. In the final step, the differentiation phase, recruited OPCs exit the mitotic cell cycle and change their phenotype to mature oligodendrocytes, and then, newly created oligodendrocytes establish contact with unmyelinated axons and form functional myelin sheaths around them [25, 28, 29]. The mechanisms underlying this complex process have been the subject of several recent studies, in which the role of cell–cell interaction in remyelination has been increasingly recognized [30, 31]. As noted, some populations of oligodendrocyte lineage cells are located closely to cerebral endothelial cells, and these cells may communicate with each other via secreting soluble factors [10, 11]. In this chapter, we attempt to overview key findings for the crosstalk between cerebral endothelial cells and oligodendrocyte lineage cells, focusing on oligodendrocyte damage and repair after stroke.

2 OPC Differentiation to Oligodendrocytes Under Normal and Pathological Conditions

Oligodendrocytes are one of the major glial cells in the CNS, and produce a lipid-rich membrane called myelin. Each oligodendrocyte can enwrap up to 60 axonal segments, thereby enabling fast and salutatory nerve impulse conduction [32]. During development, OPCs are first generated in the germinal zones, where they will proliferate. They then migrate to both gray and white matter areas, where most will differentiate into mature oligodendrocytes and form myelin sheaths.

Although myelinated tracts are formed early in life, renewal of myelin/oligodendrocyte continues throughout adult life [33–35]. Myelin in the adult CNS maintains some plasticity in response to changes in neural activity [36] and brain injury [37].

Under normal conditions in the adult brain, most subventricular zone (SVZ) progenitor cells give rise to neuronal linage cells. They migrate along the rostral migratory stream (RMS) to the olfactory bulbs, where they terminate and differentiate into mature interneurons [38–40]. Oligodendrocytes can also be generated from SVZ cells in the adult brain, and newly generated OPCs migrate towards the corpus callosum and the white matter tracts of striatum and fimbria fornix [41]. However, the proportion of SVZ progenitor cells that differentiate into oligodendrocyte linage cells decrease after the early postnatal period [39]. Interestingly, in the SVZ, neuronal and oligodendroglial progenies constitute separate lineages under physiological conditions. Using continuous live imaging and single-cell tracking of NSPCs, Ortega et al. have demonstrated that a single NSPC and its offsprings in the SVZ would not result in both neuronal and oligodendroglial progenies [42]. Furthermore, the adult SVZ is highly regionalized. The neuronal progeny with distinct identity is generated in different areas along the dorsoventral and rostrocaudal axes [43, 44]. In addition, clones fated to generate oligodendrocytes are prevalent in NSPCs isolated from dorsolateral SVZ. On the contrary, ventrolateral SVZ regions consist of both neuronal and astroglial progenies with few oligodendroglial progeny [42, 45, 46].

SVZ progenitor cells in the adult brain show some lineage plasticity under pathological conditions. After CNS damage, a number of progenitors migrate out of the RMS to the injured site. The fate of these progenitor cells can be dynamically altered according to the disease type. The fate of SVZ progenitor cells can shift from NSPCs to OPCs after demyelination, and these newly generated OPCs proliferate and migrate to the lesion areas [39, 47–49]. In a model of experimental autoimmune encephalomyelitis (EAE), enhanced proliferation and migration of SVZ NSPCs are observed, and these mobilized cells give rise to oligodendrocytes and astrocytes without neurons in the injured white matter [49]. In addition, demyelination would change the fate of glutamic acid decarboxylase 65 (GAD65)/doublecortin (Dcx)-expressing NSPCs derived from the adult SVZ to generate oligodendrocytes, rather than neurons, in corpus callosum [47]. This process may restore developmental myelination to some extent; NSPCs that generate oligodendrocytes migrate from SVZ to developing white matter, where they stop dividing to differentiate and myelinate axons [47, 50].

Although the most studied disease with oligodendrogenesis after white matter injury is multiple sclerosis (MS), there are some findings reported for research studies using stroke models. After brain ischemia, immature oligodendrocytes proliferate in the regions surrounding the lateral ventricles [51] and the infarction site (peri-infarct areas) [52], with a delayed increase in the number of mature oligodendrocytes in peri-infarct areas [51]. As discussed, under normal conditions, oligodendrocytes can be generated in the SVZ and migrate to white matter tracts of the corpus callosum, fimbria fornix, and striatum [41]. After demyelination, SVZ-generated OPCs proliferate and migrate to peri-lesional areas, attempting to differentiate into mature oligodendrocyte and remyelinate axons in response to numerous mediators [37, 47]. This finding has been reported in rodent models of ischemic brain injury, in which OPCs are generated by SVZ neuronal progenitor cells and later become mature myelinating oligodendrocytes, which help restore damaged

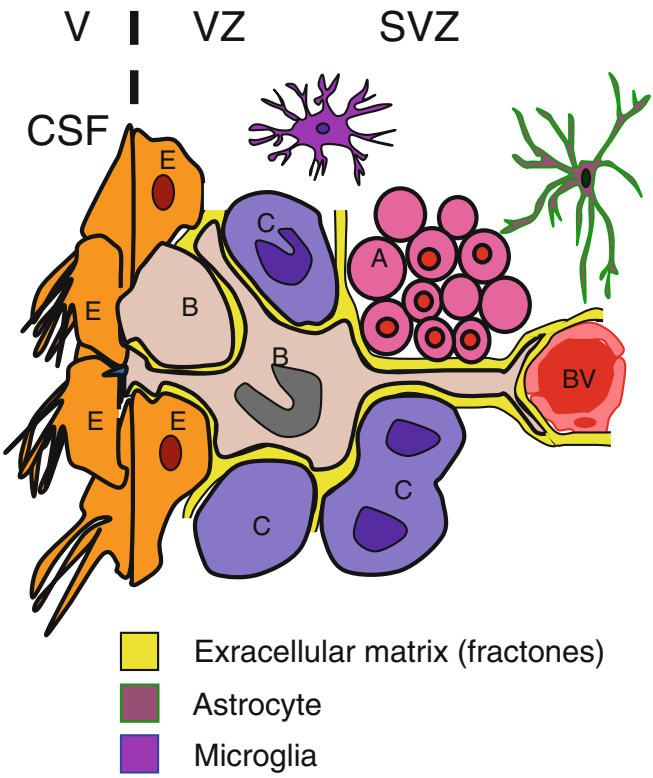

Fig. 2 Schematic of interplay for SVZ cells. The subventricular zone (SVZ) and ventricular zone (VZ) line the lateral ventricles (V) in the brain. Type B cells (B) contact the ventricle (V) containing cerebrospinal fluid (CSF) through specialized apical processes. The processes contain a single primary cilium, which is surrounded by a rosette of ependymal cells (E) with large apical surfaces forming pinwheel-like structures. On the other side, the type B cells have long basal processes with specialized endings that frequently contact blood vessels (BV). The type B cells also contact their progeny, i.e., type C cells (C) and the chains of migrating type A neuroblasts (A). The V-SVZ includes extracellular matrix (fractones) that contacts all the cell types including blood vessels, microglia, and astrocytes in this region. This figure was drawn from the authors' previous review article [59]

white matter [53–56]. Thus, increasing the recruitment of immature oligodendrocytes to the injured site and promoting their progression to mature oligodendrocytes will enhance white matter repair after injury. The process of oligodendrogenesis is influenced by many intrinsic and extrinsic factors from various types of cells, thus offering a number of pathways for potential therapeutic interventions [57].

NSPCs in the SVZ display diverse interactions with their neighboring environments [38, 58] (Fig. 2). On one side of SVZ, type B cells are surrounded by multi-ciliated non-dividing ependymal cells, which form pinwheel-like structures on the ventricular surface. These cells are in direct contact with the cerebrospinal fluid (CSF) through a short non-motile primary cilium that extends towards the

ventricle. On the other side, type B cells interact with the extensive network of blood vessels with a long basal process. Type B cells also attach to type C cells and chains of young neurons (type A cells) by the extracellular matrix. Proliferating type C cells are closely located to their progenitors, and are also often in close proximity to blood vessels [60]. Type B cells interact with one another by gap and adherens junctions, the same as in ependymal cells [61]. Furthermore, the adult SVZ possesses a highly organized basement membrane, which is absent in other areas of the brain. Overall, the SVZ is poised to receive informational inputs via cell–cell and cell–matrix contacts. The integration of these multifaceted external cues (e.g., extracellular signals from the vasculature, extracellular matrix, and the cerebrospinal fluid) to intrinsic factors leads to the determination of the fate and behavior of each cell lineage. In the next section, we will discuss how cerebral endothelial cells (and blood vessels) support oligodendrocyte function, including OPC generation from NSPCs.

3 Roles of Cerebral Endothelium on NSPC/OPC Function

The vasculature is an integral component of the SVZ stem cell niche that possesses specialized properties in regulating stem cell proliferation and regeneration [60, 62]. Endothelial cells secrete factors that contribute to stem cell self-renewal or proliferation. Co-culture of endothelial cells with NPSCs enhance the in vitro neurosphere generation from embryonic progenitors [63]. NSPCs were shown to have direct coupling with cerebral endothelial cells [19], and various kinds of perivascular regulators, including growth factors, purinergic signaling, nitric oxide signaling, and chemokines, contribute to cell genesis and fate determination in the SVZ [64].

Dividing progenitor cells (type B cells) and their transit-amplifying type C cells lie adjacent to the extensive planar vascular plexus in the SVZ. Approximately 47 % of dividing type B cells and 46 % of type C cells are found within 5 microns of the vasculature. During homeostasis and regeneration, type B cells and type C cells directly contact SVZ blood vessel sites devoid of astrocyte end-feet and pericyte coverage [60, 62]. Most dividing type B and type C cells are close to these sites, highlighting the importance of vasculature in supporting progenitor cell function. By contrast, most migrating neuroblasts are more distal to the vasculature (only 14 % are within 5 μm) compared to type B and type C cells, even though blood vessels run parallel to the aggregates of migrating neuroblast chains in the dorsal aspect of the SVZ and in the RMS. However, it still remains to be understood whether neuronal differentiation occurs in response to leaving the vascular bed or whether cells leave the vasculature after they are differentiated [62].

Blood vessels in the SVZ region also serve as a scaffold for long-distance migration of neuroblasts from SVZ to the olfactory bulb, potentially through the release of chemoattractant (e.g., brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF)), and chemorepulsive factors (e.g., semaphorins, ephrins) [65–68]. Migrating neuroblasts are ensheathed by a layer of astrocyte

processes and use each other as guides in the migration process toward the olfactory bulb. Similarly, in animal stroke models [18] and human stroke patients [69], long-distance migration of newly born immature neurons from SVZ to peri-infarct cortex is observed. Stromal cell-derived factor 1 (SDF-1)/C-X-C chemokine receptor type 4 (CXCR4) signaling assists blood vessel- and astrocyte-associated migration of adult SVZ progenitors after cortical injury [70]. Recent studies have identified that SDF-1/CXCR4-mediated signaling is a critical homing factor in the SVZ niche. CXCR4 is expressed by all progenitor cells in the SVZ. SDF-1 is expressed in the SVZ blood vessels, but the ependymal cells that line the lateral ventricles express higher levels of SDF-1 to create a concentration gradient. SDF-1 increases integrin α6 and epidermal growth factor receptor expression in activated type B and type C cells, enhancing their activated states and ability to bind laminin in the vascular niche [20]. SDF-1 also increases the motility of type A neuroblasts. These type A cells express lower levels of integrin α6, which might promote evacuating from the vascular niche.

As noted, cerebral endothelial cells within the cerebral blood vessels support neighboring cells by secreting trophic factors. Recent studies confirmed that cerebral endothelial cells regulate the function of oligodendrocyte lineage cells. In a co-culture system of endothelium with NSPCs, the chemokine CCL2/MCP-1 mediates the interaction between endothelium and neural precursor cells to promote the differentiation of NSPCs into oligodendrocytes [71]. Another study used the in vitro media-transfer system to show that conditioned medium from endothelial cells promotes the differentiation of NSPCs into oligodendrocyte lineage cells [72]. In addition, cerebral endothelial cells and OPCs may provide an oligovascular niche to promote the proliferation and migration of OPCs [73–75]. This endothelium-to-OPC supportive signaling would be attenuated by excessive oxidative stress [76], supporting the idea that oligodendrocyte/myelin maintenance and renewal is disturbed under the acute phase of stroke conditions. The detailed mechanism (e.g., mediators, etc.) from endothelial cells to OPCs will be discussed in the Sect. 5.1 and 5.5 below.

4 Roles of Oligodendrocyte Lineage Cells in Vascular Remodeling After Stroke

Oligodendrocyte lineage cells are essential for oligodendrogenesis and remyelination after white matter damage. However, the role of oligodendrocyte lineage cells in post-stroke repair is not restricted to restoration of damaged myelin. Oligodendrocyte lineage cells may give support to other cell types during the recovery process after ischemic injury. While there are only a limited number of studies describing this process, we will briefly introduce how oligodendrocyte lineage cells contribute to vascular remodeling after brain damage including ischemic conditions.

Cell–cell signaling between cerebral endothelium and OPCs contribute to vascular remodeling after stroke, in processes mediated by secretion of trophic factors and extracellular matrix proteins such as matrix metalloproteinases (MMPs) [77]. BDNF is one such trophic factor, which promotes endothelial cell survival and angiogenesis [78]. It has been suggested that BDNF derived from oligodendrocyte lineage cells do support angiogenesis, since BDNF mRNA is present in oligodendrocytes, and the physical proximity of oligodendrocytes to cerebral endothelial cells would allow oligodendrocyte-secreted BDNF to interact with the endothelial cells [77, 79]. Transforming growth factor (TGF-β) is another factor that is essential for vascular development and maturation. OPCs are one of the cell types in the neurovascular unit secreting TGF-β1 and this OPC-derived TGF-β1 has a role in maintaining BBB integrity [11]. Treating endothelial cells with OPC-conditioned media increased tight junction proteins in endothelial cells; while OPC-specific TGF-β1 knock-out mice exhibited cerebral hemorrhage, indicating loss of BBB function [11]. Recent research examining OPC contribution to postnatal angiogenesis showed that OPCs regulate angiogenesis within the white matter through OPC-encoded hypoxia-inducible factor (HIF) signaling. OPC-specific HIF stabilization resulted in increased expression of the proangiogenic genes Wnt7a/7b, and promoted angiogenesis and endothelial proliferation in vivo [80]. Further studies are needed to determine whether this OPC-specific HIF pathway plays a role in recovery after CNS injury. In addition to OPCs, mature oligodendrocytes would promote vascular remodeling after white matter damage. Cultured rat oligodendrocytes secrete MMP-9 in response to inflammatory responses. Importantly, conditioned media from IL-1β-stimulated oligodendrocytes would promote in vitro angiogenesis compared to normal oligodendrocyte-conditioned-media [81]. Similarly, in a mouse model of focal stroke, oligodendrocytes were shown to produce MMP-9 during the recovery phase, which may lead to vascular remodeling in white matter [81].

5 Possible Mediators for Endothelium–Oligodendrocyte Interaction

Crosstalk between the vascular and neuronal compartments in the neurovascular niche is mediated by an exchange of soluble signals [3, 6, 7, 17, 63, 82–84]. Many of these trophic factors from cerebral endothelial cells may also affect oligodendrocyte lineage cells. Moreover, a growing literature suggests that similar to cerebral endothelium, oligodendrocyte lineage cells could work as a “bank” for trophic factors. Therefore, it would be reasonable to hypothesize that the endothelium–oligodendrocyte interaction is also extensively mediated by secreting factors. Although the precise regulatory mechanisms that underlie angiogenesis and oligodendrocytes in the oligovascular niche still require further research to be elucidated, we will summarize several candidates of mediators for the crosstalk between cerebral endothelium and oligodendrocytes in this section.

5.1 BDNF

BDNF is a member of the "neurotrophin" family of growth factors, which are related to the canonical nerve growth factor (NGF). BDNF is well-known to act on neurons to support the survival of existing neurons and enhance the growth and differentiation of new neurons and synapses [85, 86]. BDNF can also modulate non-neuronal cell types. Irrefutably, BDNF is one of the major mediators for endothelium–oligodendrocyte interaction.

BDNF is known to regulate oligodendrocyte function in several ways. Firstly, BDNF promotes OPC proliferation and differentiation into mature oligodendrocytes. Cell culture and in vivo animal studies suggested that Trk-B receptor and ERK pathway mediated the supportive effects of BDNF [87–90]. Secondly, even under pathological conditions, BDNF can be supportive for oligodendrocyte lineage cells. In in vivo white matter injury models (spinal cord injury or cuprizone-treated model), BDNF was shown to play an important role in regulating the number of oligodendrocyte lineage cells after demyelination [91, 92]. Thirdly, BDNF may also work on NSPCs to trigger their differentiation into oligodendrocyte lineage cells. Chen et al. reported that BDNF enhanced the cell commitment of NSPCs to neuronal and oligodendrocytic fates by activating Wnt/β-catenin signaling pathway in vitro [93]. Since cerebral endothelial cells are the major BDNF-producing cell types, cerebral endothelial cells may support oligodendrocyte lineage cells via BDNF signaling. As introduced above, endothelial-derived BDNF was shown to promote the proliferation of OPCs in vitro [94]. But importantly, stressed endothelial cells secrete less BDNF and can no longer support OPCs [94], indicating that endothelial–oligodendrocyte interaction is highly dependent on their cellular conditions.

BDNF also promotes endothelial cell survival and induces angiogenesis in the brain [95]. While cell–cell trophic interactions are generally considered as "two-way," there have been still no direct proofs that BDNF mediates signals from oligodendrocytes to endothelial cells. Nevertheless, several lines of evidence strongly support the idea that oligodendrocyte lineage cells may produce BDNF to support endothelial cells. In situ hybridization and immunocytochemical studies identified expressions of BDNF mRNA/protein in cultured basal forebrain oligodendrocytes [79, 96]. The physiological relevance of these in vitro studies is supported by detection of BDNF in oligodendrocytes in vivo. For example, BDNF mRNAs are localized in subpopulations of myelin-basic-protein-positive mature oligodendrocytes in the basal forebrain, cingulate cortex, and corpus callosum of postnatal day 7 rats [79]. Similarly, BDNF mRNA and protein are also expressed in subsets of APC (adenomatous polyposis coli)-positive oligodendrocytes of adult spinal cords [97, 98]. The oligodendrocytic BDNF is bioactive because conditioned media from oligodendrocyte cultures increased cholinergic neuronal function and the effect was partially blocked by co-treatment with anti-BDNF neutralizing antibody [79]. Considering the fact that oligodendrocyte linage cells are often located close to cerebral endothelial cells, it would be reasonable to think that oligodendrocytic BDNF may interact to some extent with the cerebral vascular system.

5.2 Fibroblast Growth Factor-2

Similar to BDNF, fibroblast growth factor-2 (FGF-2/bFGF) can be proposed as an important mediator for endothelium–oligodendrocyte interactions. FGF-2 is a potent stimulator of endothelial cell migration, proliferation, sprouting, and tube formation. In addition, past studies have substantially revealed the effects of FGF-2 on oligodendrocyte function. FGF-2 by itself stimulates proliferation of late-stage OPCs and blocks their terminal differentiation into mature oligodendrocytes in vitro. Upon removal of FGF-2 from the cell culture medium of OPCs, the cells readily enter the terminal differentiation [99–101]. In addition, FGF-2 cooperates with platelet-derived growth factor (PDGF) to upregulate the expression of PDGF-receptor-alpha for OPC proliferation [101, 102]. As for OPC migration, the importance of FGF signaling in early-stage OPCs has been evaluated both in vivo and in vitro. Using an in vivo transplantation approach, Osterhout et al. demonstrated that OPCs with dominant-negative FGF receptor 1 failed to migrate [103]. Subsequent in vitro study showed that in response to FGF-2 stimulation, OPCs growing in an agarose drop successfully move out from the drop [104]. Another study suggested that FGF-2 also enhanced the migration of pre-OPCs from oligospheres, but might not induce the migration of late-stage OPCs [105]. While still controversial, FGF-2 may affect OPC survival. FGF-2 can prevent OPCs from apoptotic stress [106], but this protective effect was not observed in optic nerve OPCs [107]. Since the cellular localization of FGF-2 is observed in cerebral endothelium both in normal and pathological conditions [108], future studies are warranted to examine how FGF-2 modulates the dynamics of endothelium–oligodendrocyte crosstalk under normal conditions as well as in the acute and chronic phases after brain injury.

5.3 TGF-β

TGF-β is a prototypic member of a large family of pleiotropic cytokines, and may also be a potent modulator in the oligovascular niche. Many cells have been reported to produce TGF-β, and TGF-β is known to modulate a diverse array of cellular function [109]. In terms of its involvement in endothelium–oligodendrocyte interactions, oligodendrocytes are reported to express TGF-β in vitro in cell culture systems [110] and in vivo in spinal cords [111]. TGF-β contributes to angiogenesis by stabilizing newly formed capillary sprouts [112]. Thus far, many studies in mouse and human have demonstrated its pivotal roles in modulating angiogenesis after brain injury, such as stroke [113–118]. In addition, dysregulation of TGF-β signaling may cause hereditary vascular disorders [109]. For example, mutations in TGF-β receptors lead to hereditary hemorrhagic telangiectasia [119], and in the cerebral white matter, accumulation of TGF-β1 due to HtrA1 mutation is associated with a hereditary disorder CRASIL (cerebral autosomal recessive arteriopathy with

subcortical infarcts and leukoencephalopathy) [120]. In contrast, the roles of TGF-β on oligodendrocytic function are still understudied. An early report suggests that TGF-β affects the migration of cultured OPCs [121]. Using the oligodendrocyte precursor cell line OLI-neu, TGF-β was shown to upregulate a chondroitin sulfate proteoglycan DSD-1-PG on the cell surface [122], indicating that TGF-β may modulate the cell adhesion property of OPCs to change cell motility.

5.4 Adrenomedullin

Adrenomedullin (AM) was originally isolated from pheochromocytoma cells [123]. AM has a variety of actions on the vascular systems, such as endothelial survival/proliferation, vasodilatation, regulation of BBB permeability, and modulation of oxidative stress levels in endothelium [124, 125]. AM is secreted from various organs, and in the CNS, AM is mainly expressed in neurons and cerebral endothelium [123, 126]. AM has been shown to reduce infarct volumes in transient stroke models [127, 128]. Under stroke conditions, AM expression is markedly increased via the hypoxia-inducible factor-1 signaling [129], indicating that AM might support vascular remodeling/repairing after brain injury. Interestingly, in the mouse model of chronic cerebral hypoperfusion (i.e., vascular dementia model), AM was demonstrated to be protective toward cerebral white matter [130], where most oligodendrocytes are populated. In vitro experiments also confirmed that AM promoted OPC maturation under prolonged hypoxic conditions [131]. These reports suggest that AM may be important in oligodendrocyte regeneration after white matter damage. On the contrary, AM is released by oligodendrocyte lineage cells as well as cerebral endothelial cells. Uezono et al. detected AM mRNAs in human oligodendroglial cell line, and showed the potential effects of AM in modulating oligodendrocyte function through AM receptors in oligodendrocytes [132]. Importantly, past studies have demonstrated that AM increases several growth factors such as VEGF and FGF [133–136]. Taken together, AM might work as a “master” modulator for growth factors in the oligovascular niche.

5.5 VEGF

VEGF is a primary regulator of angiogenesis by stimulating endothelial cell proliferation, migration, and tube formation [83]. But it is now well recognized that VEGF is not solely an endothelial mediator, rather it may represent one of the major mediators that signal to more than one type of cell in the neurovascular unit [137–139]. For example, VEGF signaling plays a key role in neuronal migration and CNS development [140]. In addition, VEGF may also affect oligodendrocyte lineage cells. OPCs express the VEGF receptor, Flk-1 (also known as KDR or VEGF-receptor-2) [74], which is primarily responsible for VEGF-induced angiogenesis.

The same study suggested that VEGF-A significantly accelerated the motility of OPCs through Flk-1, and this effect was partly mediated by ROS production [74]. Notably, conditioned media from cerebral endothelial cells promoted both OPC proliferation and migration. However, endothelial-derived VEGF-A may participate in the OPC migration but not proliferation [141]. In addition to VEGF-A, other VEGF families and VEGF receptors may also be involved in oligodendrocyte function. Le Bras et al. demonstrated that VEGF-C promoted OPC proliferation through VEGF-receptor-3 [142]. Taken together, these findings indicate that VEGFs/VEGF receptors may play a central role in the endothelial–oligodendrocyte trophic coupling.

If VEGF is an important modulator for the oligovascular niche, we may need to consider the "biphasic actions" of VEGF to comprehend the dynamics of trophic coupling between cerebral endothelium and oligodendrocytes. VEGF is known to act as a deleterious factor during the acute phase of stroke. For example, VEGF administration during ischemic insults worsens BBB leakage [143, 144]. On the other hand, VEGF can be a primary regulator of angiogenesis. VEGF would trigger remodeling responses in endothelial cells (i.e., accelerating angiogenesis) after brain damage, such as stroke. In fact, infusing VEGF into the lateral ventricles stimulated angiogenesis and decreased infarct volumes in rodent models of focal cerebral ischemia [145]. An increase in angiogenesis by VEGF in rats was also associated with reduced neurological deficits after stroke [146]. Similar effects were also reported in neonatal focal rodent stroke [147]. Moreover, in transgenic mice that overexpressed human VEGF165, brain microvessel density was significantly elevated compared to wild-type mice before ischemia, and the microvessel density was higher 3 days after stroke onset [148]. Therefore, depending on the context of the microenvironment in the oligovascular niche, VEGF may exert different effects on endothelium–oligodendrocyte interactions that maintain and remodel white matter function.

5.6 MMPs

MMPs comprise a family of zinc endopeptidases, and their activities are tightly regulated by tissue inhibitor of metalloproteinases (TIMPs). This large MMP network plays major roles in the physiology and pathology of the mammalian CNS, including stroke (MMP-2/3/7/9/13, TIMP-1/2 [149]), vascular dementia (MMP-2, 3, 9 [150, 151]), and CADASIL (cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy: TIMP-3 [152]). Similar to VEGF, MMPs are well-known biphasic modulators in the CNS. During the acute phase of stroke, MMP-2/3/9/13 are deleterious, i.e., they degrade the extracellular matrix that comprises the basal lamina, which could directly damage BBB [153]. In addition, proteolysis of the neurovascular matrix by MMP-9 after stroke can trigger anoikis-like neuronal death [154]. However, during delayed phases of stroke recovery, some of these proteinases (MMP-2/3/9) may play beneficial roles [149, 153, 155]. In a mouse stroke model, peri-infarct cortical areas demonstrate a secondary

elevation in MMP-9 in endothelial and glial cells within networks of regrowing microvessels [156]. Inhibition of MMPs during this delayed phase disrupted brain repairing with the induction of hemorrhagic and malformed blood vessels. Moreover, signals of MMP-9 were observed in the migrating neuroblasts from the subventricular zone after brain injury, and inhibition of these MMPs also blocked the movement of these neuroblasts originally headed towards damaged brain [157].

As MMPs play multiple roles in the neurovascular unit, MMPs may mediate the crosstalk between cerebral endothelium and oligodendrocyte. Thus far, several studies imply the essential roles of MMP network in oligodendrocytic function. For example, MMP-9 mediated the extension of oligodendrocytic processes in cell culture [158], and MMP-9 expression level was increased during myelin formation in the optic nerve in vivo [159]. Moreover, after white matter injury, MMP-9 removed injury-induced deposition of inhibitory NG2 proteoglycan, which is an essential step for OPCs to differentiate into mature oligodendrocytes for remyelination [160]. In addition, MMP-12 was shown to cause demyelination, macrophage infiltration, and motor deficits in a mouse model of virus-induced multiple sclerosis [161]. Tissue inhibitor of metalloproteinases might be also involved in cellular function/survival of oligodendrocyte lineage cells. In a mouse model of focal stroke ischemia, mice with TIMP-3 deficiency had a higher number of immature oligodendrocytes after injury [162]. In addition to being the recipient cell for MMP actions, oligodendrocyte lineage cells may in turn produce MMPs to send signals to cerebral endothelial cells. As noted, OPCs respond quickly to a stress stimulation after white matter damage and release MMP-9 which induces early BBB leakage in a mouse model of prolonged hypoperfusion model [10]. On the other hand, during the chronic phase of white matter injury, oligodendrocytic MMP-9 may promote vascular remodeling [81]. Hence, as in the neurovascular unit, MMPs contribute different effects to the dynamics of cellular interactions in the oligovascular niche due to their biphasic property.

6 Conclusion Remarks

The concept of neurovascular unit emphasizes that cell–cell interaction is critical to maintain normal brain function as well as brain remodeling after injury. Within the conceptual framework of neurovascular unit, cerebral endothelial cells are particularly important in releasing soluble factors to nourish neighboring cells, such as astrocytes or neurons. As we discussed in this chapter, cerebral endothelial cells may also support oligodendrocyte lineage cells in the white matter. On the other hand, oligodendrocyte-derived factors can in turn modulate cerebral vascular systems. In the so-called oligovascular niche (i.e., microenvironment between cerebral endothelium and oligodendrocytes), cerebral endothelium and oligodendrocyte lineage cells may cooperate in maintaining white matter homeostasis. Although some key mediators for endothelium–oligodendrocyte crosstalk were briefly discussed here, the precise underlying mechanisms still remain to be elucidated.

Importantly, there may be overlap between factors in the oligovascular niche and the well-established neurovascular niche. Therefore, a deeper analysis, perhaps using subtractive approaches, may be required in order to rigorously define the regulatory signals that are truly unique to the oligovascular niche. As white matter injury is a key part of most CNS diseases, understanding the cell–cell interaction between cerebral endothelium and oligodendrocytes may lead to effective therapeutic approaches for white matter-related diseases, such as stroke and vascular dementia.

Acknowledgments and Funding Supported in part by the National Institutes of Health. Materials including figures in this chapter have been extensively drawn from previously published reviews from the authors: Maki et al., Front Cell Neurosci 2013, Miyamoto et al., Cell Mol Life Sci 2014, Shindo et al., J Cereb Blood Flow Metab 2015, Itoh et al., Brain Res 2015.

References

1. del Zoppo GJ. Inflammation and the neurovascular unit in the setting of focal cerebral ischemia. Neuroscience. 2009;158:972–82.
2. Hawkins BT, Davis TP. The blood-brain barrier/neurovascular unit in health and disease. Pharmacol Rev. 2005;57:173–85.
3. Iadecola C. Neurovascular regulation in the normal brain and in Alzheimer's disease. Nat Rev Neurosci. 2004;5:347–60.
4. Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci. 2003;4:399–415.
5. Lo EH, Broderick JP, Moskowitz MA. tPA and proteolysis in the neurovascular unit. Stroke. 2004;35:354–6.
6. Zacchigna S, Lambrechts D, Carmeliet P. Neurovascular signalling defects in neurodegeneration. Nat Rev Neurosci. 2008;9:169–81.
7. Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 2008;57:178–201.
8. Shindo A et al. Subcortical ischemic vascular disease: roles of oligodendrocyte function in experimental models of subcortical white-matter injury. J Cereb Blood Flow Metab. 2015.
9. Wilkins A, et al. Oligodendrocytes promote neuronal survival and axonal length by distinct intracellular mechanisms: a novel role for oligodendrocyte-derived glial cell line-derived neurotrophic factor. J Neurosci. 2003;23:4967–74.
10. Seo JH, et al. Oligodendrocyte precursors induce early blood-brain barrier opening after white matter injury. J Clin Invest. 2013;123:782–6.
11. Seo JH, et al. Oligodendrocyte precursor cells support blood-brain barrier integrity via TGF-beta signaling. PLoS One. 2014;9, e103174.
12. Bell RD, et al. Pericytes control key neurovascular functions and neuronal phenotype in the adult brain and during brain aging. Neuron. 2010;68:409–27.
13. Zlokovic BV. Neurodegeneration and the neurovascular unit. Nat Med. 2010;16:1370–1.
14. Zlokovic BV. Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. Nat Rev Neurosci. 2011;12:723–38.
15. Makita T, et al. Endothelins are vascular-derived axonal guidance cues for developing sympathetic neurons. Nature. 2008;452:759–63.
16. Dugas JC, et al. A novel purification method for CNS projection neurons leads to the identification of brain vascular cells as a source of trophic support for corticospinal motor neurons. J Neurosci. 2008;28:8294–305.

17. Guo S, et al. Neuroprotection via matrix-trophic coupling between cerebral endothelial cells and neurons. Proc Natl Acad Sci U S A. 2008;105:7582–7.
18. Ohab JJ, et al. A neurovascular niche for neurogenesis after stroke. J Neurosci. 2006;26: 13007–16.
19. Teng H, et al. Coupling of angiogenesis and neurogenesis in cultured endothelial cells and neural progenitor cells after stroke. J Cereb Blood Flow Metab. 2008;28:764–71.
20. Kokovay E, et al. Adult SVZ lineage cells home to and leave the vascular niche via differential responses to SDF1/CXCR4 signaling. Cell Stem Cell. 2010;7:163–73.
21. Taguchi A, et al. Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. J Clin Invest. 2004;114:330–8.
22. Thored P, et al. Long-term neuroblast migration along blood vessels in an area with transient angiogenesis and increased vascularization after stroke. Stroke. 2007;38:3032–9.
23. Franklin RJ. Why does remyelination fail in multiple sclerosis? Nat Rev Neurosci. 2002;3:705–14.
24. Zhang R, Chopp M, Zhang ZG. Oligodendrogenesis after cerebral ischemia. Front Cell Neurosci. 2013;7:201.
25. Zhao C, et al. Mechanisms of CNS remyelination—the key to therapeutic advances. J Neurol Sci. 2005;233:87–91.
26. Franklin RJ, Ffrench-Constant C. Remyelination in the CNS: from biology to therapy. Nat Rev Neurosci. 2008;9:839–55.
27. Franklin RJ, Kotter MR. The biology of CNS remyelination: the key to therapeutic advances. J Neurol. 2008;1:19–25.
28. Fancy SPJ, et al. Myelin regeneration: a recapitulation of development? Ann Rev Neurosci. 2011;34:21–43.
29. Miron VE, Kuhlmann T, Antel JP. Cells of the oligodendroglial lineage, myelination, and remyelination. Biochim Biophys Acta. 2011;2:184–93.
30. Miron VE, et al. M2 microglia and macrophages drive oligodendrocyte differentiation during CNS remyelination. Nat Neurosci. 2013;16:1211–8.
31. Moore CS, et al. How factors secreted from astrocytes impact myelin repair. J Neurosci Res. 2011;89:13–21.
32. Baumann N, Pham-Dinh D. Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol Rev. 2001;81:871–927.
33. Dimou L, et al. Progeny of Olig2-expressing progenitors in the gray and white matter of the adult mouse cerebral cortex. J Neurosci. 2008;28:10434–42.
34. Paus T, et al. Structural maturation of neural pathways in children and adolescents: in vivo study. Science. 1999;283:1908–11.
35. Young KM, et al. Oligodendrocyte dynamics in the healthy adult CNS: evidence for myelin remodeling. Neuron. 2013;77:873–85.
36. Scholz J, et al. Training induces changes in white-matter architecture. Nat Neurosci. 2009;12: 1370–1.
37. Nait-Oumesmar B, et al. The role of SVZ-derived neural precursors in demyelinating diseases: from animal models to multiple sclerosis. J Neurol Sci. 2008;265:26–31.
38. Falcao AM, et al. The path from the choroid plexus to the subventricular zone: go with the flow! Front Cell Neurosci. 2012;6:34.
39. Gonzalez-Perez O, Alvarez-Buylla A. Oligodendrogenesis in the subventricular zone and the role of epidermal growth factor. Brain Res Rev. 2011;67:147–56.
40. Ihrie RA, Alvarez-Buylla A. Lake-front property: a unique germinal niche by the lateral ventricles of the adult brain. Neuron. 2011;70:674–86.
41. Menn B, et al. Origin of oligodendrocytes in the subventricular zone of the adult brain. J Neurosci. 2006;26:7907–18.
42. Ortega F, et al. Oligodendrogliogenic and neurogenic adult subependymal zone neural stem cells constitute distinct lineages and exhibit differential responsiveness to Wnt signalling. Nat Cell Biol. 2013;15:602–13.

43. Hack MA, et al. Neuronal fate determinants of adult olfactory bulb neurogenesis. Nat Neurosci. 2005;8:865–72.
44. Merkle FT, Mirzadeh Z, Alvarez-Buylla A. Mosaic organization of neural stem cells in the adult brain. Science. 2007;317:381–4.
45. Costa MR, et al. Continuous live imaging of adult neural stem cell division and lineage progression in vitro. Development. 2011;138:1057–68.
46. Ortega F, et al. Using an adherent cell culture of the mouse subependymal zone to study the behavior of adult neural stem cells on a single-cell level. Nat Protoc. 2011;6:1847–59.
47. Jablonska B, et al. Chordin-induced lineage plasticity of adult SVZ neuroblasts after demyelination. Nat Neurosci. 2010;13:541–50.
48. Nait-Oumesmar B, et al. Progenitor cells of the adult mouse subventricular zone proliferate, migrate and differentiate into oligodendrocytes after demyelination. Eur J Neurosci. 1999;11: 4357–66.
49. Picard-Riera N, et al. Experimental autoimmune encephalomyelitis mobilizes neural progenitors from the subventricular zone to undergo oligodendrogenesis in adult mice. Proc Natl Acad Sci U S A. 2002;99:13211–6.
50. John GR, et al. Multiple sclerosis: re-expression of a developmental pathway that restricts oligodendrocyte maturation. Nat Med. 2002;8:1115–21.
51. Mandai K, et al. Ischemic damage and subsequent proliferation of oligodendrocytes in focal cerebral ischemia. Neuroscience. 1997;77:849–61.
52. Tanaka K, et al. Activation of NG2-positive oligodendrocyte progenitor cells during postischemic reperfusion in the rat brain. Neuroreport. 2001;12:2169–74.
53. Bain JM, et al. Vascular endothelial growth factors A and C are induced in the SVZ following neonatal hypoxia-ischemia and exert different effects on neonatal glial progenitors. Transl Stroke Res. 2013;4:158–70.
54. Kim HJ, Chuang DM. HDAC inhibitors mitigate ischemia-induced oligodendrocyte damage: potential roles of oligodendrogenesis, VEGF, and anti-inflammation. Am J Transl Res. 2014;6:206–23. eCollection 2014.
55. Zhang L, et al. Erythropoietin amplifies stroke-induced oligodendrogenesis in the rat. PLoS One. 2010;5, e11016.
56. Zhang RL, et al. Sildenafil enhances neurogenesis and oligodendrogenesis in ischemic brain of middle-aged mouse. PLoS One. 2012;7:31.
57. Boulanger JJ, Messier C. From precursors to myelinating oligodendrocytes: contribution of intrinsic and extrinsic factors to white matter plasticity in the adult brain. Neuroscience. 2014;269:343–66.
58. Fuentealba LC, Obernier K, Alvarez-Buylla A. Adult neural stem cells bridge their niche. Cell Stem Cell. 2012;10:698–708.
59. Maki T, et al. Mechanisms of oligodendrocyte regeneration from ventricular-subventricular zone-derived progenitor cells in white matter diseases. Front Cell Neurosci. 2013;7:275.
60. Shen Q, et al. Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell-cell interactions. Cell Stem Cell. 2008;3:289–300.
61. Mirzadeh Z, et al. Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain. Cell Stem Cell. 2008;3:265–78.
62. Tavazoie M, et al. A specialized vascular niche for adult neural stem cells. Cell Stem Cell. 2008;3:279–88.
63. Shen Q, et al. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science. 2004;304:1338–40.
64. Goldman SA, Chen Z. Perivascular instruction of cell genesis and fate in the adult brain. Nat Neurosci. 2011;14:1382–9.
65. Bovetti S, et al. Blood vessels form a scaffold for neuroblast migration in the adult olfactory bulb. J Neurosci. 2007;27:5976–80.
66. Kojima T, et al. Subventricular zone-derived neural progenitor cells migrate along a blood vessel scaffold toward the post-stroke striatum. Stem Cells. 2010;28:545–54.

67. Snapyan M, et al. Vasculature guides migrating neuronal precursors in the adult mammalian forebrain via brain-derived neurotrophic factor signaling. J Neurosci. 2009;29:4172–88.
68. Whitman MC, et al. Blood vessels form a migratory scaffold in the rostral migratory stream. J Comp Neurol. 2009;516:94–104.
69. Jin K, et al. Evidence for stroke-induced neurogenesis in the human brain. Proc Natl Acad Sci U S A. 2006;103:13198–202.
70. Saha B, et al. Cortical lesion stimulates adult subventricular zone neural progenitor cell proliferation and migration to the site of injury. Stem Cell Res. 2013;11:965–77.
71. Chintawar S, et al. Blood-brain barrier promotes differentiation of human fetal neural precursor cells. Stem Cells. 2009;27:838–46.
72. Plane JM, et al. Intact and injured endothelial cells differentially modulate postnatal murine forebrain neural stem cells. Neurobiol Dis. 2010;37:218–27.
73. Arai K, Lo EH. An oligovascular niche: cerebral endothelial cells promote the survival and proliferation of oligodendrocyte precursor cells. J Neurosci. 2009;29:4351–5.
74. Hayakawa K, et al. Vascular endothelial growth factor regulates the migration of oligodendrocyte precursor cells. J Neurosci. 2011;31:10666–70.
75. Hayakawa K, et al. Cerebral endothelial derived vascular endothelial growth factor promotes the migration but not the proliferation of oligodendrocyte precursor cells in vitro. Neurosci Lett. 2012;513:42–6.
76. Arai K, Lo EH. Oligovascular signaling in white matter stroke. Biol Pharm Bull. 2009;32: 1639–44.
77. Miyamoto N, et al. Crosstalk between cerebral endothelium and oligodendrocyte. Cell Mol Life Sci. 2014;71:1055–66.
78. Kermani P, Hempstead B. BDNF: a newly described mediator of angiogenesis. Trends Cardiovasc Med. 2007;17:140–3.
79. Dai X, et al. The trophic role of oligodendrocytes in the basal forebrain. J Neurosci. 2003;23: 5846–53.
80. Yuen TJ, et al. Oligodendrocyte-encoded HIF function couples postnatal myelination and white matter angiogenesis. Cell. 2014;158:383–96.
81. Pham LD, et al. Crosstalk between oligodendrocytes and cerebral endothelium contributes to vascular remodeling after white matter injury. Glia. 2012;60:875–81.
82. Chopp M, Zhang ZG, Jiang Q. Neurogenesis, angiogenesis, and MRI indices of functional recovery from stroke. Stroke. 2007;38:827–31.
83. Greenberg DA, Jin K. From angiogenesis to neuropathology. Nature. 2005;438:954–9.
84. Leventhal C, et al. Endothelial trophic support of neuronal production and recruitment from the adult mammalian subependyma. Mol Cell Neurosci. 1999;13:450–64.
85. Acheson A, et al. A BDNF autocrine loop in adult sensory neurons prevents cell death. Nature. 1995;374:450–3.
86. Huang EJ, Reichardt LF. Neurotrophins: roles in neuronal development and function. Annu Rev Neurosci. 2001;24:677–736.
87. Van't Veer A, et al. Brain-derived neurotrophic factor effects on oligodendrocyte progenitors of the basal forebrain are mediated through trkB and the MAP kinase pathway. J Neurosci Res. 2009;87:69–78.
88. Vondran MW, et al. BDNF+/− mice exhibit deficits in oligodendrocyte lineage cells of the basal forebrain. Glia. 2010;58:848–56.
89. Xiao J, et al. Brain-derived neurotrophic factor promotes central nervous system myelination via a direct effect upon oligodendrocytes. Neurosignals. 2010;18:186–202.
90. Xiao J, et al. Extracellular signal-regulated kinase 1/2 signaling promotes oligodendrocyte myelination in vitro. J Neurochem. 2012;122:1167–80.
91. Nakajima H, et al. Targeted retrograde gene delivery of brain-derived neurotrophic factor suppresses apoptosis of neurons and oligodendroglia after spinal cord injury in rats. Spine (Phila Pa 1976). 2010;35:497–504.
92. VonDran MW, et al. Levels of BDNF impact oligodendrocyte lineage cells following a cuprizone lesion. J Neurosci. 2011;31:14182–90.

93. Chen BY, et al. Brain-derived neurotrophic factor stimulates proliferation and differentiation of neural stem cells, possibly by triggering the Wnt/beta-catenin signaling pathway. J Neurosci Res. 2013;91:30–41.
94. Arai K, Lo E. An oligovascular niche: cerebral endothelial cells promote the survival and proliferation of oligodendrocyte precursor cells. J Neurosci. 2009;29:4351–6.
95. Kermani P, Hempstead B. Brain-derived neurotrophic factor: a newly described mediator of angiogenesis. Trends Cardiovasc Med. 2007;17:140–3.
96. Dai X, Qu P, Dreyfus CF. Neuronal signals regulate neurotrophin expression in oligodendrocytes of the basal forebrain. Glia. 2001;34:234–9.
97. Dougherty KD, Dreyfus CF, Black IB. Brain-derived neurotrophic factor in astrocytes, oligodendrocytes, and microglia/macrophages after spinal cord injury. Neurobiol Dis. 2000;7: 574–85.
98. Wetmore C, Olson L. Neuronal and nonneuronal expression of neurotrophins and their receptors in sensory and sympathetic ganglia suggest new intercellular trophic interactions. J Comp Neurol. 1995;353:143–59.
99. Bansal R, Pfeiffer SE. Inhibition of protein and lipid sulfation in oligodendrocytes blocks biological responses to FGF-2 and retards cytoarchitectural maturation, but not developmental lineage progression. Dev Biol. 1994;162:511–24.
100. Gard AL, Pfeiffer SE. Glial cell mitogens bFGF and PDGF differentially regulate development of O4+GalC- oligodendrocyte progenitors. Dev Biol. 1993;159:618–30.
101. McKinnon RD, et al. FGF modulates the PDGF-driven pathway of oligodendrocyte development. Neuron. 1990;5:603–14.
102. Bogler O, et al. Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells. Proc Natl Acad Sci U S A. 1990;87:6368–72.
103. Osterhout DJ, et al. Transplanted oligodendrocyte progenitor cells expressing a dominant-negative FGF receptor transgene fail to migrate in vivo. J Neurosci. 1997;17:9122–32.
104. Milner R, et al. Contrasting effects of mitogenic growth factors on oligodendrocyte precursor cell migration. Glia. 1997;19:85–90.
105. Decker L, et al. Oligodendrocyte precursor migration and differentiation: combined effects of PSA residues, growth factors, and substrates. Mol Cell Neurosci. 2000;16:422–39.
106. Yasuda T, et al. Apoptosis occurs in the oligodendroglial lineage, and is prevented by basic fibroblast growth factor. J Neurosci Res. 1995;40:306–17.
107. Barres BA, et al. Multiple extracellular signals are required for long-term oligodendrocyte survival. Development. 1993;118:283–95.
108. Issa R, et al. Expression of basic fibroblast growth factor mRNA and protein in the human brain following ischaemic stroke. Angiogenesis. 2005;8:53–62.
109. ten Dijke P, Arthur HM. Extracellular control of TGFbeta signalling in vascular development and disease. Nat Rev Mol Cell Biol. 2007;8:857–69.
110. McKinnon RD, et al. A role for TGF-beta in oligodendrocyte differentiation. J Cell Biol. 1993;121:1397–407.
111. Hinks GL, Franklin RJ. Distinctive patterns of PDGF-A, FGF-2, IGF-I, and TGF-beta1 gene expression during remyelination of experimentally-induced spinal cord demyelination. Mol Cell Neurosci. 1999;14:153–68.
112. Bottner M, Krieglstein K, Unsicker K. The transforming growth factor-betas: structure, signaling, and roles in nervous system development and functions. J Neurochem. 2000;75: 2227–40.
113. Ata AK, Funa K, Olsson Y. Expression of various TGF-beta isoforms and type I receptor in necrotizing human brain lesions. Acta Neuropathol. 1997;93:326–33.
114. Krupinski J, et al. Increased expression of TGF-beta 1 in brain tissue after ischemic stroke in humans. Stroke. 1996;27:852–7.
115. Krupinski J, et al. Inducible nitric oxide production and expression of transforming growth factor-beta1 in serum and CSF after cerebral ischaemic stroke in man. Nitric Oxide. 1998;2:442–53.

116. Slevin M, et al. Serial measurement of vascular endothelial growth factor and transforming growth factor-beta1 in serum of patients with acute ischemic stroke. Stroke. 2000;31: 1863–70.
117. Vivien D, Ali C. Transforming growth factor-beta signalling in brain disorders. Cytokine Growth Factor Rev. 2006;17:121–8.
118. Zhang ZG, et al. Bone marrow-derived endothelial progenitor cells participate in cerebral neovascularization after focal cerebral ischemia in the adult mouse. Circ Res. 2002; 90:284–8.
119. Govani FS, Shovlin CL. Hereditary haemorrhagic telangiectasia: a clinical and scientific review. Eur J Hum Genet. 2009;17:860–71.
120. Hara K, et al. Association of HTRA1 mutations and familial ischemic cerebral small-vessel disease. N Engl J Med. 2009;360:1729–39.
121. Fok-Seang J, et al. Cytokine-induced changes in the ability of astrocytes to support migration of oligodendrocyte precursors and axon growth. Eur J Neurosci. 1998;10:2400–15.
122. Schnadelbach O, Mandl C, Faissner A. Expression of DSD-1-PG in primary neural and glial-derived cell line cultures, upregulation by TGF-beta, and implications for cell-substrate interactions of the glial cell line Oli-neu. Glia. 1998;23:99–119.
123. Kitamura K, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun. 1993;192:553–60.
124. Kato J, et al. Adrenomedullin: a protective factor for blood vessels. Arterioscler Thromb Vasc Biol. 2005;25:2480–7.
125. Kitamura K, et al. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. 1993. Biochem Biophys Res Commun. 2012;425:548–55.
126. Serrano J, et al. Adrenomedullin in the central nervous system. Microsc Res Tech. 2002;57: 76–90.
127. Dogan A, et al. Intravenous infusion of adrenomedullin and increase in regional cerebral blood flow and prevention of ischemic brain injury after middle cerebral artery occlusion in rats. J Cereb Blood Flow Metab. 1997;17:19–25.
128. Watanabe K, et al. Adrenomedullin reduces ischemic brain injury after transient middle cerebral artery occlusion in rats. Acta Neurochir (Wien). 2001;143:1157–61.
129. Eto T. A review of the biological properties and clinical implications of adrenomedullin and proadrenomedullin N-terminal 20 peptide (PAMP), hypotensive and vasodilating peptides. Peptides. 2001;22:1693–711.
130. Maki T, et al. Angiogenic and vasoprotective effects of adrenomedullin on prevention of cognitive decline after chronic cerebral hypoperfusion in mice. Stroke. 2011;42:1122–8.
131. Maki T, et al. Adrenomedullin promotes differentiation of oligodendrocyte precursor cells into myelin-basic-protein expressing oligodendrocytes under pathological conditions in vitro. Stem Cell Res. 2015;15:68–74.
132. Uezono Y, et al. Production of cAMP by adrenomedullin in human oligodendroglial cell line KG1C: comparison with calcitonin gene-related peptide and amylin. Brain Res Mol Brain Res. 2001;97:59–69.
133. Ichikawa-Shindo Y, et al. The GPCR modulator protein RAMP2 is essential for angiogenesis and vascular integrity. J Clin Invest. 2008;118:29–39.
134. Iimuro S, et al. Angiogenic effects of adrenomedullin in ischemia and tumor growth. Circ Res. 2004;95:415–23.
135. Maki T, et al. Angiogenic roles of adrenomedullin through vascular endothelial growth factor induction. Neuroreport. 2011;22:442–7.
136. Schwarz N, et al. Adrenomedullin increases the expression of calcitonin-like receptor and receptor activity modifying protein 2 mRNA in human microvascular endothelial cells. J Endocrinol. 2006;190:505–14.
137. Chow J, et al. Astrocyte-derived VEGF mediates survival and tube stabilization of hypoxic brain microvascular endothelial cells in vitro. Brain Res Dev Brain Res. 2001;130:123–32.
138. Lambrechts D, Carmeliet P. VEGF at the neurovascular interface: therapeutic implications for motor neuron disease. Biochim Biophys Acta. 2006;1762:1109–21.

139. Rosenstein JM, Krum JM. New roles for VEGF in nervous tissue—beyond blood vessels. Exp Neurol. 2004;187:246–53.
140. Carmeliet P, Storkebaum E. Vascular and neuronal effects of VEGF in the nervous system: implications for neurological disorders. Semin Cell Dev Biol. 2002;13:39–53.
141. Hayakawa K, et al. Cerebral endothelial derived vascular endothelial growth factor promotes the migration but not the proliferation of oligodendrocyte precursor cells in vitro. Neurosci Lett. 2012;513(1):42–6.
142. Le Bras B, et al. VEGF-C is a trophic factor for neural progenitors in the vertebrate embryonic brain. Nat Neurosci. 2006;9:340–8.
143. Fagan SC, et al. Targets for vascular protection after acute ischemic stroke. Stroke. 2004;35:2220–5.
144. Hansen TM, Moss AJ, Brindle NP. Vascular endothelial growth factor and angiopoietins in neurovascular regeneration and protection following stroke. Curr Neurovasc Res. 2008;5: 235–44.
145. Sun Y, et al. VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. J Clin Invest. 2003;111:1843–51.
146. Zhang ZG, et al. VEGF enhances angiogenesis and promotes blood-brain barrier leakage in the ischemic brain. J Clin Invest. 2000;106:829–38.
147. Dzietko M, et al. Delayed VEGF treatment enhances angiogenesis and recovery after neonatal focal rodent stroke. Transl Stroke Res. 2013;4:189–200.
148. Wang Y, et al. VEGF overexpression induces post-ischaemic neuroprotection, but facilitates haemodynamic steal phenomena. Brain. 2005;128:52–63.
149. Rosell A, Lo EH. Multiphasic roles for matrix metalloproteinases after stroke. Curr Opin Pharmacol. 2008;8:82–9.
150. Nakaji K, et al. Matrix metalloproteinase-2 plays a critical role in the pathogenesis of white matter lesions after chronic cerebral hypoperfusion in rodents. Stroke. 2006;37:2816–23.
151. Rosenberg GA, Sullivan N, Esiri MM. White matter damage is associated with matrix metalloproteinases in vascular dementia. Stroke. 2001;32:1162–8.
152. Monet-Lepretre M, et al. Abnormal recruitment of extracellular matrix proteins by excess Notch3 ECD: a new pathomechanism in CADASIL. Brain. 2013;136:1830–45.
153. Morancho A, et al. Metalloproteinase and stroke infarct size: role for anti-inflammatory treatment? Ann N Y Acad Sci. 2010;1207:123–33.
154. Gu Z, et al. S-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. Science. 2002;297:1186–90.
155. Zhao BQ, Tejima E, Lo EH. Neurovascular proteases in brain injury, hemorrhage and remodeling after stroke. Stroke. 2007;38:748–52.
156. Zhao BQ, et al. Role of matrix metalloproteinases in delayed cortical responses after stroke. Nat Med. 2006;12:441–5.
157. Lee SR, et al. Involvement of matrix metalloproteinase in neuroblast cell migration from the subventricular zone after stroke. J Neurosci. 2006;26:3491–5.
158. Oh LY, et al. Matrix metalloproteinase-9/gelatinase B is required for process outgrowth by oligodendrocytes. J Neurosci. 1999;19:8464–75.
159. Larsen PH, et al. Myelin formation during development of the CNS is delayed in matrix metalloproteinase-9 and -12 null mice. J Neurosci. 2006;26:2207–14.
160. Larsen PH, et al. Matrix metalloproteinase-9 facilitates remyelination in part by processing the inhibitory NG2 proteoglycan. J Neurosci. 2003;23:11127–35.
161. Hansmann F, et al. Matrix metalloproteinase-12 deficiency ameliorates the clinical course and demyelination in Theiler's murine encephalomyelitis. Acta Neuropathol. 2012;124: 127–42.
162. Yang Y, et al. Tissue inhibitor of metalloproteinases-3 mediates the death of immature oligodendrocytes via TNF-alpha/TACE in focal cerebral ischemia in mice. J Neuroinflammation. 2011;8:108.

Part III
Peripheral Immune Cells in Stroke

The Peripheral Immune Response to Stroke

Josef Anrather

1 Introduction

Historically, the central nervous system (CNS) has been viewed as a place with limited immune surveillance and capacity to mount immune responses. This has led to attribute "immunological privilege" to the CNS, a term first coined by Billingham and Boswell [1]. Already in the nineteenth century the Dutch ophthalmologist van Dooremaal [2] described the phenomenon of immune privilege by demonstrating the long-term survival of mouse skin grafts placed in the anterior chamber of a dog's eye. However, within the brain, immune privilege does not apply to all structures. Murphy and Sturm [3] observed that allogeneic tumor transplants were readily rejected when they came in contact with the cerebral ventricles while transplants that were embedded within the brain parenchyma did not elicit an immune response and showed prolonged survival times. These studies were later extended by Medawar [4] to show that allogeneic skin grafts transplanted into the anterior chamber of the eye or the brain of rabbits were efficiently rejected when the host was previously immunized with donor-derived cells and that this rejection was dependent on vascularization of the graft. The resistance of the CNS to mount an effective immune response also extends to the innate immune system. Bacterial endotoxin injected into the brain parenchyma triggers less inflammatory response as measured by immune cell infiltration, than injection of the same endotoxin dose into the skin [5]. Similar to observations with the adaptive immune response, the reduction in endotoxin-mediated inflammation was not found when endotoxin was injected into the ventricles. These studies collectively suggest that the CNS is not immune privileged per se, but there are mechanisms in place to suppress both the innate and

J. Anrather (✉)
Feil Family Brain and Mind Research Institute, Weill Cornell Medical College,
407 East 61st Street, RR 409, New York, NY 10065, USA
e-mail: joa2006@med.cornell.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_9

adaptive immune responses. These include the blood–brain barrier (BBB) and the glia limitans, which minimize the exchange of potentially antigenic macromolecules and pro-inflammatory mediators (e.g., cytokines, chemokines, DAMP) and pose a barrier for easy immune cell entry from the circulation. In addition, the brain features a generally anti-inflammatory milieu under homeostatic conditions sustained by the expression by highly immunosuppressive cytokines such as transforming growth factor-β (TGF-β) and insulin-like growth factor-1 (IGF-1). Once these structural and molecular barriers are removed, as it is the case during ischemic brain injury, inflammation can proceed "as planned" and contribute to the outcome of stroke. This chapter will briefly discuss immune cells participating in the response to cerebral ischemia, deliberate potential entry points of these cells to gain access to the ischemic tissue, and outline the role of the ischemic brain in shaping the peripheral immune response.

2 Participating Immune Cells

Post-ischemic inflammation is characterized by activation of brain-resident leukocytes, microglia, astroglia, and endothelium, and the orchestrated recruitment of various blood-borne immune cells.

Microglia, *monocytes/macrophages*, *and dendritic cells*—Historically it has been difficult to separate the relative contribution of resident (microglia) and blood (monocyte)-derived macrophages to ischemic brain injury. Because these cells are not readily distinguishable by morphology or marker gene expression, most results derived from in vivo studies could either implicate microglia or macrophages or both. Only recently, multi-label flow cytometry of brain immune cells and bone marrow chimeric animals that express discernable markers (fluorescent proteins or CD45 alloantigen) in have allowed a clear distinction of microglia and blood-derived myeloid cells. A recently developed mouse model, that expresses myeloid-restricted tamoxifen-inducible Cre-recombinase, opens the possibility to generate microglia-selective gene deletions to further dissect the contribution of brain resident and hematogenous myeloid cells in stroke [6]. Development of mice expressing fluorescent proteins in different classes or functionally distinct subsets of hematogenous immune cells should also benefit studies that investigate origin, trafficking, and fate of immune cells after cerebral ischemia. Analysis of microglia/macrophages in brain ischemia is further complicated by the fact that the cellular and functional phenotype of these cells in the post-ischemic microenvironment is not static but fluent as exemplified by the rapid transformation of microglia after cerebral ischemia. Microglial activation occurs before the appearance of neuronal cell death [7] and this early response is characterized by increased arborisation and exploratory behavior giving way to de-arborisation and amoeboid transformation within the first 24 h after stroke while the cells remain stationary [8, 9]. In a study in bone marrow chimeric mice expressing green fluorescent protein (GFP) Schilling et al. [10] found that microglia contributed to increased numbers of macrophage-like

cells during the first days after cerebral ischemia while GFP$^+$ blood-borne cells appeared in robust numbers only after day 2. While pro-inflammatory and cytotoxic activities of microglia have been demonstrated in vitro, eliminating proliferating microglia/macrophages by ganciclovir treatment in mice that express herpes simplex virus-1-thymidine kinase in the myeloid lineage increased ischemic brain injury and reduced expression of the neurotrophic cytokine IGF-1 [11]. Similar to microglia, monocyte-derived macrophages can exhibit various phenotypes. Blood monocytes exist as two functionally distinct subpopulations (inflammatory and patrolling) best characterized by their expression of C-C chemokine receptor 2 (CCR2) and C-X3-C chemokine receptor 1 (CX3CR1), both of which are important for monocyte entry into the ischemic brain [12–14], and the relative abundance of these subsets in the blood has been linked to clinical stroke outcome [15, 16]. Initially, infiltrating monocytes are of the "inflammatory" subtype while "patrolling" monocytes are prevalent at later time points [17]. The pathophysiological importance of this finding, however, has yet to be determined. In experimental stroke, monocytes/macrophages infiltrate the brain parenchyma 48–72 h after ischemia and can persist for several weeks [10]. There is also evidence that inhibition of adhesion molecules involved in lymphocyte and monocyte trafficking, such as the very late activation antigen-4 (VLA-4) or the lymphocyte function antigen-1 (LIF-1), can reduce infarct volumes in stroke models [18–21]. These studies suggest that lymphocytes and monocytes, in addition to neutrophils, participate in post-ischemic brain inflammation [22]. However, the precise role of monocyte-derived macrophages in the deleterious effects of post-ischemic inflammation remains to be defined. In addition, depending on molecular cues encountered in the post-ischemic brain parenchyma, monocytes/macrophages can undergo classical (M1, pro-inflammatory) or alternative (M2, anti-inflammatory in most settings) activation, thus contributing to the resolution of inflammation [8, 23]. Phagocytosis, which is mainly seen in microglia and not macrophages and is tied to MER proto-oncogene tyrosine kinase (MerTK), milk fat globule-EGF factor 8 protein (MFG-E8) and triggering receptor expressed on myeloid cells-2 (TREM2) expression [24, 25], can contribute to delayed neuronal cell death after ischemia [24, 26]. A subset of microglia and infiltrating macrophages express the dendritic cell (DC) marker CD11c and MHC class-II molecules [27]. Using chimeric animals that express yellow fluorescent protein (YFP) under the control of the CD11c promoter in bone marrow-derived cells, the same study showed that DCs originate from brain-resident myeloid cells early after ischemia, while peripheral immune cells contribute the majority of CD11c$^+$/YFP$^+$ cells 3 days after stroke [27]. Although brain DCs have the capacity to present antigen and induce T cell proliferation in vitro [28], it remains to be shown whether these cells engage in antigen presentation after brain ischemia as predicted by some studies [29].

Neutrophils—Although intravascular adhesion of neutrophils is a relatively early post-ischemic event, parenchymal accumulation is generally observed later. Nevertheless, neutrophils are among the first hematogenous immune cells found in the brain after experimental stroke peaking at 48–72 h in most models and declining rapidly afterwards. Although it is not clear whether they enter the brain parenchyma

under all circumstances [30], there is evidence that neutrophils contribute to post-ischemic inflammation by limiting tissue perfusion due to intravascular clogging [31, 32], destabilizing the BBB by releasing matrix-metalloproteinases (MMP) [33, 34], and by generating reactive oxygen and nitrogen species [35, 36]. However, a cause-and-effect relationship between the extent of neutrophil trafficking and the severity of ischemic damage has not been firmly established [37]. Attesting to the complex role of neutrophils in cerebral ischemic injury, a recent study postulates a protective role of neutrophils, that have undergone N2 polarization as characterized by Ym1 (chitinase 3-like 3, *Chi3l3*) expression, in stroke pathology [38].

Mast cells—Are brain resident immune cells located in the perivascular space surrounding brain parenchymal vessel and in meninges that are rich in peptidases and vasoactive molecules. Mast cells are activated early after cerebral ischemia and contribute to the BBB breakdown and brain edema by releasing gelatinase and vasoactive mediators [39, 40].

Lymphocytes—T cells are detrimental in the early phase of ischemia and lymphocyte-deficient mice are protected in models of focal ischemia [41, 42]. The mechanism does not involve classical antigen-mediated T cell activation and the cytotoxic activity might be tied to innate T cell functions [42]. Accordingly, IL-17 secreting γδT cells, that do not undergo classical antigen-dependent T cell activation, have been shown to contribute to ischemic injury [43, 44]. While effector T lymphocytes may contribute to focal ischemic injury [41, 45], regulatory T cells (Treg) could have a protective effect by downregulating post-ischemic inflammation. Tregs appear in the ischemic tissue after the acute phase and confer neuroprotection by IL-10 secretion, an effect that might be antigen-independent [46–49]. Similarly, regulatory B cells (Breg) confer neuroprotection through an IL-10 dependent mechanism, but do not enter the ischemic brain [50–52]. Recent work that has extended the range of stroke-related inflammation beyond innate immune mechanisms to include adaptive immunity [53, 54]. Disruption of the blood–brain barrier during acute stroke releases novel CNS antigens that are normally sequestered in the brain and exposes them to the systemic immune system. Meningeal lymphatic vessels that run along the venous sinus and terminate in the deep cervical lymph nodes [55], are likely to be involved in the cranial export of antigenic macromolecules and antigen-presenting cells (APC) after ischemic brain injury (Fig. 1). Evidence for antigen-specific T cell reactivity has been found in animal models of stroke. There is an ever increasing list of CNS-derived peptides that can induce a peripheral T cell response after stroke including—but not limited to—peptides derived from myelin basic protein (MBP), neuron-specific enolase (NSE), proteolipid protein (PLP), NMDA receptor 2A (NR2A), and microtubule-associated protein (MAP) [54, 56]. Peptide-reactive B and T cells can be found in cervical lymph nodes and spleen as early as 4 days after transient focal ischemia in mice [56]. Among the T cells, the response was similar in memory ($CD4^+$) and effector ($CD8^+$) T cells. In human stroke, APCs loaded with CNS-derived peptides have been found in T cell zones of cervical lymph nodes and palate tonsils [57]. Association studies of this small patient cohort indicated that increased reactivity to neuronal-derived antigens was correlated with smaller infarct size and better long-term outcome,

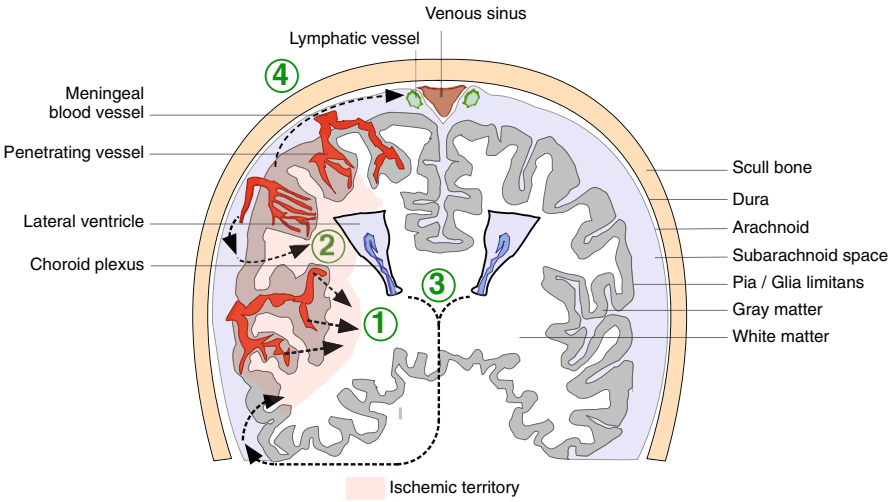

Fig. 1 Possible immune cell entry points after ischemic brain injury. (*1*) Circulating immune cells adhere to the activated endothelium within the ischemic territory and migrate into the ischemic tissue across the damaged BBB. (*2*) Blood leukocytes can extravasate through meningeal vessel to enter the subarachnoid space. From there they might infiltrate the brain parenchyma along chemotactic gradients produced within the ischemic territory by activated endothelial cells, glia, and neurons. Impairment of the surface glia limitans covering the ischemic tissue facilitates the exchange of such inflammatory mediators between the brain parenchyma and the subarachnoid space. (*3*) Immune cells can gain access to the brain by entering the ventricular space through the choroid plexus, where adhesion molecules are upregulated in response to ischemic brain injury. Once in the CSF they can enter the brain parenchyma by penetrating the epithelial cell layer of the ventricles or migrate from the ventricles into the subarachnoid space via the foramina of Luschka and of Magendie located in the fourth ventricle. (*4*) Antigenic macromolecules or activated antigen-presenting cells can exit the cranium through meningeal lymphatic vessel terminating at deep cervical lymph nodes

whereas greater reactivity to MBP was correlated with larger infarcts and worse outcome. This dichotomy might be an indication that the adaptive immune response to ischemic brain injury can be skewed towards reactive (Th1/Th17) or tolerogenic (Th2) phenotypes. This interpretation is supported by studies in rodent stroke models that indicate a beneficial effect of a Th2 immune response on stroke outcome [58, 59]. Future studies will have to address whether a tolerogenic immune response is linked to favorable stroke outcome in humans.

3 Immune Cell Entry Points

The forebrain with exception of the olfactory bulb is covered by a continuous layer of astrocyte cell bodies in rodents or by cytoplasmic processes of marginal astrocytes in primates known as the glia limitans [60]. The astrocytes are in close contact

with the basal lamina which is in intimate contact with the pia mater, the innermost leptomeningeal membrane. This structure forms a tight barrier to the subarachnoid space and limits the exchange of solutes and cells between the brain parenchyma and the cerebrospinal fluid (CSF). The glia limitans extends along penetrating blood vessels and together with pericytes, the basement membrane and interendothelial tight junctions form the BBB that renders the blood vessel of the cerebral vasculature impermeable to macromolecules and circulating cells. In the course of inflammation these barriers are altered and leukocytes penetrate into the CNS via multiple routes including (1) blood-to-parenchyma through a weakened BBB, (2) blood-to-CSF via the choroid plexus, (3) blood-to-CSF via leptomeningeal vessel, and (4) CSF-to-parenchyma across the glia limitans (Fig. 1).

Parenchymal entry—In the course of cerebral ischemia the BBB undergoes several changes that are reversible at first but become permanent at later time points of the ischemic injury. The first opening of the BBB is due to loss of endothelial tight junctions with concomitant transient downregulation or redistribution of junction proteins such as occludins, claudin, and zonula occludens-1. While the impairment of the BBB caused by endothelial tight-junction dysfunction occurs within hours after cerebral ischemia, a second opening of the BBB occurs in later phases of the ischemic insult and is characterized by loss of vascular cells, including endothelial cells and pericytes, proteolytic degradation of the basal membrane and retraction of astrocytic endfeet from the gliovascular unit [61]. It is during this phase that substantial infiltration of peripheral immune cells is observed in the ischemic territory. Within the reperfused territory leukocytes adhere to the endothelium of arterioles and post-capillary venules via endothelial upregulation of selectin-family adhesion molecules (P- and E-selectin) which results in a slow-down and rolling of intravascular leukocytes [62]. Firm attachment to endothelial cells is achieved through integrin-mediated interaction with endothelial-expressed intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), among others. Interestingly, the interaction with the vasculature does not result in leukocyte extravasation and adherence is a relatively short-lived event that, depending on the experimental model, reverts back to pre-ischemia levels within hours after reperfusion [63, 64]. Leukocyte clogging at the time of reperfusion is thought to contribute to "no-reflow" phenomena [32], but leukocyte trapping in capillaries has not been consistently found in cerebral ischemia [63, 65]. Eventually leukocytes extravasate from the circulation into the brain parenchyma although this event is not synchronized with endothelial expression of adhesion molecules and generation of parenchymal chemokines, which in most transient focal cerebral ischemia models in rodents peak well before sustained numbers of parenchymal leukocytes are observed. The reasons for this remain elusive, but the existence of a double barrier—endothelial and glial basement membrane—could contribute to this effect [66]. As a result, blood-borne immune cells, specifically neutrophils, are often found in the ischemic territory forming cuffs that surround the vessel, without entering the neuropil. This has led to the assumption that the contribution of peripheral immune cells to ischemic damage might be marginal. However, "injury at a distance" due to release of neurotoxic factors from perivascular leukocytes has to be

considered as a contributing factor during the early stages of ischemia-reperfusion. Activated leukocytes express several proteases, which are released extracellularly and matrix metalloproteases (MMPs) have been consistently linked to ischemic brain injury. Increased levels of such proteases should aid the degradation of the extracellular matrix including the basement membranes and expose the leukocytes to parenchymal chemokine gradients that enhance leukocyte infiltration. Overall, although immune cell entry from the vasculature within the ischemic parenchyma is likely, hard evidence for this route of entry is still missing.

Choroid plexus—The choroid plexus is a highly vascularized structure that attaches to the ependymal cells lining the cerebral ventricles and separates the ventricles from the subarachnoid space. In contrast to the brain parenchymal and meningeal blood vessel, the endothelial cells of the choroid plexus are fenestrated allowing solutes and intravascular cells to cross the endothelial cell layer. Instead, the choroid epithelial cells are interconnected by continuous tight junctions similar to the ones found in endothelial cells of parenchymal blood vessels. The highly polarized epithelium forms villous structures, which increase its ventricular surface area, and is able to actively produce large quantities of CSF. The stroma of the choroid plexus contains a sizable number of tissue-resident macrophages and dendritic cells that express MHC class-II molecules and may present antigens to T cells entering the CSF [67]. In the healthy brain the choroid plexus is the entry site for patrolling lymphocytes—mostly $CD4^+$ central memory T cells [68]. To facilitate leukocyte trafficking through the choroid plexus, the choroid plexus epithelium expresses constitutively ICAM-1 and VCAM-1, which together with mucosal vascular-addressing cell adhesion molecule-1 (MAdCAM-1), are upregulated under inflammatory conditions [69, 70]. The choroid plexus epithelial cells also constitutively express the chemokine CCL20 that acts on the CCR6 receptor on the surface of IL-17 secreting lymphocytes to promote their CSF entry [71]. Given that IL-17 γδT cells have been implicated in stroke pathophysiology, the selective recruitment of IL-17^+ T cells across the choroid plexus might be relevant for the outcome of ischemic brain injury. CD73, an ecto-ATPase expressed on choroid plexus epithelial cells and lymphocytes, has been shown to mediate monocyte/macrophage migration across the choroid plexus in a model of spinal cord injury [72]. Deletion of CD73 exacerbated ischemic injury in mice undergoing focal ischemia even when wild-type leukocytes were engrafted in the $CD73^{-/-}$ host after bone marrow ablation [73]. The results indicate that immune cells with an anti-inflammatory phenotype or immune cells contributing to tissue repair [72] may use this route of entry. Taken together, more studies are needed to address the likely role of the choroid plexus as an entry point for leukocytes after stroke.

Meninges—The brain and the spinal cord are enclosed by three membranes. The fibrous dura mater, the highly vascularized arachnoid, that with its trabecular structure spans the subarachnoid space filled with CSF, and the pia mater, which covers the brain parenchyma and is in close contact with astrocytes forming the glia limitans at the surface of the brain. The arachnoid is highly vascularized and cerebral vessels are embedded within the arachnoid trabeculae before they enter the brain parenchyma. Similarly to the choroid plexus, the meninges are populated by bone

marrow-derived perivascular myeloid cells that express the microglia/macrophage marker IbaI and MHC class-II molecules while a subpopulation of meningeal macrophages expresses the dendritic cell marker CD11c [67]. Little is known about the physiological role of meningeal macrophages but given their proximity to blood vessels and their possible interaction with CSF lymphocytes, it is predicted that these cells play a role in the CNS immune surveillance. The other constitutive meningeal immune cell population identified in humans and rodents are mast cells primarily located in the dura mater [74]. Because mast cells contain granules with vasoactive molecules and proteases, they have been implicated in BBB disruption and in promoting neutrophil extravasation in the course of cerebral ischemia [74, 75]. Several studies have addressed the role of leptomeningeal vessel as a source of blood-borne immune cells after stroke. Consistent with a meningeal origin, neutrophils are found on the abluminal site of leptomeningeal vessel within hours after stroke in permanent and transient ischemia models in rodents. A strong association of neutrophils with leptomeningeal vessel has also been observed in tissue samples from human stroke victims [76]. Whether neutrophils that extravasated into the subarachnoid space go on to infiltrate the ischemic territory remains to be established, but the fact that accumulation in the meninges precedes the appearance of neutrophils in the brain parenchyma supports such a scenario [30, 76, 77].

4 Alterations of the Peripheral Immune System After Stroke

While peripheral immune cells contribute to ischemic brain injury, the ischemic brain in turn has a profound effect on the composition and behavior of the peripheral immune system. This effect is mediated by the systemic release of danger-associated molecular pattern (DAMP) molecules from the ischemic territory, humoral signals generated by hypothalamic stress response centers, and neural signals of the autonomous nervous system. The response is characterized by an early state of hyperinflammation, followed by a phase of immunosuppression with increased susceptibility to infection. Parts of this response are not specific to ischemic brain injury and are also observed in other conditions associated with severe tissue injury such as burns and traumatic organ damage [78, 79]. Of note, in a mouse model of transient focal ischemia, anesthesia and surgical trauma itself led to early changes in the composition of blood immune cell populations independent of actual middle cerebral artery occlusion [80]. Therefore, observed changes in the peripheral immune response after experimental stroke have to be interpreted in the context of the occlusion model, anesthesia, surgical manipulation, and time points of analysis. Moreover, many of the changes to the peripheral immune system discussed below are only observed in stroke models that result in large ischemic injuries (>30–40 % of the hemispheric volume), a correlation also observed in human stroke [81]. Accordingly, distal occlusion of the middle cerebral artery in rodents, which produces infarcts <20 % of the hemispheric volume, does not elicit a peripheral immune response and does not cause immunosuppression. In addition, stroke-induced changes in the

peripheral immune system show lateralization [82, 83] and the net immunomodulatory autonomic output after ischemia might be dependent on brain structures damaged [84]. Consequently, electrolytic lesion experiments showed that lesions in the hypothalamus were immunosuppressive while similar lesions in the hippocampus and amygdala resulted in immunostimulation [85, 86].

Hyperinflammation—the early response to ischemic brain injury. In experimental stroke, the earliest peripheral immune response to ischemic brain injury is characterized by elevated serum cytokine levels (IL-6, interferon-γ, CXCL1) and increased production of inflammatory mediators in circulating and splenic immune cells (TNF, IL-6, IL-2, CCL2, and CXCL2) within hours after ischemia [87, 88]. The response is generally transient and most parameters return to baseline levels 24 h after stroke. Comparable changes can be observed in human stroke patients. Tumor necrosis factor (TNF) and IL-6 are increased in patients at stroke onset (<24 h) [89] and IL-6 serum levels are positively correlated with stroke severity and unfavorable outcomes [90]. However, this early activation of the immune system is superseded by a state of systemic immunodepression that predisposes to post-stroke infections [91, 92]. Accordingly, complications from pulmonary or urinary tract infections have been observed in ~20 % of stroke patients [93].

Stroke-induced immunodeficiency syndrome—Early studies on the immune status of stroke patients found prolonged peripheral lymphopenia and reduced T cell responsiveness [82, 94]. Ischemic brain injury leads to sustained decrease in blood and splenic B, T, and NK cells in mice undergoing transient middle cerebral artery occlusion [92, 95, 96]. These changes are observed 12 h after ischemia and persist for several weeks. Importantly, the immunosuppression leads to increased susceptibility to nosocomial bacterial infection and to higher mortality rate [92]. The decrease in lymphocytes was correlated with increased splenocyte apoptosis, spleen atrophy, and Treg expansion [92, 95]. The sympathetic nervous system was fundamentally involved in this response. Treatment of animals with the β-adrenergic receptor antagonist propranolol or chemical ablation of sympathetic noradrenergic terminals by 6-hydroxydopamine was sufficient to lower bacteremia and bacterial colonization of the lungs and increased significantly survival rates along with preservation of splenic and blood lymphocyte populations [92]. Interestingly, the glucocorticoid receptor antagonist RU-486 reversed the lymphopenia in the blood, but not in the spleen, and did not impact bacterial colonization or survival rates after stroke. Whether the splenic response to cerebral ischemia is induced by similar mechanisms in all stroke models and in humans remains to be determined. Studies in rats concluded that the loss of splenic lymphocytes was not due to increased apoptosis but due to activation of α-adrenergic receptors on trabecular and capsular smooth muscle cells that leads to spleen contraction and expels immune cells into circulation [97] (Fig. 2). The study found that although spleen size was decreased 1 day after stroke, spleen volume was restored 3 days thereafter arguing against long-lasting effects of cerebral ischemia on spleen physiology. As in rats, splenic size loss in humans is transient and there is a tendency of increased splenic volume 4 days after stroke [98]. The immunosuppressive effects of ischemic brain injury are not limited to the spleen. In the bone marrow, tyrosine hydroxylase and

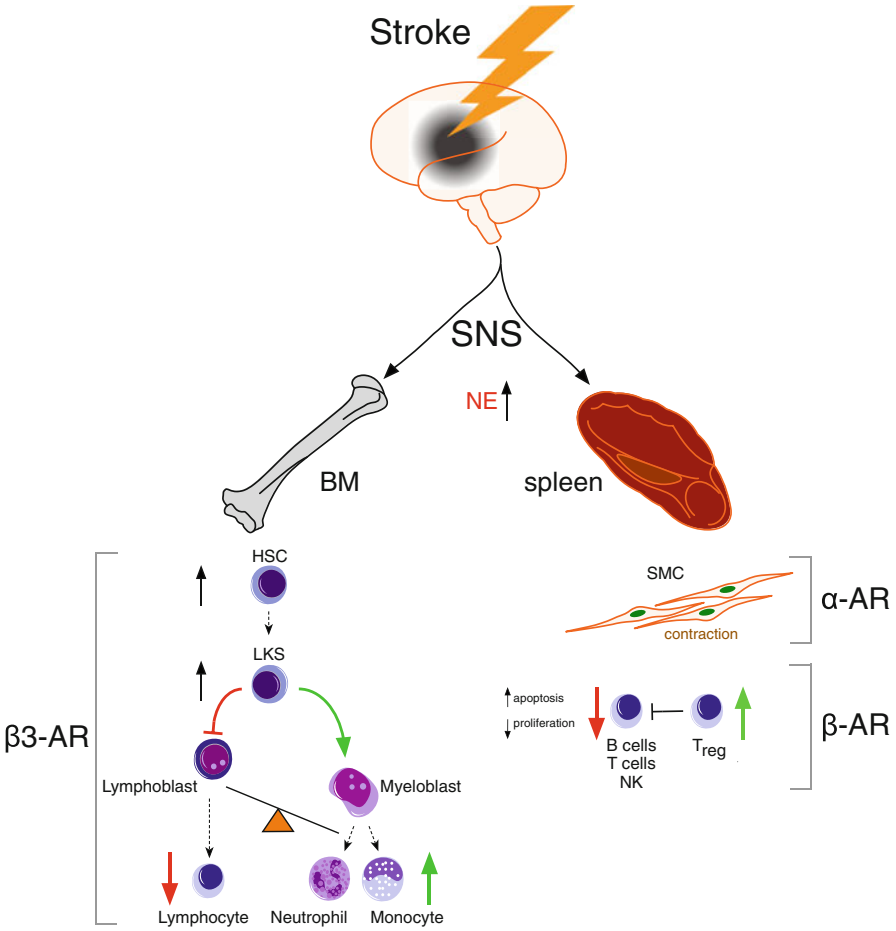

Fig. 2 Stroke-induced immunodeficiency syndrome. Stroke increases activity of the sympathetic nervous system (SNS) resulting in the release of norepinephrine (NE) from sympathetic terminals in bone marrow (BM) and spleen. In the spleen activation of α-adrenergic receptors (α-AR) on capsular and trabecular smooth muscle cells (SMC) results in contraction of the splenic pulp, decrease in organ size, and reduction in cellular density. Furthermore, stimulation of β-adrenergic receptors (β-AR) on splenic lymphocytes results in Treg expansion and B cell, T cell, and NK cell apoptosis. In the BM, NE activates β3-adrenergic receptors (β3-AR) located on mesenchymal stromal cells, which trigger the downregulation of homeostatic factors including CXCL12 (SDF-1), resulting in increased hematopoietic stem cell (HSC) and Lin^{-}/c-Kit^{+}/$Sca\text{-}1^{+}$ (LKS) stem cell proliferation. LKS stem cells are located immediately before the commitment to lymphoid or myeloid lineages. Adrenergic signaling skews LKS cells to the myeloid lineage resulting in increased neutrophil and monocyte production while decreasing the generation of lymphocytes

norepinephrine (NE) levels increase 1 day after transient middle cerebral artery occlusion in mice [99]. This triggers a response in mesenchymal stromal cells, probably through activation of β3-adrenergic receptors (β3-AR), resulting in the reduction of homeostatic and cell retention factors such as interleukin-7, C-X-C

motif chemokine 12 (stromal cell-derived factor 1), VCAM-1, stem cell factor, and angiopoietin-1. Downregulation of these factors increases hematopoietic stem cell (HSC) proliferation (Fig. 2). This proliferative response, however, does not profit all arms of leukocyte lineages equally [99]. The hematopoietic system becomes skewed towards the myeloid lineage. This switch is accomplished by increased expression of transcription factors associated with myeloid lineage progression such as the ETS-domain transcription factor PU.1 and CCAAT/enhancer-binding protein β (C/EBPβ). Collectively the data suggest that the brain is a strong regulator of the peripheral immune system by regulating development and homeostasis of splenic and BM immune cell populations and that increased sympathetic output after stroke is the main efferent branch responsible for these effects.

5 Epilog

Bidirectional interactions between the injured brain and the peripheral immune system are not only important for the development of the ischemic injury but also strongly affect the immune status of the organism as a whole. Whereas the initial immune response triggered by stroke is largely pro-inflammatory, stroke-induced immunodeficiency syndrome, while being desirable for limiting the deleterious effects of post-ischemic inflammation, poses a severe risk for bacterial infections and unfavorable outcome. Because inflammation and peripheral immune cells that infiltrate the ischemic brain might also be involved in repair processes, it will be of importance to determine how the functionality of these systems is affected by stroke. In conclusion, immunomodulation by enhancing the activity of reparatory neutrophils and macrophages or by skewing the adaptive immune system to a "tolerized" state might constitute a more promising approach than indiscriminate anti-inflammatory therapies to limit the deleterious effects of post-stroke inflammation.

References

1. Billingham RE, Boswell T. Studies on the problem of corneal homografts. Proc R Soc Lond B Biol Sci. 1953;141:392–406.
2. Van Dooremaal JC. De ontwikkeling van levende veefsels op vreemden boden geent. Onderz physiol Lab Rijksuniv Utrechsche Loogeschool. 1873;3:277.
3. Murphy JB, Sturm E. Conditions determining the transplantability of tissues in the brain. J Exp Med. 1923;38:183–97.
4. Medawar PB. Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. Br J Exp Pathol. 1948;29:58–69.
5. Andersson PB, Perry VH, Gordon S. The acute inflammatory response to lipopolysaccharide in CNS parenchyma differs from that in other body tissues. NSC. 1992;48:169–86.
6. Goldmann T, Wieghofer P, Müller PF, Wolf Y, Varol D, Yona S, Brendecke SM, Kierdorf K, Staszewski O, Datta M, Luedde T, Heikenwalder M, Jung S, Prinz M. A new type of microglia

gene targeting shows TAK1 to be pivotal in CNS autoimmune inflammation. Nat Neurosci. 2013;16:1618–26.
7. Rupalla K, Allegrini PR, Sauer D, Wiessner C. Time course of microglia activation and apoptosis in various brain regions after permanent focal cerebral ischemia in mice. Acta Neuropathol (Berl). 1998;96:172–8.
8. Fumagalli S, Perego C, Ortolano F, De Simoni M-G. CX3CR1 deficiency induces an early protective inflammatory environment in ischemic mice. Glia. 2013;61:827–42.
9. Morrison HW, Filosa JA. A quantitative spatiotemporal analysis of microglia morphology during ischemic stroke and reperfusion. J Neuroinflammation. 2013;10:4.
10. Schilling M, Besselmann M, Leonhard C, Mueller M, Ringelstein EB, Kiefer R. Microglial activation precedes and predominates over macrophage infiltration in transient focal cerebral ischemia: a study in green fluorescent protein transgenic bone marrow chimeric mice. Exp Neurol. 2003;183:25–33.
11. Lalancette-Hebert M, Gowing G, Simard A, Weng YC, Kriz J. Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. J Neurosci. 2007;27:2596–605.
12. Denes A, Ferenczi S, Halász J, Környei Z, Kovács KJ. Role of CX3CR1 (fractalkine receptor) in brain damage and inflammation induced by focal cerebral ischemia in mouse. J Cereb Blood Flow Metab. 2008;28:1707–21.
13. Schilling M, Strecker J-K, Ringelstein EB, Kiefer R, Schäbitz W-R. Turn-over of meningeal and perivascular macrophages in the brain of MCP-1-, CCR-2- or double knockout mice. Exp Neurol. 2009;219:583–5.
14. Schilling M, Strecker J-K, Ringelstein EB, Schäbitz W-R, Kiefer R. The role of CC chemokine receptor 2 on microglia activation and blood-borne cell recruitment after transient focal cerebral ischemia in mice. Brain Res. 2009;1289:79–84.
15. Urra X, Villamor N, Amaro S, Gómez-Choco M, Obach V, Oleaga L, Planas AM, Chamorro A. Monocyte subtypes predict clinical course and prognosis in human stroke. J Cereb Blood Flow Metab. 2009;29:994–1002.
16. Kaito M, Araya S-I, Gondo Y, Fujita M, Minato N, Nakanishi M, Matsui M. Relevance of distinct monocyte subsets to clinical course of ischemic stroke patients. PLoS One. 2013;8, e69409.
17. Gliem M, Mausberg AK, Lee J-I, Simiantonakis I, van Rooijen N, Hartung H-P, Jander S. Macrophages prevent hemorrhagic infarct transformation in murine stroke models. Ann Neurol. 2012;71:743–52.
18. Arumugam TV, Salter JW, Chidlow JH, Ballantyne CM, Kevil CG, Granger DN. Contributions of LFA-1 and Mac-1 to brain injury and microvascular dysfunction induced by transient middle cerebral artery occlusion. Am J Physiol Heart Circ Physiol. 2004;287:H2555–60.
19. Becker KJ. Activation of immune responses to brain antigens after stroke. J Neurochem. 2012;123 Suppl 2:148–55.
20. Prestigiacomo CJ, Kim SC, Connolly ES, Liao H, Yan SF, Pinsky DJ. CD18-mediated neutrophil recruitment contributes to the pathogenesis of reperfused but not nonreperfused stroke. Stroke. 1999;30:1110–7.
21. Relton JK, Sloan KE, Frew EM, Whalley ET, Adams SP, Lobb RR. Inhibition of alpha4 integrin protects against transient focal cerebral ischemia in normotensive and hypertensive rats. Stroke. 2001;32:199–205.
22. Campanella M, Sciorati C, Tarozzo G, Beltramo M. Flow cytometric analysis of inflammatory cells in ischemic rat brain. Stroke. 2002;33:586–92.
23. Hu X, Li P, Guo Y, Wang H, Leak RK, Chen S, Gao Y, Chen J. Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. Stroke. 2012;43:3063–70.
24. Neher JJ, Emmrich JV, Fricker M, Mander PK, Théry C, Brown GC. Phagocytosis executes delayed neuronal death after focal brain ischemia. Proc Natl Acad Sci U S A. 2013;110: E4098–107.
25. Schilling M, Besselmann M, Müller M, Strecker JK, Ringelstein EB, Kiefer R. Predominant phagocytic activity of resident microglia over hematogenous macrophages following transient

focal cerebral ischemia: an investigation using green fluorescent protein transgenic bone marrow chimeric mice. Exp Neurol. 2005;196:290–7.
26. Sieber MW, Jaenisch N, Brehm M, Guenther M, Linnartz-Gerlach B, Neumann H, Witte OW, Frahm C. Attenuated inflammatory response in triggering receptor expressed on myeloid cells 2 (TREM2) knock-out mice following stroke. PLoS One. 2013;8, e52982.
27. Felger JC, Abe T, Kaunzner UW, Gottfried-Blackmore A, Gal-Toth J, McEwen BS, Iadecola C, Bulloch K. Brain dendritic cells in ischemic stroke: time course, activation state, and origin. Brain Behav Immun. 2010;24:724–37.
28. Gottfried-Blackmore A, Kaunzner UW, Idoyaga J, Felger JC, McEwen BS, Bulloch K. Acute in vivo exposure to interferon-gamma enables resident brain dendritic cells to become effective antigen presenting cells. Proc Natl Acad Sci U S A. 2009;106:20918–23.
29. Liesz A, Karcher S, Veltkamp R. Spectratype analysis of clonal T cell expansion in murine experimental stroke. J Neuroimmunol. 2013;257(1–2):46–52.
30. Enzmann G, Mysiorek C, Gorina R, Cheng Y-J, Ghavampour S, Hannocks M-J, Prinz V, Dirnagl U, Endres M, Prinz M, Beschorner R, Harter PN, Mittelbronn M, Engelhardt B, Sorokin L. The neurovascular unit as a selective barrier to polymorphonuclear granulocyte (PMN) infiltration into the brain after ischemic injury. Acta Neuropathol. 2012;125:395–412.
31. Dawson DA, Ruetzler CA, Carlos TM, Kochanek PM, Hallenbeck JM. Polymorphonuclear leukocytes and microcirculatory perfusion in acute stroke in the SHR. Keio J Med. 1996;45: 248–52; discussion 252–3.
32. del Zoppo GJ, Schmid-Schönbein GW, Mori E, Copeland BR, Chang CM. Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. Stroke. 1991;22:1276–83.
33. Ludewig P, Sedlacik J, Gelderblom M, Bernreuther C, Korkusuz Y, Wagener C, Gerloff C, Fiehler J, Magnus T, Horst AK. Carcinoembryonic antigen-related cell adhesion molecule 1 inhibits MMP-9-mediated blood-brain-barrier breakdown in a mouse model for ischemic stroke. Circ Res. 2013;113:1013–22.
34. Rosell A, Cuadrado E, Ortega-Aznar A, Hernández-Guillamon M, Lo EH, Montaner J. MMP-9-positive neutrophil infiltration is associated to blood-brain barrier breakdown and basal lamina type IV collagen degradation during hemorrhagic transformation after human ischemic stroke. Stroke. 2008;39:1121–6.
35. Forster C, Clark HB, Ross ME, Iadecola C. Inducible nitric oxide synthase expression in human cerebral infarcts. Acta Neuropathol (Berl). 1999;97:215–20.
36. Garcia-Bonilla L, Moore JM, Racchumi G, Zhou P, Butler JM, Iadecola C, Anrather J. Inducible nitric oxide synthase in neutrophils and endothelium contributes to ischemic brain injury in mice. J Immunol. 2014;193:2531–7.
37. Emerich DF, Dean RL, Bartus RT. The role of leukocytes following cerebral ischemia: pathogenic variable or bystander reaction to emerging infarct? Exp Neurol. 2002;173:168–81.
38. Cuartero MI, Ballesteros I, Moraga A, Nombela F, Vivancos J, Hamilton JA, Corbí AL, Lizasoain I, Moro MA. N2 neutrophils, novel players in brain inflammation after stroke: modulation by the PPARγ agonist rosiglitazone. Stroke. 2013;44(12):3498–508.
39. Mattila OS, Strbian D, Saksi J, Pikkarainen TO, Rantanen V, Tatlisumak T, Lindsberg PJ. Cerebral mast cells mediate blood-brain barrier disruption in acute experimental ischemic stroke through perivascular gelatinase activation. Stroke. 2011;42:3600–5.
40. Strbian D, Kovanen PT, Karjalainen-Lindsberg M-L, Tatlisumak T, Lindsberg PJ. An emerging role of mast cells in cerebral ischemia and hemorrhage. Ann Med. 2009;41:438–50.
41. Hurn PD, Subramanian S, Parker SM, Afentoulis ME, Kaler LJ, Vandenbark AA, Offner H. T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation. J Cereb Blood Flow Metab. 2007;27:1798–805.
42. Kleinschnitz C, Schwab N, Kraft P, Hagedorn I, Dreykluft A, Schwarz T, Austinat M, Nieswandt B, Wiendl H, Stoll G. Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. Blood. 2010;115: 3835–42.

43. Gelderblom M, Weymar A, Bernreuther C, Velden J, Arunachalam P, Steinbach K, Orthey E, Arumugam TV, Leypoldt F, Simova O, Thom V, Friese MA, Prinz I, Hölscher C, Glatzel M, Korn T, Gerloff C, Tolosa E, Magnus T. Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke. Blood. 2012;120:3793–802.
44. Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, Iwaki T, Okada Y, Iida M, Cua DJ, Iwakura Y, Yoshimura A. Pivotal role of cerebral interleukin-17-producing γδT cells in the delayed phase of ischemic brain injury. Nat Med. 2009;15:946–50.
45. Yilmaz G, Arumugam TV, Stokes KY, Granger DN. Role of T lymphocytes and interferon-gamma in ischemic stroke. Circulation. 2006;113:2105–12.
46. Li P, Gan Y, Sun B-L, Zhang F, Lu B, Gan Y, Liang W, Thomson AW, Chen J, Hu X. Adoptive regulatory T cell therapy protects against cerebral ischemia. Ann Neurol. 2012;74:458–71.
47. Liesz A, Suri-Payer E, Veltkamp C, Doerr H, Sommer C, Rivest S, Giese T, Veltkamp R. Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. Nat Med. 2009;15:192–9.
48. Liesz A, Zhou W, Na S-Y, Hämmerling GJ, Garbi N, Karcher S, Mracsko E, Backs J, Rivest S, Veltkamp R. Boosting regulatory T cells limits neuroinflammation in permanent cortical stroke. J Neurosci. 2013;33:17350–62.
49. Planas AM, Chamorro A. Regulatory T cells protect the brain after stroke. Nat Med. 2009;15: 138–9.
50. Abete P, Testa G, Cacciatore F, Della-Morte D, Galizia G, Langellotto A, Rengo F. Ischemic preconditioning in the younger and aged heart. Aging Dis. 2011;2:138–48.
51. Offner H, Hurn PD. A novel hypothesis: regulatory B lymphocytes shape outcome from experimental stroke. Transl Stroke Res. 2012;3:324–30.
52. Ren X, Akiyoshi K, Dziennis S, Vandenbark AA, Herson PS, Hurn PD, Offner H. Regulatory B cells limit CNS inflammation and neurologic deficits in murine experimental stroke. J Neurosci. 2011;31:8556–63.
53. Becker KJ. Sensitization and tolerization to brain antigens in stroke. NSC. 2009;158:1090–7.
54. Urra X, Miró F, Chamorro A, Planas AM. Antigen-specific immune reactions to ischemic stroke. Front Cell Neurosci. 2014;8:278.
55. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, Derecki NC, Castle D, Mandell JW, Lee KS, Harris TH, Kipnis J. Structural and functional features of central nervous system lymphatic vessels. Nature. 2015;523:337–41.
56. Ortega SB, Noorbhai I, Poinsatte K, Kong X, Anderson A, Monson NL, Stowe AM. Stroke induces a rapid adaptive autoimmune response to novel neuronal antigens. Discov Med. 2015;19:381–92.
57. Planas AM, Gómez-Choco M, Urra X, Gorina R, Caballero M, Chamorro A. Brain-derived antigens in lymphoid tissue of patients with acute stroke. J Immunol. 2012;188:2156–63.
58. Korhonen P, Kanninen KM, Lehtonen S, Lemarchant S, Puttonen KA, Oksanen M, Dhungana H, Loppi S, Pollari E, Wojciechowski S, Kidin I, García-Berrocoso T, Giralt D, Montaner J, Koistinaho J, Malm T. Immunomodulation by interleukin-33 is protective in stroke through modulation of inflammation. Brain Behav Immun. 2015;49:322–36.
59. Xiong X, Barreto GE, Xu L, Ouyang YB, Xie X, Giffard RG. Increased brain injury and worsened neurological outcome in interleukin-4 knockout mice after transient focal cerebral ischemia. Stroke. 2011;42:2026–32.
60. Liu X, Zhang Z, Guo W, Burnstock G, He C, Xiang Z. The superficial glia limitans of mouse and monkey brain and spinal cord. Anat Rec (Hoboken). 2013;296:995–1007.
61. Yang Y, Rosenberg GA. Blood-brain barrier breakdown in acute and chronic cerebrovascular disease. Stroke. 2011;42:3323–8.
62. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. Nat Med. 2011;17:796–808.
63. Dirnagl U, Niwa K, Sixt G, Villringer A. Cortical hypoperfusion after global forebrain ischemia in rats is not caused by microvascular leukocyte plugging. Stroke. 1994;25:1028–38.
64. Hudetz AG, Oliver JA, Wood JD, Newman PJ, Kampine JP. Leukocyte adhesion in pial cerebral venules after PMA stimulation and ischemia/reperfusion in vivo. Adv Exp Med Biol. 1997;411:513–8.

65. Ritter LS, Orozco JA, Coull BM, McDonagh PF, Rosenblum WI. Leukocyte accumulation and hemodynamic changes in the cerebral microcirculation during early reperfusion after stroke. Stroke. 2000;31:1153–61.
66. Owens T, Bechmann I, Engelhardt B. Perivascular spaces and the two steps to neuroinflammation. J Neuropathol Exp Neurol. 2008;67:1113–21.
67. Chinnery HR, Ruitenberg MJ, McMenamin PG. Novel characterization of monocyte-derived cell populations in the meninges and choroid plexus and their rates of replenishment in bone marrow chimeric mice. J Neuropathol Exp Neurol. 2010;69:896–909.
68. Kivisäkk P, Mahad DJ, Callahan MK, Trebst C, Tucky B, Wei T, Wu L, Baekkevold ES, Lassmann H, Staugaitis SM, Campbell JJ, Ransohoff RM. Human cerebrospinal fluid central memory CD4+ T cells: evidence for trafficking through choroid plexus and meninges via P-selectin. Proc Natl Acad Sci U S A. 2003;100:8389–94.
69. Engelhardt B, Wolburg-Buchholz K, Wolburg H. Involvement of the choroid plexus in central nervous system inflammation. Microsc Res Tech. 2001;52:112–29.
70. Steffen BJ, Breier G, Butcher EC, Schulz M, Engelhardt B. ICAM-1, VCAM-1, and MAdCAM-1 are expressed on choroid plexus epithelium but not endothelium and mediate binding of lymphocytes in vitro. Am J Pathol. 1996;148:1819–38.
71. Reboldi A, Coisne C, Baumjohann D, Benvenuto F, Bottinelli D, Lira S, Uccelli A, Lanzavecchia A, Engelhardt B, Sallusto F. CC chemokine receptor 6–regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. Nat Immunol. 2009;10:514–23.
72. Shechter R, Miller O, Yovel G, Rosenzweig N, London A, Ruckh J, Kim K-W, Klein E, Kalchenko V, Bendel P, Lira SA, Jung S, Schwartz M. Recruitment of beneficial M2 macrophages to injured spinal cord is orchestrated by remote brain choroid plexus. Immunity. 2013;38:555–69.
73. Petrovic-Djergovic D, Hyman MC, Ray JJ, Bouis D, Visovatti SH, Hayasaki T, Pinsky DJ. Tissue-resident ecto-5′ nucleotidase (CD73) regulates leukocyte trafficking in the ischemic brain. J Immunol. 2012;188:2387–98.
74. Lindsberg PJ, Strbian D, Karjalainen-Lindsberg M-L. Mast cells as early responders in the regulation of acute blood-brain barrier changes after cerebral ischemia and hemorrhage. J Cereb Blood Flow Metab. 2010;30:689–702.
75. Strbian D, Karjalainen-Lindsberg M-L, Tatlisumak T, Lindsberg PJ. Cerebral mast cells regulate early ischemic brain swelling and neutrophil accumulation. J Cereb Blood Flow Metab. 2006;26:605–12.
76. Pérez-de Puig I, Miró-Mur F, Ferrer-Ferrer M, Gelpi E, Pedragosa J, Justicia C, Urra X, Chamorro A, Planas AM. Neutrophil recruitment to the brain in mouse and human ischemic stroke. Acta Neuropathol (Berl). 2015;129:239–57.
77. Garcia JH, Liu KF, Yoshida Y, Lian J, Chen S, del Zoppo GJ. Influx of leukocytes and platelets in an evolving brain infarct (Wistar rat). Am J Pathol. 1994;144:188–99.
78. Barlow Y. T lymphocytes and immunosuppression in the burned patient: a review. Burns. 1994;20:487–90.
79. Lenz A, Franklin GA, Cheadle WG. Systemic inflammation after trauma. Injury. 2007;38:1336–45.
80. Denes A, Pradillo JM, Drake C, Buggey H, Rothwell NJ, Allan SM. Surgical manipulation compromises leukocyte mobilization responses and inflammation after experimental cerebral ischemia in mice. Front Neurosci. 2013;7:271.
81. Hug A, Dalpke A, Wieczorek N, Giese T, Lorenz A, Auffarth G, Liesz A, Veltkamp R. Infarct volume is a major determiner of post-stroke immune cell function and susceptibility to infection. Stroke. 2009;40:3226–32.
82. Czlonkowska A, Korlak J, KUCZYŃSKA A. Lymphocyte subsets after stroke. Ann N Y Acad Sci. 1988;540:608–10.
83. Gendron A, Teitelbaum J, Cossette C, Nuara S, Dumont M, Geadah D, du Souich P, Kouassi E. Temporal effects of left versus right middle cerebral artery occlusion on spleen lymphocyte subsets and mitogenic response in Wistar rats. Brain Res. 2002;955:85–97.

84. Meyer S, Strittmatter M, Fischer C, Georg T, Schmitz B. Lateralization in autonomic dysfunction in ischemic stroke involving the insular cortex. Neuroreport. 2004;15:357–61.
85. Brooks WH, Cross RJ, Roszman TL, Markesbery WR. Neuroimmunomodulation: neural anatomical basis for impairment and facilitation. Ann Neurol. 1982;12:56–61.
86. Cross RJ, Markesbery WR, Brooks WH, Roszman TL. Hypothalamic-immune interactions. I. The acute effect of anterior hypothalamic lesions on the immune response. Brain Res. 1980;196:79–87.
87. Chapman KZ, Dale VQ, Denes A, Bennett G, Rothwell NJ, Allan SM, McColl BW. A rapid and transient peripheral inflammatory response precedes brain inflammation after experimental stroke. J Cereb Blood Flow Metab. 2009;29:1764–8.
88. Offner H, Subramanian S, Parker SM, Afentoulis ME, Vandenbark AA, Hurn PD. Experimental stroke induces massive, rapid activation of the peripheral immune system. J Cereb Blood Flow Metab. 2005;26:654–65.
89. Ferrarese C, Mascarucci P, Zoia C, Cavarretta R, Frigo M, Begni B, Sarinella F, Frattola L, De Simoni MG. Increased cytokine release from peripheral blood cells after acute stroke. J Cereb Blood Flow Metab. 1999;19:1004–9.
90. Basic Kes V, Simundic A-M, Nikolac N, Topic E, Demarin V. Pro-inflammatory and anti-inflammatory cytokines in acute ischemic stroke and their relation to early neurological deficit and stroke outcome. Clin Biochem. 2008;41:1330–4.
91. Meisel C, Schwab JM, Prass K, Meisel A, Dirnagl U. Central nervous system injury-induced immune deficiency syndrome. Nat Rev Neurosci. 2005;6:775–86.
92. Prass K, Meisel C, Höflich C, Braun J, Halle E, Wolf T, Ruscher K, Victorov IV, Priller J, Dirnagl U, Volk H-D, Meisel A. Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. J Exp Med. 2003;198:725–36.
93. Langhorne P, Stott DJ, Robertson L, MacDonald J, Jones L, McAlpine C, Dick F, Taylor GS, Murray G. Medical complications after stroke: a multicenter study. Stroke. 2000;31:1223–9.
94. Czlonkowska A, Cyrta B, Korlak J. Immunological observations on patients with acute cerebral vascular disease. J Neurol Sci. 1979;43:455–64.
95. Offner H, Subramanian S, Parker SM, Wang C, Afentoulis ME, Lewis A, Vandenbark AA, Hurn PD. Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. J Immunol. 2006;176:6523–31.
96. Offner H, Vandenbark AA, Hurn PD. Effect of experimental stroke on peripheral immunity: CNS ischemia induces profound immunosuppression. Neuroscience. 2009;158:1098–111.
97. Seifert HA, Hall AA, Chapman CB, Collier LA, Willing AE, Pennypacker KR. A transient decrease in spleen size following stroke corresponds to splenocyte release into systemic circulation. J Neuroimmune Pharmacol. 2012;7(4):1017–24.
98. Sahota P, Vahidy F, Nguyen C, Bui T-T, Yang B, Parsha K, Garrett J, Bambhroliya A, Barreto A, Grotta JC, Aronowski J, Rahbar MH, Savitz S. Changes in spleen size in patients with acute ischemic stroke: a pilot observational study. Int J Stroke. 2013;8:60–7.
99. Courties G, Herisson F, Sager HB, Heidt T, Ye Y, Wei Y, Sun Y, Severe N, Dutta P, Scharff J, Scadden DT, Weissleder R, Swirski FK, Moskowitz MA, Nahrendorf M. Ischemic stroke activates hematopoietic bone marrow stem cells. Circ Res. 2015;116:407–17.

The Role of Spleen-Derived Immune Cells in Ischemic Brain Injury

Heng Zhao

1 Introduction

Despite numerous extensive studies on stroke therapy in the past decades, few have successfully translated to the clinic and many limitations and potential problems remain. One major limitation is that the brain has been studied in isolation, as if tissue injury in the brain is unrelated to what occurs in peripheral organs. We now realize that stroke not only injures the brain, but it also affects multiple organs, including the skeletal muscle [1–3], heart [4], liver [5], lung [6], and peripheral immune system, including the spleen [7–9]. As a result, it has been speculated that modulating peripheral organs may protect against brain injury. And in fact, we have reported that repetitive ischemia performed in the hind limbs of rats reduces brain infarction after focal ischemia—a phenomenon defined as remote preconditioning [10]. Other important facts are that the spleen interacts with the ischemic brain, as stroke results in spleen atrophy, while spleen removal (splenectomy) performed before stroke onset robustly reduces post-stroke brain injury [11, 12]. Therefore, the spleen may be an alternative avenue or target for stroke treatment. In this Chapter, we discuss how the spleen and its immune cells contribute to brain injury induced by stroke.

H. Zhao, Ph.D. (✉)
Department of Neurosurgery, Stanford University School of Medicine,
MSLS Bldg, P306, 1201 Welch Rd., Stanford, CA 94305-5327, USA
e-mail: hzhao@stanford.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_10

2 The Structure and Function of the Spleen

The spleen is a unique organ belonging to the super immune system, which consists of many lymphatic organs and multiple immune cells [13–15]. The immune system includes primary and secondary lymphoid organs. The primary lymphoid organs mainly include the bone marrow and thymus, which are the sites of lymphocyte development and maturation. The secondary lymphoid tissues include the spleen, lymph nodes, and mucosa- and skin-associated lymphoid tissues, within which mature lymphocytes exert their immune response, such as antigen recognition and activation, clonal selection and proliferation, as well as phagocytosis [16–19].

The spleen, as the largest secondary lymphoid organ, plays important roles in the immune response. It is composed of red and white pulp with distinct morphologies and functions [16–19]. Blood circulation in the spleen is supplied by a single artery called the splenic artery, which branches into a network of smaller arteries that travel through the spleen, forming arterioles, and connecting with sinuses [14]. The small arterioles travel to and end in a venous sinusoidal system in the red pulp, which serves as a blood filter by trapping old or damaged red blood cells that are phagocytosed by red pulp macrophages. Another major function of the red pulp is for iron recycling [14]. White pulp is a lymphoid tissue surrounding the small branches of arterials, and is composed of T cell, B cell (follicle), and marginal zones, where the immune response occurs [16–19].

Taken together, the primary function of the spleen is to monitor circulating blood, filter blood by trapping damaged or old red blood cells, and initiate the immune response against pathogens found in the circulation. Both innate and adaptive immune responses can be easily mounted in the spleen, as the unique pattern of blood supply and structure or organization of the immune compartments allow various immune responses to occur. However, the spleen also releases lymphocytes into the blood circulation in response to stress and inflammation, which travel to other organs, including the brain. Therefore, the spleen is also an important organ for modulating brain functions and neuroinflammation.

3 The Spleen Is Involved in Brain Infarction Induced by Stroke

It is well known that bidirectional effects occur between the injured brain and peripheral immune system after stroke, and the spleen has a unique role in such bidirectional effects. On one hand, stroke causes peripheral immune suppression [9], which results in post-stroke infection and mortality [20–23]. The immune suppression is manifested by a reduction in lymphocyte activation and spleen atrophy after stroke [9]. As a major secondary lymphocyte organ, the spleen functions to maintain lymphocyte and erythrocyte populations. Splenic atrophy is a typical

marker of the immune suppression induced by stroke [9]. On the other hand, the peripheral immune system increases local brain inflammation via recruitment and infiltration of circulating neutrophils [24–27], monocytes/macrophages [28, 29], and T cells [30–35], thus exacerbating ischemic injury.

The most direct evidence that spleen is involved in brain injury is shown in splenectomy studies. Ajmo and colleagues showed in their first study that splenectomy performed 2 weeks before stroke reduced infarction by 80 % [11, 12]. This study is supported by Li et al., who found that splenectomy performed immediately before traumatic brain injury decreases animal mortality and improves cognitive function in rats [36, 37]. In addition, Ostrowski et al. reported that acute splenic irradiation, which results in lymphocyte death in the spleen, reduces brain injury in rat focal cerebral ischemia [38]. They found that the therapeutic time window extends to 3–4 h after stroke onset, suggesting that spleen modulation post-stroke can provide neuroprotection [38]. More recently, Seifert et al. and Zhang et al. also confirmed the neuroprotective effect of splenectomy against stroke [39–41]. In contrast, Kim et al. failed to see any neuroprotective effect of splenectomy immediately before stroke on reducing infarction, although splenectomy did reduce the accumulation of inflammatory macrophages in the ischemic brain [42]. The explanation for this controversial result is unknown. Although the authors argue that the timing of splenectomy immediately before stroke is a factor [42], others have shown that spleen irradiation performed even 3–4 h after stroke can generate protection [38].

In addition to the protective effects of splenectomy against stroke, evidence exists to suggest that the spleen modulates brain injury induced by stroke. First, there is a correlation between reductions in spleen size after stroke and the extent of infarction in animal studies, as well as between spleen size and brain injury in stroke patients [43]. Second, a number of studies suggest that neuroprotectants attenuate the reduction in spleen size and splenocyte numbers. As we reported, moderate hypothermia attenuates the reduction in spleen size and lymphocyte numbers, and this correlates with the robust protective effects of hypothermia on brain infarction [44]. In addition, cord blood injection inhibited brain injury, which was associated with less reduced spleen sizes [45]. Taken together, the spleen contributes to brain injury induced by stroke.

Nevertheless, the underlying mechanisms involved in the cross talk between the spleen and post-stroke ischemic brain, and how immune cells derived from the spleen modulate ischemic brain injury are unclear. In the next two sections I will identify potential mechanisms based on previous studies about spleen function, as well as the possible roles of splenic immune cells in neuroinflammation.

4 Communication Between the Spleen and Brain

Bidirectional communication between the spleen and brain occurs via the immune system and central nervous system (CNS). The peripheral lymphoid organs, including the spleen, are hardwired to the autonomous nervous system [46]. In addition,

lymphocytes in the spleen and other peripheral lymphoid organs express receptors for neurotransmitters released from the nervous system [23]. Other sensors, such as cytokine receptors in the CNS, relay information from the CNS to peripheral organs. This information is mainly processed in the brain by the frontal premotor cortex, hypothalamus, pituitary, and brain stem, which forms three major pathways: the hypothalamus–pituitary–adrenal (HPA) axis, the sympathetic nervous system (SNS), and the parasympathetic nervous system (PNS) [23]. These three major systems bridge the peripheral lymphoid organs, including the spleen, with the brain.

The SNS innervates almost all lymphoid organs, including the bone marrow, thymus, lymphoid nodes, and spleen [23]. In addition, almost all leukocytes express adrenergic receptors, which react with the neurotransmitter catecholamine released from the SNS [47–51]. Activation of the SNS due to inflammatory stimulation in the brain results in a large release of catecholamine, which influences immune functions in the peripheral lymphoid organs [23]. It is known that catecholamine releases from the SNS results in a transient and rapid release of leukocytes from the lymphoid organs, including the spleen [52, 53]. This results in a rapid increase of leukocytes into the circulation, which migrate to the brain and exacerbate the brain's inflammatory response [52]. Indeed, Ajmo and colleagues demonstrated that treatment with either prazosin, an α1 receptor blocker, or carvedilol, a pan adrenergic receptor blocker, prevented the reduction in spleen size, and carvedilol significantly reduced infarct volume, suggesting that SNS-mediated catecholamines regulate the splenic response to stroke through the activation of adrenergic receptors [12].

In addition to the SNS, PNS and HPA are also involved in the crosstalk between the CNS and peripheral lymphoid organs [23]. PNS activity modulated by the release of acetylcholine promotes the anti-inflammatory response, and reduces cytokine production, such as IL-1β and TNFα, in the peripheral organs [54]. In addition, HPA activity leads to the release of glucocorticoids, which are also anti-inflammatory and immune-suppressive [55, 56]. Nevertheless, whether or not PNS and HPA activity results in leukocyte release from the spleen is not clear.

Although it is clear that the spleen contributes to brain injury induced by stroke, and communication between the spleen and CNS is modulated by the three pathways previously discussed, the role of the SNS, PNS, and HPA in the worsening effects of spleen on brain injury has not been well studied. It is likely that stroke results in neuroinflammation in the ischemic brain, which produces various inflammatory cytokines, such as IL-1β, IL-6, and TNF α [23]. These released cytokines may secret into the CSF or diffuse within the ischemic brain, thus stimulating the SNS and resulting in the release of leukocytes from the spleen. Another possibility is that inflammatory cytokines are released from the ischemic brain into the circulation, and these cytokines act directly on the spleen via the circulating blood [23]. The released leukocytes could then infiltrate into the ischemic brain, and result in a larger infarction.

5 Neuroinflammation and Spleen Immune-Cell Trafficking to the Brain After Stroke

Inflammation plays several critical roles in stroke and stroke-induced brain injury. For example, systemic infection-induced inflammation correlates with stroke [57–63], and surgery-induced inflammation also increases the risk of stroke [64–66]. Second, after ischemic stroke there is an immediate onset of neuroinflammation, which involves multiple facets, including brain damage, tissue clearance, and functional recovery [67]. The spleen may be involved in these aforementioned neuroinflammation and stroke, although there is no clear evidence indicating how spleen immune cells modulate stroke, and the role of splenocytes on post-stroke brain recovery has not been studied.

Upon stroke, cerebral blood vessel(s) is occluded and become hypoxic, causing reduced nitric oxide and further constriction of blood vessels, which increases production of reactive oxygen species and platelet activity [67]. These intravascular changes cause the adhesion of leukocytes to the blood vessel walls, and increases in blood–brain barrier permeability and leukocyte infiltrate to the ischemic brain. Therefore, in addition to resident microglia in the brain, which are activated and transformed into macrophages, other leukocytes from circulating blood, including neutrophils, monocytes/macrophages, B cells, T cells, NK, and NKT cells, play important roles in neuroinflammation and brain injury induced by stroke [67].

It is known that T cells modulate brain injury [31, 32, 67]. T cells include the subsets of CD4 T cells, CD8 T cells, and γδT cells. Functionally, activated CD4 T cells during immune responses can be further differentiated into Th1, Th2, and Th17 subsets. It has been reported that the lack of total T cells, or a subset of CD4, CD8, or γδT cells, results in a smaller infarction compared with immune intact animals [68]. In addition, the lack of Th1 cells results in neuroprotection, while the lack of Th2 cells exacerbates brain injury [31]. We also observed that the lack of Th17 was neuroprotective against stroke (unpublished observation). Th17 cells function by secreting IL-17. A previous study has shown that neutralization of IL-17 attenuates neuroinflammation [69]. Among these cell types, CD4+CD25+ regulatory T cells (Treg) have been the most widely studied, with results showing that Treg cells have anti-inflammatory functions and their activity attenuates delayed brain injury induced by stroke [68, 70, 71]. B cells have been less studied in stroke. The first study on B cells suggested that B cells do not have an effect on brain injury induced by stroke [33]. More recently, regulatory B cells were found to inhibit brain injury [72].

Monocytes/macrophages are also important for brain injury induced by stroke. Macrophages can be derived from both brain resident microglia and peripheral blood monocytes. The latter can be released from bone marrow and other secondary lymphoid organs, including the spleen. Functionally, macrophages are polarized into M1 and M2 phenotypes, with pro- and anti-inflammatory roles, respectively [73, 74].

It has been reported that the M1 phenotype is detrimental, while M2 phenotype is beneficial, in ischemic stroke [75–77]. In particular, M2 macrophages may play a critical role in promoting brain repair and recovery after stroke [75].

As we discussed, the spleen contains most of the cell types in the immune system, including T cells, B cells, monocytes, and macrophages, and stroke results in the contraction of the spleen, which leads to the release of leukocytes into the blood circulation. The released leukocytes then migrate to the brain modulating acute brain injury. Nevertheless, as discussed, leukocytes recruited into the ischemic brain include those released not only from the spleen and other secondary lymphoid nodes, but also from the primary lymphoid organs, such as bone marrow. It still remains unclear how much the spleen contributes to or modulates brain injury induced by stroke among these various lymphoid organs.

Although the spleen contributes to brain injury induced by stroke, few studies have examined the exact roles of the individual cell types that make up splenocytes or the underlying mechanisms of spleen-induced brain injury. Nevertheless, several lines of evidence from previous studies indeed suggest that the involvement of spleen in ischemic brain injury is associated with splenocytes and neuroinflammation. First, one recent study suggests that spleen contraction induced by stroke correlates with reduced cell numbers of monocytes in the spleen, including pro-inflammatory $Ly6C^{hi}$ and anti-inflammatory $Ly6c^{low}$ monocytes [42]. The study further showed that the deployment of these monocyte subsets coincided with respective increases in the ischemic brain [42]. In contrast, splenectomy reduced leukocyte infiltrations into the ischemic brain [42], suggesting that monocytes are released from the spleen and migrate to the ischemic brain. Second, there is additional direct evidence that splenocytes infiltrate into the ischemic brain. In one study, splenocytes were labeled with CFSE, and it was found that CFSE-positive cells were released into the blood from the spleen, including T cells, neutrophils, and monocytes. The presence of CFSE-positive monocytes and NK cells in the cerebral blood vessels of the ischemic brain [39] suggests that splenocytes migrate into the ischemic brain. Third, splenocyte infiltrations are associated with the expression of inflammatory factors. Splenectomy results in reductions in T cells, neutrophils, and macrophages in the ischemic brain [41], and this is associated with decreases in pro-inflammatory cytokines, such as IL-1β and TNFα, and with increases in anti-inflammatory cytokines, including IL-10, in the brain [41]. Another study suggests that the protective effect of splenectomy is associated with IFNγ, as IFNγ was found to be increased in the spleen in early stroke followed by increases in the brain [40]. Fourth, the protective effects of neuroprotectants are linked to spleen functions. For instance, agmatine treatment reduced the contraction of white pulp, and inhibited the accumulation of CD11b macrophages and Treg cells in the spleen [78]. In addition, MFX treatment attenuated Ly6C expression in pro-inflammatory macrophage subsets and CCR2 expression in the spleen tissues [79]. Furthermore, the injection of cord blood reversed the reduction in spleen size and concomitant reductions in CD8 T cells after stroke, as well as increased IL-10 while inhibiting IFN-gamma [45].

6 Problems and Future Research Directions

It is well established that the spleen contributes to acute brain injury after stroke, but there are many questions raised from previous studies. First, the underlying mechanisms of the spleen's contribution to brain injury are not understood. Future studies can address the following questions: How are the nerve pathways, including SNS, PNS, and HPA involved in the interaction between the spleen and brain after stroke? How are immune cells released from the spleen? Which splenocytes migrate to the ischemic brain, and which cell types play the most important roles? Second, the spleen is only one of the immune organs, and how much it contributes to ischemic brain injury is unknown. Therefore, one may ask, what is the relative contribution of the spleen, in comparison to the other lymphoid organs, to brain injury? Third, previous studies have focused on studying the relationship between acute brain injury and the spleen; whether the spleen plays an important role in brain repair and recovery has not been studied. More scientific issues include: Does the spleen play a critical role in brain recovery after stroke? If so, what is the underlying mechanism?

7 Conclusions

Stroke results in the release of inflammatory factors that stimulate nerve tissue sensors inside and outside of the brain as well as sensors expressed on peripheral organs, such as the spleen. The contraction of the spleen after stroke leads to the release of splenocytes, which migrate to the ischemic brain and exacerbate brain injury. Taken together, the spleen plays an important role in acute brain injury induced by stroke, but the underlying mechanisms require more study, and the importance of the spleen to brain repair and recovery is not clear.

Acknowledgement I wish to thank Ms. Cindy H. Samos for editorial assistance. This study was supported by NINDS grants 2R56NS06413606, 2 R01 NS064136-06.

References

1. Knops M, Werner CG, Scherbakov N, Fiebach J, Dreier JP, Meisel A, Heuschmann PU, Jungehulsing GJ, von Haehling S, Dirnagl U, Anker SD, Doehner W. Investigation of changes in body composition, metabolic profile and skeletal muscle functional capacity in ischemic stroke patients: the rationale and design of the Body Size in Stroke Study (BoSSS). J Cachex Sarcopenia Muscle. 2013;4:199–207.
2. Scherbakov N, von Haehling S, Anker SD, Dirnagl U, Doehner W. Stroke induced sarcopenia: muscle wasting and disability after stroke. Int J Cardiol. 2013;170:89–94.
3. Springer J, Schust S, Peske K, Tschirner A, Rex A, Engel O, Scherbakov N, Meisel A, von Haehling S, Boschmann M, Anker SD, Dirnagl U, Doehner W. Catabolic signaling and muscle wasting after acute ischemic stroke in mice: indication for a stroke-specific sarcopenia. Stroke. 2014;45:3675–83.

4. Ishikawa H, Tajiri N, Vasconcellos J, Kaneko Y, Mimura O, Dezawa M, Borlongan CV. Ischemic stroke brain sends indirect cell death signals to the heart. Stroke. 2013;44:3175–82.
5. Ottani A, Giuliani D, Mioni C, Galantucci M, Minutoli L, Bitto A, Altavilla D, Zaffe D, Botticelli AR, Squadrito F, Guarini S. Vagus nerve mediates the protective effects of melanocortins against cerebral and systemic damage after ischemic stroke. J Cereb Blood Flow Metab. 2009;29:512–23.
6. Wu S, Fang CX, Kim J, Ren J. Enhanced pulmonary inflammation following experimental intracerebral hemorrhage. Exp Neurol. 2006;200:245–9.
7. Offner H. Modeling immunity and inflammation in stroke: don't be afraid of mice? Stroke. 2014;45:e181–2.
8. Offner H, Subramanian S, Parker SM, Afentoulis ME, Vandenbark AA, Hurn PD. Experimental stroke induces massive, rapid activation of the peripheral immune system. J Cereb Blood Flow Metab. 2006;26:654–65.
9. Offner H, Subramanian S, Parker SM, Wang C, Afentoulis ME, Lewis A, Vandenbark AA, Hurn PD. Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. J Immunol. 2006;176:6523–31.
10. Ren C, Yan Z, Wei D, Gao X, Chen X, Zhao H. Limb remote ischemic postconditioning protects against focal ischemia in rats. Brain Res. 2009;1288:88–94.
11. Ajmo Jr CT, Vernon DO, Collier L, Hall AA, Garbuzova-Davis S, Willing A, Pennypacker KR. The spleen contributes to stroke-induced neurodegeneration. J Neurosci Res. 2008;86:2227–34.
12. Ajmo Jr CT, Collier LA, Leonardo CC, Hall AA, Green SM, Womble TA, Cuevas J, Willing AE, Pennypacker KR. Blockade of adrenoreceptors inhibits the splenic response to stroke. Exp Neurol. 2009;218:47–55.
13. Chaplin DD. Regulation of spleen white pulp structure and function by lymphotoxin. Adv Exp Med Biol. 2002;512:49–56.
14. Mebius RE, Kraal G. Structure and function of the spleen. Nat Rev Immunol. 2005;5:606–16.
15. Scothorne RJ. The spleen: structure and function. Histopathology. 1985;9:663–9.
16. Bronte V, Pittet MJ. The spleen in local and systemic regulation of immunity. Immunity. 2013;39:806–18.
17. den Haan JM, Kraal G. Innate immune functions of macrophage subpopulations in the spleen. J Innate Immun. 2012;4:437–45.
18. Hey YY, O'Neill HC. Murine spleen contains a diversity of myeloid and dendritic cells distinct in antigen presenting function. J Cell Mol Med. 2012;16:2611–9.
19. Zhao L, Liu L, Guo B, Zhu B. Regulation of adaptive immune responses by guiding cell movements in the spleen. Front Microbiol. 2015;6:645.
20. Braun JS, Prass K, Dirnagl U, Meisel A, Meisel C. Protection from brain damage and bacterial infection in murine stroke by the novel caspase-inhibitor Q-VD-OPH. Exp Neurol. 2007;206:183–91.
21. Engel O, Meisel A. Models of infection before and after stroke: investigating new targets. Infect Disord Drug Targets. 2010;10:98–104.
22. Meisel A, Meisel C, Harms H, Hartmann O, Ulm L. Predicting post-stroke infections and outcome with blood-based immune and stress markers. Cerebrovasc Dis. 2012;33:580–8.
23. Meisel C, Schwab JM, Prass K, Meisel A, Dirnagl U. Central nervous system injury-induced immune deficiency syndrome. Nat Rev Neurosci. 2005;6:775–86.
24. Arakawa K, Yasuda S, Hao H, Kataoka Y, Morii I, Kasahara Y, Kawamura A, Ishibashi-Ueda H, Miyazaki S. Significant association between neutrophil aggregation in aspirated thrombus and myocardial damage in patients with ST-segment elevation acute myocardial infarction. Circ J. 2009;73:139–44.
25. Ikegame Y, Yamashita K, Hayashi S, Yoshimura S, Nakashima S, Iwama T. Neutrophil elastase inhibitor prevents ischemic brain damage via reduction of vasogenic edema. Hypertens Res. 2010;33:703–7.
26. Perez-de-Puig I, Miro-Mur F, Ferrer-Ferrer M, Gelpi E, Pedragosa J, Justicia C, Urra X, Chamorro A, Planas AM. Neutrophil recruitment to the brain in mouse and human ischemic stroke. Acta Neuropathol. 2015;129:239–57.

27. Zhao X, Sun G, Zhang H, Ting SM, Song S, Gonzales N, Aronowski J. Polymorphonuclear neutrophil in brain parenchyma after experimental intracerebral hemorrhage. Transl Stroke Res. 2014;5:554–61.
28. Hammond MD, Taylor RA, Mullen MT, Ai Y, Aguila HL, Mack M, Kasner SE, McCullough LD, Sansing LH. CCR2+ Ly6C(hi) inflammatory monocyte recruitment exacerbates acute disability following intracerebral hemorrhage. J Neurosci. 2014;34:3901–9.
29. Joo SP, Xie W, Xiong X, Xu B, Zhao H. Ischemic postconditioning protects against focal cerebral ischemia by inhibiting brain inflammation while attenuating peripheral lymphopenia in mice. Neuroscience. 2013;243:149–57.
30. Dziennis S, Mader S, Akiyoshi K, Ren X, Ayala P, Burrows GG, Vandenbark AA, Herson PS, Hurn PD, Offner HA. Therapy with recombinant T-cell receptor ligand reduces infarct size and infiltrating inflammatory cells in brain after middle cerebral artery occlusion in mice. Metab Brain Dis. 2011;26:123–33.
31. Gu L, Xiong X, Zhang H, Xu B, Steinberg GK, Zhao H. Distinctive effects of T cell subsets in neuronal injury induced by cocultured splenocytes in vitro and by in vivo stroke in mice. Stroke. 2012;43:1941–6.
32. Gu L, Xiong X, Wei D, Gao X, Krams S, Zhao H. T cells contribute to stroke-induced lymphopenia in rats. PLoS One. 2013;8:e59602.
33. Hurn PD, Subramanian S, Parker SM, Afentoulis ME, Kaler LJ, Vandenbark AA, Offner H. T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation. J Cereb Blood Flow Metab. 2007;27:1798–805.
34. Ren X, Akiyoshi K, Vandenbark AA, Hurn PD, Offner H. CD4 + FoxP3+ regulatory T-cells in cerebral ischemic stroke. Metab Brain Dis. 2011;26:87–90.
35. Xiong X, Gu L, Zhang H, Xu B, Zhu S, Zhao H. The protective effects of T cell deficiency against brain injury are ischemic model-dependent in rats. Neurochem Int. 2013;62: 265–70.
36. Chu W, Li M, Li F, Hu R, Chen Z, Lin J, Feng H. Immediate splenectomy down-regulates the MAPK-NF-kappaB signaling pathway in rat brain after severe traumatic brain injury. J Trauma Acute Care Surg. 2013;74:1446–53.
37. Li M, Li F, Luo C, Shan Y, Zhang L, Qian Z, Zhu G, Lin J, Feng H. Immediate splenectomy decreases mortality and improves cognitive function of rats after severe traumatic brain injury. J Trauma. 2011;71:141–7.
38. Ostrowski RP, Schulte RW, Nie Y, Ling T, Lee T, Manaenko A, Gridley DS, Zhang JH. Acute splenic irradiation reduces brain injury in the rat focal ischemic stroke model. Transl Stroke Res. 2012;3:473–81.
39. Seifert HA, Hall AA, Chapman CB, Collier LA, Willing AE, Pennypacker KR. A transient decrease in spleen size following stroke corresponds to splenocyte release into systemic circulation. J Neuroimmune Pharmacol. 2012;7:1017–24.
40. Seifert HA, Leonardo CC, Hall AA, Rowe DD, Collier LA, Benkovic SA, Willing AE, Pennypacker KR. The spleen contributes to stroke induced neurodegeneration through interferon gamma signaling. Metab Brain Dis. 2012;27:131–41.
41. Zhang BJ, Men XJ, Lu ZQ, Li HY, Qiu W, Hu XQ. Splenectomy protects experimental rats from cerebral damage after stroke due to anti-inflammatory effects. Chin Med J (Engl). 2013;126:2354–60.
42. Kim E, Yang J, Beltran CD, Cho S. Role of spleen-derived monocytes/macrophages in acute ischemic brain injury. J Cereb Blood Flow Metab. 2014;34:1411–9.
43. Sahota P, Vahidy F, Nguyen C, Bui TT, Yang B, Parsha K, Garrett J, Bambhroliya A, Barreto A, Grotta JC, Aronowski J, Rahbar MH, Savitz S. Changes in spleen size in patients with acute ischemic stroke: a pilot observational study. Int J Stroke. 2013;8:60–7.
44. Gu LJ, Xiong XX, Ito T, Lee J, Xu BH, Krams S, Steinberg GK, Zhao H. Moderate hypothermia inhibits brain inflammation and attenuates stroke-induced immunodepression in rats. CNS Neurosci Ther. 2014;20:67–75.
45. Golden JE, Shahaduzzaman M, Wabnitz A, Green S, Womble TA, Sanberg PR, Pennypacker KR, Willing AE. Human umbilical cord blood cells alter blood and spleen cell populations after stroke. Transl Stroke Res. 2012;3:491–9.

46. Huston JM, Ochani M, Rosas-Ballina M, Liao H, Ochani K, Pavlov VA, Gallowitsch-Puerta M, Ashok M, Czura CJ, Foxwell B, Tracey KJ, Ulloa L. Splenectomy inactivates the cholinergic antiinflammatory pathway during lethal endotoxemia and polymicrobial sepsis. J Exp Med. 2006;203:1623–8.
47. Galant SP, Underwood S, Allred S, Hanifin JM. Beta adrenergic receptor binding on polymorphonuclear leukocytes in atopic dermatitis. J Invest Dermatol. 1979;72:330–2.
48. Heijnen CJ, Rouppe van der Voort C, Wulffraat N, van der Net J, Kuis W, Kavelaars A. Functional alpha 1-adrenergic receptors on leukocytes of patients with polyarticular juvenile rheumatoid arthritis. J Neuroimmunol. 1996;71:223–6.
49. Kozlik R, Kramer HH, Wicht H, Bircks W, Reinhardt D. Beta-adrenoceptor density on mononuclear leukocytes and right atrial myocardium in infants and children with congenital heart disease. Klin Wochenschr. 1991;69:910–6.
50. Nwosu UC, Rice PJ. Beta-adrenoceptor changes in mononuclear leukocytes during pregnancy. Proc Soc Exp Biol Med. 1995;209:157–62.
51. Prengel AW, Lindner KH, Anhaupl T, Trunk E, Georgieff M, Lurie KG. Regulation of beta 2-adrenergic receptors on mononuclear leukocytes in patients with acute ischemic heart disease. Crit Care Med. 1997;25:646–51.
52. Hanna RN, Hedrick CC. Stressing out stem cells: linking stress and hematopoiesis in cardiovascular disease. Nat Med. 2014;20:707–8.
53. Scheiermann C, Kunisaki Y, Lucas D, Chow A, Jang JE, Zhang D, Hashimoto D, Merad M, Frenette PS. Adrenergic nerves govern circadian leukocyte recruitment to tissues. Immunity. 2012;37:290–301.
54. Cheyuo C, Jacob A, Wu R, Zhou M, Coppa GF, Wang P. The parasympathetic nervous system in the quest for stroke therapeutics. J Cereb Blood Flow Metab. 2011;31:1187–95.
55. Melief J, de Wit SJ, van Eden CG, Teunissen C, Hamann J, Uitdehaag BM, Swaab D, Huitinga I. HPA axis activity in multiple sclerosis correlates with disease severity, lesion type and gene expression in normal-appearing white matter. Acta Neuropathol. 2013;126:237–49.
56. Silverman MN, Sternberg EM. Glucocorticoid regulation of inflammation and its functional correlates: from HPA axis to glucocorticoid receptor dysfunction. Ann N Y Acad Sci. 2012;1261:55–63.
57. Bova IY, Bornstein NM, Korczyn AD. Acute infection as a risk factor for ischemic stroke. Stroke. 1996;27:2204–6.
58. Emsley HC, Tyrrell PJ. Inflammation and infection in clinical stroke. J Cereb Blood Flow Metab. 2002;22:1399–419.
59. Galante A, Pietroiusti A, Silvestrini M, Stanzione P, Bernardi G. Leukocyte aggregation: a possible link between infection and ischemic stroke. Stroke. 1992;23:1533.
60. Jeerakathil T, Lo W. Infection as cause of stroke: a contagious idea that may explain racial disparity. Neurology. 2014;82:908–9.
61. Mattila KJ, Valtonen VV, Nieminen MS, Asikainen S. Role of infection as a risk factor for atherosclerosis, myocardial infarction, and stroke. Clin Infect Dis. 1998;26:719–34.
62. Palm F, Urbanek C, Grau A. Infection, its treatment and the risk for stroke. Curr Vasc Pharmacol. 2009;7:146–52.
63. Worthmann H, Tryc AB, Deb M, Goldbecker A, Ma YT, Tountopoulou A, Lichtinghagen R, Weissenborn K. Linking infection and inflammation in acute ischemic stroke. Ann N Y Acad Sci. 2010;1207:116–22.
64. Dacey LJ, Likosky DS, Leavitt BJ, Lahey SJ, Quinn RD, Hernandez Jr F, Quinton HB, Desimone JP, Ross CS, O'Connor GT, Northern New England Cardiovascular Disease Study Group. Perioperative stroke and long-term survival after coronary bypass graft surgery. Ann Thorac Surg. 2005;79:532–6. discussion 537.
65. McAlister FA, Jacka M, Graham M, Youngson E, Cembrowski G, Bagshaw SM, Pannu N, Townsend DR, Srinathan S, Alonso-Coello P, Devereaux PJ. The prediction of postoperative stroke or death in patients with preoperative atrial fibrillation undergoing non-cardiac surgery: a VISION sub-study. J Thromb Haemost. 2015;13(10):1768–75.

66. Wu JC, Chen YC, Liu L, Chen TJ, Huang WC, Thien PF, Cheng H, Lo SS. The risk of stroke after spinal fusion surgery: a national cohort study. Spine J. 2012;12:492–9.
67. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. Nat Med. 2011;17:796–808.
68. Kleinschnitz C, et al. Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature. Blood. 2013;121:679–91.
69. Gelderblom M, Weymar A, Bernreuther C, Velden J, Arunachalam P, Steinbach K, Orthey E, Arumugam TV, Leypoldt F, Simova O, Thom V, Friese MA, Prinz I, Holscher C, Glatzel M, Korn T, Gerloff C, Tolosa E, Magnus T. Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke. Blood. 2012;120:3793–802.
70. Liesz A, Suri-Payer E, Veltkamp C, Doerr H, Sommer C, Rivest S, Giese T, Veltkamp R. Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. Nat Med. 2009;15:192–9.
71. Planas AM, Chamorro A. Regulatory T cells protect the brain after stroke. Nat Med. 2009;15: 138–9.
72. Ren X, Akiyoshi K, Dziennis S, Vandenbark AA, Herson PS, Hurn PD, Offner H. Regulatory B cells limit CNS inflammation and neurologic deficits in murine experimental stroke. J Neurosci. 2011;31:8556–63.
73. Gordon S, Martinez FO. Alternative activation of macrophages: mechanism and functions. Immunity. 2010;32:593–604.
74. Van Dyken SJ, Locksley RM. Interleukin-4- and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease. Annu Rev Immunol. 2013;31:317–43.
75. Jin Q, Cheng J, Liu Y, Wu J, Wang X, Wei S, Zhou X, Qin Z, Jia J, Zhen X. Improvement of functional recovery by chronic metformin treatment is associated with enhanced alternative activation of microglia/macrophages and increased angiogenesis and neurogenesis following experimental stroke. Brain Behav Immun. 2014;40:131–42.
76. Pan J, Jin JL, Ge HM, Yin KL, Chen X, Han LJ, Chen Y, Qian L, Li XX, Xu Y. Malibatol a regulates microglia M1/M2 polarization in experimental stroke in a PPARgamma-dependent manner. J Neuroinflammation. 2015;12:51.
77. Suenaga J, Hu X, Pu H, Shi Y, Hassan SH, Xu M, Leak RK, Stetler RA, Gao Y, Chen J. White matter injury and microglia/macrophage polarization are strongly linked with age-related long-term deficits in neurological function after stroke. Exp Neurol. 2015;272:109–19.
78. Uranchimeg D, Kim JH, Kim JY, Lee WT, Park KA, Batbaatar G, Tundevrentsen S, Amgalanbaatar D, Lee JE. Recovered changes in the spleen by agmatine treatment after transient cerebral ischemia. Anat Cell Biol. 2010;43:44–53.
79. Bao Y, Kim E, Bhosle S, Mehta H, Cho S. A role for spleen monocytes in post-ischemic brain inflammation and injury. J Neuroinflammation. 2010;7:92.

Regulatory T Cells in Ischemic Brain Injury

Arthur Liesz

1 Introduction

Post-stroke neuroinflammation has come into the focus of current preclinical stroke research [1]. Among the pathophysiological mechanisms of microglial activation, brain leukocyte invasion and secretion of pro-inflammatory factors, lymphocytes have been uncovered as the key leukocyte subpopulation determining the neuroinflammatory outcome. Several studies have shown that pro-inflammatory lymphocytes such as TH1, TH17, and γδ-T cells worsen stroke outcome and that blocking their brain invasion is neuroprotective [2–5]. Contrary to pro-inflammatory lymphocytes, regulatory T cells have been characterized in primary inflammatory diseases as disease-limiting protective cells [6]. Finding this key role of regulatory T cells in other T cell-driven pathologies has initiated productive research efforts on the role of regulatory T cells also in ischemic brain injury over the past years. Due to the complex function of regulatory cells in immune homeostasis and disease as well as partially divergent findings using different stroke models, uncertainty has emerged about the pathophysiological function of regulatory lymphocytes in stroke.

2 Regulatory T Cells in Post-Stroke Neuroinflammation

The immune system has evolved several regulatory mechanisms to inhibit an overshooting immune reaction to tissue damage including cell depletion, anergy, and unresponsiveness to self-antigens. The presence of regulatory T cells (Treg)

A. Liesz, M.D. (✉)
Institute for Stroke and Dementia Research, Klinikum der Universität München,
Feodor-Lynen-Strasse 17, 81377 Munich, Germany
e-mail: Arthur.Liesz@med.uni-muenchen.de

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_11

suppressing actively (self-reactive) immunity is one of the key mechanisms of preserving immune homeostasis and limiting inflammatory collateral damage [7]. For example, depletion of CD25+ CD4+ Treg cells naturally arising in the immune system induces autoimmune diseases and reconstitution of this cell population prevents disease development [8]. Lack of Treg has been shown to be a primary cause of autoimmune diseases in humans [9]. In addition to sustaining self-tolerance, Treg are also intricate in suppressive control of a broad spectrum of immune responses including those against autologous tumor cells [10], allergens [11] and organ transplantation [12]. Although Treg might be simplistically defined as immunosuppressive T cells, several phenotypically and functionally distinct Treg subpopulations have been defined, such as induced Treg populations, Tr1, TH3, and various others [13]. Yet, the best investigated population of regulatory lymphocytes are CD4+ CD25+ Foxp3+ naturally occurring regulatory T cells, which are physiologically produced in the thymus as a mature and functional cell population [14]. While it is still unknown at which specific site Treg act as immunomodulators after stroke, their kinetics and magnitude of brain infiltration have been characterized in several studies using models of experimental middle cerebral artery occlusion (MCAO). One of the first studies systematically analyzing brain leukocyte invasion after transient brain ischemia (filament-induced MCAO resulting in large hemispheric lesions) by flow cytometry was performed by Gelderblom and colleagues [15]. In this study only a very low number of CD25+ Foxp3+ Treg cells at a frequency of less than 5 % of all CD4+ T cells was observed within the first week after transient ischemia. In contrast, using a permanent MCA occlusion model we detected substantial T cell and Treg counts in the ischemic hemisphere (Fig. 1) with Foxp3+ Treg cells constituting approx. 20 % of all CD4+ cells [16]. Correspondingly, it was previously shown that distal, permanent occlusion induces a significantly stronger neuroinflammatory reaction with manifold higher T cell counts in the ischemic hemisphere than in proximal, transient occlusion models [17]. Also in contrast to the initial study by Gelderblom et al., a more recent work by Stubbe et al. has investigated cerebral Treg counts also at later stages after large hemispheric lesions and detected only negligible Foxp3+ cells in ischemic hemispheres early after MCAO

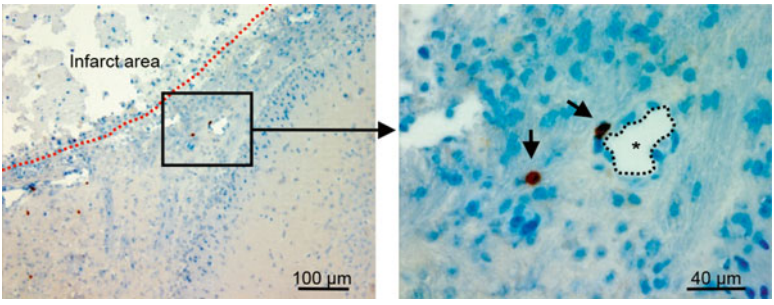

Fig. 1 Regulatory T cells invade the ischemic brain. Immunohistochemical sections showing Treg cells predominantly in the peri-infarct area. Arrows indicate Foxp3+ Treg cells; the asterisk marks a blood vessel (Liesz et al., Nature Medicine, 2009)

but substantial brain Treg invasion at 14days and 30days after MCAO. Additionally, the percentage of Treg cells within the T_{helper} cell population was increased in brains after stroke compared to peripheral lymphatic organs [18].

Apart from the evidence of variable Treg cell invasion depending of the used stroke model, it is feasible to assume that Treg cells might execute their function on target cells in the peripheral immune system. The majority of studies has detected the effector function of Treg cells in depletion or therapeutic paradigms (see below) already within the first days after brain injury before a considerable amount of Treg have even invaded the brain. Likewise, delayed deletion of Treg cells by antibodies or in inducible Foxp3-KO mice at 3days after MCAO was not effective in altering stroke outcome [18, 19]. Hence, although the definite site of action for Treg after stroke is still unsolved, currently available data suggests that Treg fulfill their immunomodulatory function within the first 3days after stroke and most likely outside the CNS. Potential peripheral target sites of Treg cells might include suppression of peripheral effector T cell activation, inhibition of autoantigen-specific clonal expansion or priming of transendothelial effector T cell-migration via currently unknown mechanisms.

3 Treg Depletion in Experimental Stroke

Depletion of the regulatory T cell population has been used as an experimental paradigm to investigate the functional role of Treg in brain ischemia in several publications (Table 1). Methodologically, two different approaches have been used for Treg depletion, antibody-mediated cell lysis using CD25-specific antibodies or the use of transgenic mice with a diphtheria toxin receptor (DTR) transgene under the control of the Foxp3 promotor for inducible Treg depletion. Using Treg-depletion paradigms about half of the experiments performed revealed an increased infarct volume while the other half did not detect any effect on stroke outcome and one study even observed a reduction of infarct size in Treg-deficient mice (Table 1). This discrepancy of Treg depletion on stroke outcome led to an unsolved debate on their role and denomination of Treg as a "double-edged sword" in acute brain injury [20–22]. This discrepancy could not be attributed to the used depletion paradigm, since genetic deletion models can be found on both sides of the "efficacy spectrum". Notably, the three publications using inducible Foxp3-KO mice used in each case a different mouse strain: Ren et al. [23] used the original $Foxp3^{DTR}$ mouse [24], Liesz et al. [19] the Foxp3.LuciDTR.4 mouse line [25], and Kleinschnitz et al. [26] the DEREG mouse [24]. All three of these DTR-transgene-mediated Foxp3-deletion models are using different transgene constructs, are variable in Treg-depletion efficacy and the used DT treatment protocol. Nevertheless, a systematic bias by the used depletion paradigm did not become evident for the authors. In contrast, it is apparent that the utilized stroke models, and more precisely the resulting volume of the ischemic lesion, do indeed predict a deterioration of outcome versus no effect or improved lesion size after Treg depletion (Table 1). In this line, both studies from our group

Table 1 Summary of studies investigating Treg depletion paradigms [31]

Study	Model	Lesion (% hemisphere)	Latest endpoint	Depletion model	Depletion efficacy	Outcome on infarct vol.
Liesz et al. (2009) [16]	Distal, permanent	≈10 %	7days	1. Anti-CD25	90 %	Increased
				2. Adoptive cell transfer	n.a.	
Liesz et al. (2013) [19]	Distal, permanent	≈10 %	7days	Foxp3-KO	85 %	Increased
	30 min transient	≈10 %	7days	Anti-CD25	90 %	Increased
Xie et al. [27]	Rats: 90 min transient	≈40 %	3days	Anti-CD25	65 %	Increased
Liesz et al. (2009) [16]	90 min transient	≈55 %	7days	Anti-CD25	90 %	No effect
Ren et al. [23]	60 min transient	≈55 %	3days	Foxp3-KO	90 %	No effect
Li et al. (2013) [28]	60 min transient	≈50 %	3days	Anti-CD25	90 %	No effect
Stubbe et al. [63][a]	30 min transient	≈50 %	27days	Anti-CD25	75 %	No effect
Liesz et al. (2013) [19]	60 min transient	≈50 %	3days	Foxp3-KO	85 %	No effect
Kleinschnitz et al. [26]	60 min transient	≈55 %	7days	Foxp3-KO	90 %	Reduced

[a]Depletion 3days after MCAO induction; in all other studies: pretreatment or acute depletion

Table 2 Effect of Treg depletion on cerebral neuroinflammation

Study	Outcome on infarct volume	Leukocyte invasion	Cerebral cytokines	Microglia
Liesz et al. (2009)	Increased	↑	↑	(↑)
Liesz et al. (2013)	Increased	NA	↑	NA
Xie et al.	Increased	↑	↑	No effect
Liesz et al. (2009)	No effect	NA	NA	NA
Ren et al.	No effect	NA	NA	NA
Li et al. (2013)	No effect	NA	NA	NA
Stubbe et al.	No effect	No effect	No effect	No effect
Liesz et al. (2013)	No effect	NA	NA	NA
Kleinschnitz et al.	Reduced	NA	NA	NA

have detected an increase of stroke volume after Treg depletion only in small, permanent ischemia lesion models but not after extensive infarction induced by transient MCAO [16, 19]. A recent study by Xie et al. has also detected an increase of infarct volume after antibody-mediated Treg depletion in a rat model of moderate brain ischemia with rapamycin pretreatment [27]. In contrast, all studies investigating effects of Treg depletion without detection of an effect on stroke outcome [16, 18, 19, 23, 28] or even an increase of lesion volume [26] were performed in transient mechanical occlusion models with extensive brain lesions.

The immunological effects of Treg depletion on the neuroinflammatory outcome after stroke have unfortunately only been investigated in a small fraction of the studies (Table 2). All three studies detecting an exacerbation of stroke outcome after Treg depletion also measured an associated increase in neuroinflammatory biomarkers [16, 19, 27]. The most robust findings were an increase in leukocyte brain invasion, particularly of lymphocyte subpopulations, and an increase in pro-inflammatory cytokine secretion such as TNF-α, IL-1β, IL-12, and IFN-γ. Among the studies using models of extensive brain injury with no effect of Treg depletion on stroke outcome, only one study [18] has investigated neuroimmunological readouts. Correspondingly to the missing effect on lesion volume, also inflammatory markers were not altered by the antibody-mediated Treg depletion in this study.

4 Treg-Therapies for Ischemic Stroke

Despite the obvious and still unresolved discrepancies arising from studies investigating effects of Treg depletion, a rapidly increasing number of reports have analyzed different strategies to increase the Treg number and/or function for experimental stroke therapy (Table 3). Out of 15 independent experiments reported in 13 studies, 13 have detected an improvement of stroke outcome while two have demonstrated an increase in infarct volume when using two independent Treg-targeted therapies. This discrepancy could not be attributed to the used

Table 3 Studies investigating Treg-targeted therapeutic approaches (by treatment and outcome)

Study	Model	Lesion (% hemisphere)	Latest endpoint	Treatment paradigm	Outcome on infarct volume
Chen et al. [64]	Rats: distal, permanent	≈20 %	48 h	Mucosal immunization	Reduced
Gee et al. [65]	Rats: 3 h transient	Infarct vol. NA[a]	28days	Mucosal immunization	Reduced (behavior)
Ishibashi et al. [41]	Rats: distal, permanent	≈20 %	28days	Mucosal immunization	Reduced
Li et al. (2013) [28]	Rats: 2 h transient	≈40 %	3days	Adoptive transfer	Reduced
	Mouse: 60 min transient	≈35 %	3days	Adoptive transfer	Reduced
Li et al. (2013) [46]	Mouse: 60 min transient	≈35 %	3days	Adoptive transfer	Reduced
Li et al. (2014) [66]	Mouse: 60 min transient	≈35 %	3days	Adoptive transfer	Reduced
Brea et al. [67]	Rat: transient	≈30 %	28days	Adoptive transfer	Reduced
Liesz et al. (2013) [19]	Mouse: distal, permanent	≈10 %	7days	HDACi	Reduced
Brea et al. [67]	Rat: transient	≈30 %	10days	CD28SA	Reduced
Na et al. [29]	Mouse: distal, permanent	≈10 %	7days	CD28SA	Reduced
	Mouse: 60 min transient	≈50 %	3days	CD28SA	Reduced
Xie et al. [27]	Rats: 90 min transient	≈45 %	28days	mTOR Inh.	Reduced
Kleinschnitz et al. [26]	Mouse: 60 min transient	≈55 %	3days	Adoptive transfer	Increased
Schuhmann et al. [30]	Mouse: 60 min transient	≈40 %	3days	CD28SA	Increased

[a]This study analyzed the behavioral deficits as the primary outcome and did not report infarct volume

therapeutic paradigm since both studies showing an exacerbation of lesion volume (adoptive Treg transfer and CD28SA) have been respectively tested in three or more other experiments that have been showing an improved outcome (Table 3). Again, it seems that the lesion size induced by the used stroke model might be a predictor of the neuroprotective effect of Tregs: out of the 13 experiments detecting a benefit of Treg-therapy, only two have reported infarct volumes in the control group of more than 40 % of the hemisphere, while the other studies deployed models inducing small to moderate-sized lesions. Yet, particularly two studies [29, 30] using both the CD28SA treatment in transient ischemia models with similar lesion size have reported contradicting results, indicating the presence of other confounding factors independent of lesion volume. We have performed in a recent review a Meta-analysis in order to assess an approximation of the efficacy of Treg-targeted therapeutic approaches in experimental stroke models [31]. For this, all studies having investigated interventions with the aim to modulate Treg (defined at least by CD25 or Foxp3 expressing CD4+ T cells) number and/or efficacy in models of experimental brain ischemia were included in the analysis. The overall effect size estimation revealed an odds ratio favoring Treg-targeted interventions [31]. However, a large heterogeneity between the included studies regarding study design, experimental model and species (rat and mouse) has to be taken into account. Therefore, the number of studies investigating Treg interventions has to be increased to more robustly estimate a realistic effect size. Also the publication of negative results has to be encouraged to avoid a publication bias in such an emerging research field as Treg-targeted cell therapies for acute brain injuries.

5 Mechanisms of Treg Function in Post-Stroke Neuroinflammation

Basic immunological research on Treg function has identified several mechanisms by which Treg cells suppress an immune reaction [32]. These can be mainly divided into mechanisms acting on lymphocyte activation and mechanisms inhibiting antigen-presenting cells of the innate immune system. While most of these mechanisms can be recognized using in vitro model systems, the contribution of individual mechanisms such as different anti-inflammatory cytokines or cell–cell-contact dependent suppression is highly diverse in between in vivo disease models. The most prominent mechanisms of Treg function in vivo are the secretion of anti-inflammatory cytokines (IL-10, TGF-β), expression of immunosuppressant molecules (CTLA-4, CD39, PD, consumption of vital cytokines (IL-1, IL-2) and the secretion of cytolytic molecules (granzymes, perforin) [6].

In the model of experimental brain ischemia, several studies have demonstrated that IL-10 is a critical neuroprotective cytokine regulating post-stroke neuroinflammation [33–36]. The main sources of cerebral IL-10 are regulatory lymphocytes

(T and B cells) and microglia/monocytes. The key role of IL-10 as a primary effector mechanism of Treg cells in tissue injury [37] was verified in some of the experimental stroke studies [16, 19, 38]. Accordingly, strategies increasing lymphocyte-derived IL-10 production [19, 29, 35, 39] or therapeutic IL-10 administration [33, 36, 40] have been shown to be beneficial for stroke outcome. In addition, alternative Treg mechanisms have been identified also in brain ischemia models such as the expression of TGF-β and IL-35 [27] as well as a role of Treg cells in the PD-1/PD-L1 pathway. Pro-inflammatory lymphocytes and brain-resident microglia/macrophages have been characterized as the target cells for the immunosuppressive effect of Treg cells after brain injury. Studies that have identified a beneficial effect of Treg detected an inhibition of T cell brain invasion [16, 28], suppression of effector T cell proliferation [19], reduced production of lymphocytic and monocytic cerebral cytokines [27, 28, 41], suppression of microglia/monocyte activation [16] or a priming towards a M2-like microglial phenotype [27]. In contrast, studies detecting an exacerbation of stroke burden by Treg-targeted therapies [26, 30] have found an association of the increased lesion volume with amplified cerebral immune cell accumulation [30], however, it is assumable that the deleterious effects observed by Treg in these two studies might be independent of an immunological function but rather attributable to an impact on secondary microthrombosis [26].

6 Circulating Treg Cells After Stroke

Acute brain ischemia induces profound alterations of the peripheral immune reaction, encompassing peripheral immune activation in the acute phase after brain injury [42] and an immunosuppressive syndrome in the later phase [43]. In addition to the effect of Treg in modulating central neuroinflammation, their functional role has also been detected in alterations of the peripheral immune system after stroke. A consistent finding in the subacute phase after extensive experimental stroke is that cellular immunosuppression and splenic atrophy is accompanied by a relative expansion of Treg cells in spleen and blood [44, 45]. Interestingly, a recent report suggested that the adoptive transfer of Treg cells reduced on one side the systemic inflammatory reaction in the acute phase after stroke and on the other side also ameliorated the extent of immunosuppression (i.e., lymphopenia) in the later subacute phase [46]. This finding of a dualistic role of Treg in systemic immunity comparing the acute and subacute phases after stroke is well in line with the concept that the initial overactivation of the peripheral immune system might result in the later immunosuppression syndrome [47]. Thereby, the potent homeostatic function of Treg might suppress the initial systemic inflammation, attenuating the subsequent immune disturbances such as lymphopenia due to activation-induced lymphopenia cell death and exhaustion of antigen-presenting cells.

7 Regulatory T Cells in Stroke Patients

Only very few studies have investigated regulatory T cells in stroke patients. Understandably, these studies have focused on peripheral immune effects after stroke by investigating blood samples of patients with different stroke entities. The first study specifically analyzing Treg function after stroke by Hug et al. has found that Treg function is preserved in the context of post-stroke immunosuppression in contrast to the dysfunction of effector cell populations such as circulating monocytes or helper T cells [48]. In contrast, a second study has found reduced suppressive capacities of post-stroke Treg in female but not male stroke patients, proposing sex-specific effects on post-stroke peripheral immunity [49]. This difference between the studies could be explained by differing patient characteristics in terms of comorbidities and stroke severity. While the latter study has detected a robust increase of Treg cell counts after stroke in accordance with experimental studies, another report has shown the opposite. Li et al. reported a significant reduction of peripheral Treg in stroke patients [50]. In contrast to the previous studies investigating post-stroke effects on peripheral Treg function, a report by Wigren et al., analyzed the association of Treg counts in a cohort of 700 participants of the Malmö Diet and Cancer study with the prospective incidence of stroke. While low Treg counts at baseline were associated with an increased risk of myocardial infarction, this association was not present for stroke [51]. Overall, clinical data is supporting the experimental finding of substantial peripheral immunomodulation after stroke, which is also affecting the Treg population, yet, specific changes might depend on stroke entity, severity, and patient characteristics.

8 Potential Causes for Differential Effects of Treg Function in Post-Stroke Neuroinflammation

As previously discussed, considerable discrepancy on the role of Treg in stroke can be observed in the literature. Based on our current knowledge of mechanisms involved in post-stroke secondary neuroinflammation as well as the impact of acute brain injuries on the peripheral immune system [1, 43, 52], the most probable reasons for this phenomenon shall be discussed in the following paragraph.

8.1 Thromboinflammation and Secondary Microthrombosis

The commonly used transient MCA occlusion models by intraluminal filaments which were also used in the two studies detecting a deleterious role of Treg in stroke [26, 30] can be described as a transient mechanical vascular occlusion model (TMVO) with prompt revascularization. One prominent and distinct feature of this model is

the occurrence of a delayed neuronal damage due to microthrombosis [53]. Notably, the occurrence of microthrombosis is not common to human stroke and animal models of permanent or gradual reperfusion [54] and might rather represent a subpopulation of stroke patients receiving endovascular reperfusion therapy with immediate recanalization of a proximal artery. In experimental models, the presence of such microthrombosis might be determined by the extent of endothelial damage induced by the filament insertion, speed of filament retraction, and occlusion time. Finally, these at first-sight minor methodological differences might have a major pathophysiological impact by the presence or absence of secondary thrombosis [54, 55].

8.2 The Impact of Lesion Volume on Stroke-Immunology

As noted above, it seems that lesion size might be a confounder independent of reperfusion. It is well acknowledged that the phenomenon of post-stroke peripheral immunosuppression occurs in stroke patients and animal stroke models only after extensive brain tissue injury [43, 45, 52, 56]. While mice and men with substantial brain lesions develop substantial lymphopenia and alterations of the monocyte population, small ischemic injuries induce only a minor immunomodulation but no immunosuppressive syndrome. While the impact of peripheral immunosuppression after major stroke on secondary neuroinflammation was to our knowledge not systematically investigated, it is conceivable that peripheral immune alterations might affect also central neuroinflammation. Indeed, a recent previous study performing a face-to-face comparison of the extent of cerebral leukocyte invasion, microglial activation, and cytokine secretion in three common models of brain ischemia of differing lesion size detected crucial difference in between models with manifold stronger inflammatory reaction in small permanent ischemia models than in extensive hemispheric lesions after transient MCA occlusion [17]. Therefore, it is plausible that regulatory T cells have an inferior role in stroke models with only minor bystander inflammation compared to the critical role of Treg cells in lesion models with an overshooting immune reaction (Fig. 2).

8.3 Milieu-Dependent Treg Function

Moreover, the above stated methodological difference including the presence of microthrombosis/thromboinflammation, induction of peripheral immune alterations, and the extent of neuroinflammation might have a direct impact on the functional properties of natural Treg cells. It is known from other disease paradigms that Treg function in vivo might be particularly dependent of the immunological milieu. For example in cancer research this phenomenon—very much alike to stroke-immunology—has been termed as the "Janus-faced function of Treg" [57, 58]. In addition, the currently predominant perception of neuroinflammation after acute brain injury and particularly stroke research as *too much of a bad thing* should be revisited. Several elegant reports by the groups of Michal Schwartz and Jonathan

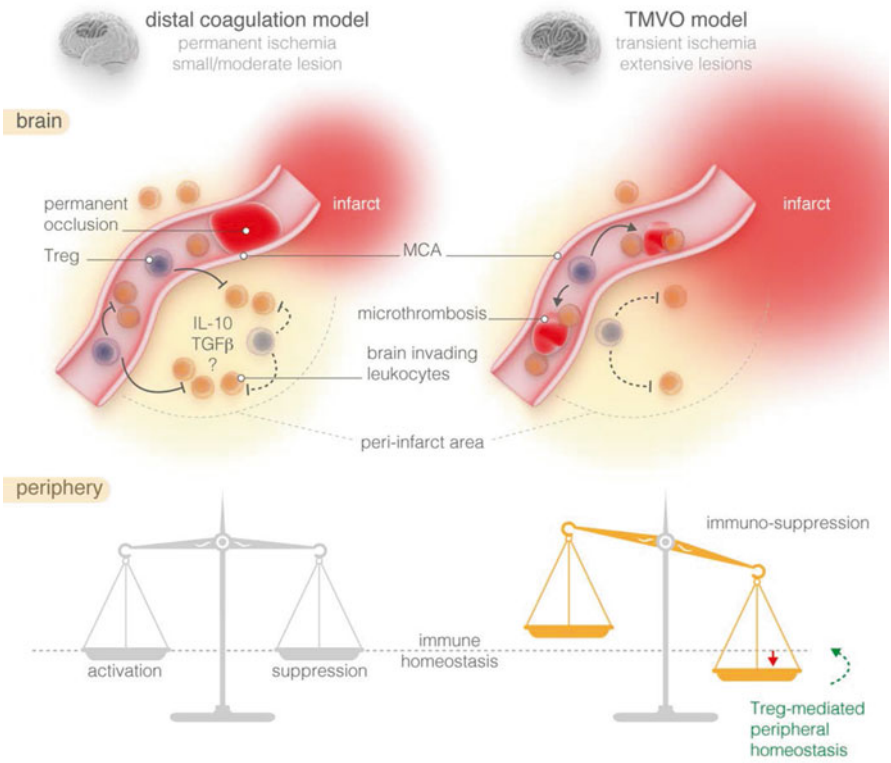

Fig. 2 Differential role of regulatory T cells in experimental stroke models. The schematic diagram illustrates the discrepancy of the observed role of Treg in the brain (*upper panels*) versus peripheral immune system (*lower panels*) in models of permanent ischemia with small- to moderate-sized lesions or after extensive brain lesions in TMVO models. Permanent occlusion of the distal middle cerebral artery (MCA; *left panels*) induces a strong neuroinflammatory reaction, but preserves peripheral immune homeostasis with only minor immunomodulation. In this context, Treg have a primary role in inhibiting an overshooting inflammatory reaction mediated by pro-inflammatory leukocytes. It is assumed that this immunosuppressive function of Treg takes place in the periphery even before brain invasion. In contrast, TMVO models with extensive brain injuries (*right panels*) have less pronounced neuroinflammation, but induce an immunosuppressive phenotype of the peripheral immune system. Here, Treg have only a minor function in suppressing the neuroinflammatory response, and might even have a non-immunologic function in the manifestation of secondary microthrombosis and thromboinflammation. The principal role of Treg in TMVO models seems rather to be in ameliorating immune disturbances by inhibiting initial immunologic activation or overactivation and later immunosuppression, thereby preserving homeostatic systemic immune function. *IL-10*, interleukin 10; *TGFβ*, tumor growth factor β (Reproduced from Liesz et al., Stroke, 2015)

Kipnis have established the concept of "protective autoimmunity" (see Schwartz and Raposo [59] for a recent review of the concept). This concept ascribes secondary inflammation as a generally physiological and protective mechanisms in which too much of immune activation as well as immunosuppression might be deleterious. Accordingly, in certain situations of acute brain lesions and neurodegeneration

models—determined by the time-dependent inflammatory milieu of the brain during disease progression—Treg depletion as well as its augmentation might negatively affect the outcome [60–62]. Therefore, the very specific properties of the used stroke model and targeted mechanism of Treg function have to be carefully investigated before considering Treg in a potentially oversimplified model as *good or bad* immune cells after stroke.

References

1. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. Nat Med. 2011;17(7):796–808.
2. Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, et al. Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury. Nat Med. 2009;15(8):946–50.
3. Gelderblom M, Weymar A, Bernreuther C, Velden J, Arunachalam P, Steinbach K, et al. Neutralization of the IL-17 axis diminishes neutrophil invasion and protects from ischemic stroke. Blood. 2012;120(18):3793–802. Epub Nov 1 2012. English.
4. Liesz A, Zhou W, Mracsko E, Karcher S, Bauer H, Schwarting S, et al. Inhibition of lymphocyte trafficking shields the brain against deleterious neuroinflammation after stroke. Brain. 2011;134(Pt 3):704–20.
5. Wei Y, Yemisci M, Kim HH, Yung LM, Shin HK, Hwang SK, et al. Fingolimod provides long-term protection in rodent models of cerebral ischemia. Ann Neurol. 2011;69(1):119–29.
6. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol. 2010;10(7):490–500.
7. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance. Cell. 2000;101(5):455–8.
8. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995;155(3):1151–64. Epub 1995/08/01. eng.
9. Ochs HD, Ziegler SF, Torgerson TR. FOXP3 acts as a rheostat of the immune response. Immunol Rev. 2005;203:156–64. Epub 2005/01/22. eng.
10. Baecher-Allan C, Anderson DE. Regulatory cells and human cancer. Semin Cancer Biol. 2006;16(2):98–105. Epub 2005/12/28. eng.
11. Chatila TA. Role of regulatory T cells in human diseases. J Allergy Clin Immunol. 2005;116(5):949–59; quiz 60. Epub 2005/11/09. eng.
12. Battaglia M, Roncarolo MG. Induction of transplantation tolerance via regulatory T cells. Inflamm Allergy Drug Targets. 2006;5(3):157–65. Epub 2006/08/22. eng.
13. Jiang H, Chess L. Regulation of immune responses by T cells. N Engl J Med. 2006;354(11):1166–76. Epub 2006/03/17. eng.
14. Shevach EM, DiPaolo RA, Andersson J, Zhao DM, Stephens GL, Thornton AM. The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells. Immunol Rev. 2006;212:60–73.
15. Gelderblom M, Leypoldt F, Steinbach K, Behrens D, Choe CU, Siler DA, et al. Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. Stroke. 2009;40(5):1849–57.
16. Liesz A, Suri-Payer E, Veltkamp C, Doerr H, Sommer C, Rivest S, et al. Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. Nat Med. 2009;15(2):192–9.

17. Zhou W, Liesz A, Bauer H, Sommer C, Lahrmann B, Valous N, et al. Postischemic brain infiltration of leukocyte subpopulations differs among murine permanent and transient focal cerebral ischemia models. Brain Pathol. 2013;23(1):34–44. Epub 2012/07/11. eng.
18. Stubbe T, Ebner F, Richter D, Randolf Engel O, Klehmet J, Royl G, et al. Regulatory T cells accumulate and proliferate in the ischemic hemisphere for up to 30 days after MCAO. J Cereb Blood Flow Metab. 2012;33(1):37–47.
19. Liesz A, Zhou W, Na SY, Hammerling GJ, Garbi N, Karcher S, et al. Boosting regulatory T cells limits neuroinflammation in permanent cortical stroke. J Neurosci. 2013;33(44):17350–62. Epub 2013/11/01. eng.
20. Kleinschnitz C, Wiendl H. Con: regulatory T cells are protective in ischemic stroke. Stroke. 2013;44(8):e87–8. Epub 2013/07/04. eng.
21. Hu X, Li P, Chen J. Pro: regulatory T cells are protective in ischemic stroke. Stroke. 2013;44(8):e85–6. Epub 2013/07/04. eng.
22. Urra X, Cervera A, Villamor N, Planas AM, Chamorro A. Harms and benefits of lymphocyte subpopulations in patients with acute stroke. Neuroscience. 2009;158(3):1174–83. Epub 2008/07/16. eng.
23. Ren X, Akiyoshi K, Vandenbark AA, Hurn PD, Offner H. CD4+ FoxP3+ regulatory T-cells in cerebral ischemic stroke. Metab Brain Dis. 2011;26(1):87–90. PMCID: 3070853. Epub 2010/11/18. eng.
24. Lahl K, Loddenkemper C, Drouin C, Freyer J, Arnason J, Eberl G, et al. Selective depletion of Foxp3+ regulatory T cells induces a scurfy-like disease. J Exp Med. 2007;204(1):57–63. PMCID: 2118432. Epub 2007/01/04. eng.
25. Suffner J, Hochweller K, Kuhnle MC, Li X, Kroczek RA, Garbi N, et al. Dendritic cells support homeostatic expansion of Foxp3+ regulatory T cells in Foxp3.LuciDTR mice. J Immunol. 2010;184(4):1810–20. Epub 2010/01/20. eng.
26. Kleinschnitz C, Kraft P, Dreykluft A, Hagedorn I, Gobel K, Schuhmann MK, et al. Regulatory T cells are strong promoters of acute ischemic stroke in mice by inducing dysfunction of the cerebral microvasculature. Blood. 2013;121(4):679–91. Epub 2012/11/20. eng.
27. Xie L, Sun F, Wang J, Mao X, Xie L, Yang SH, et al. mTOR signaling inhibition modulates macrophage/microglia-mediated neuroinflammation and secondary injury via regulatory T cells after focal ischemia. J Immunol. 2014;192(12):6009–19. PMCID: 4128178. Epub 2014/05/16. eng.
28. Li P, Gan Y, Sun BL, Zhang F, Lu B, Gao Y, et al. Adoptive regulatory T-cell therapy protects against cerebral ischemia. Ann Neurol. 2013;74(3):458–71. PMCID: 3748165. Epub 2013/05/16. eng.
29. Na SY, Mracsko E, Liesz A, Hunig T, Veltkamp R. Amplification of regulatory T cells using a CD28 superagonist reduces brain damage after ischemic stroke in mice. Stroke. 2015;46(1):212–20. Epub 2014/11/08. Eng.
30. Schuhmann MK, Kraft P, Stoll G, Lorenz K, Meuth SG, Wiendl H, et al. CD28 superagonist-mediated boost of regulatory T cells increases thrombo-inflammation and ischemic neurodegeneration during the acute phase of experimental stroke. J Cereb Blood Flow Metab. 2015;35(1):6–10. Epub 2014/10/16. Eng.
31. Liesz A, Hu X, Kleinschnitz C, Offner H. Functional role of regulatory lymphocytes in stroke: facts and controversies. Stroke. 2015;46(5):1422–30.
32. Shevach EM. Review mechanisms of Foxp3 + T regulatory cell-mediated suppression. Immunity. 2009;30(5):636–45.
33. Spera PA, Ellison JA, Feuerstein GZ, Barone FC. IL-10 reduces rat brain injury following focal stroke. Neurosci Lett. 1998;251(3):189–92.
34. Grilli M, Barbieri I, Basudev H, Brusa R, Casati C, Lozza G, et al. Interleukin-10 modulates neuronal threshold of vulnerability to ischaemic damage. Eur J Neurosci. 2000;12(7): 2265–72.
35. Bodhankar S, Chen Y, Vandenbark AA, Murphy SJ, Offner H. IL-10-producing B-cells limit CNS inflammation and infarct volume in experimental stroke. Metab Brain Dis. 2013;28(3):375–86. PMCID: 3737266. Epub 2013/05/04. eng.

36. Liesz A, Bauer A, Hoheisel JD, Veltkamp R. Intracerebral interleukin-10 injection modulates post-ischemic neuroinflammation: an experimental microarray study. Neurosci Lett. 2014;579:18–23. Epub 2014/07/16. eng.
37. O'Garra A, Vieira PL, Vieira P, Goldfeld AE. IL-10-producing and naturally occurring CD4+ Tregs: limiting collateral damage. J Clin Invest. 2004;114(10):1372–8.
38. Frenkel D, Huang Z, Maron R, Koldzic DN, Hancock WW, Moskowitz MA, et al. Nasal vaccination with myelin oligodendrocyte glycoprotein reduces stroke size by inducing IL-10-producing CD4+ T cells. J Immunol. 2003;171(12):6549–55.
39. Frenkel D, Huang Z, Maron R, Koldzic DN, Moskowitz MA, Weiner HL. Neuroprotection by IL-10-producing MOG CD4+ T cells following ischemic stroke. J Neurol Sci. 2005;233(1-2):125–32.
40. Strle K, Zhou JH, Shen WH, Broussard SR, Johnson RW, Freund GG, et al. Interleukin-10 in the brain. Crit Rev Immunol. 2001;21(5):427–49.
41. Ishibashi S, Maric D, Mou Y, Ohtani R, Ruetzler C, Hallenbeck JM. Mucosal tolerance to E-selectin promotes the survival of newly generated neuroblasts via regulatory T-cell induction after stroke in spontaneously hypertensive rats. J Cereb Blood Flow Metab. 2009;29(3): 606–20.
42. Offner H, Subramanian S, Parker SM, Afentoulis ME, Vandenbark AA, Hurn PD. Experimental stroke induces massive, rapid activation of the peripheral immune system. J Cereb Blood Flow Metab. 2006;26(5):654–65. Epub 2005/08/27. eng.
43. Meisel C, Schwab JM, Prass K, Meisel A, Dirnagl U. Central nervous system injury-induced immune deficiency syndrome. Nat Rev Neurosci. 2005;6(10):775–86.
44. Offner H, Subramanian S, Parker SM, Wang C, Afentoulis ME, Lewis A, et al. Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. J Immunol. 2006;176(11):6523–31. Epub 2006/05/20. eng.
45. Liesz A, Hagmann S, Zschoche C, Adamek J, Zhou W, Sun L, et al. The spectrum of systemic immune alterations after murine focal ischemia: immunodepression versus immunomodulation. Stroke. 2009;40(8):2849–58. Epub 2009/05/16. eng.
46. Li P, Mao L, Zhou G, Leak RK, Sun BL, Chen J, et al. Adoptive regulatory T-cell therapy preserves systemic immune homeostasis after cerebral ischemia. Stroke. 2013;44(12):3509–15. PMCID: 3895539. Epub 2013/10/05. eng.
47. Liesz A, Dalpke A, Mracsko E, Antoine DJ, Roth S, Zhou W, et al. DAMP signaling is a key pathway inducing immune modulation after brain injury. J Neurosci. 2015;35(2):583–98.
48. Hug A, Liesz A, Muerle B, Zhou W, Ehrenheim J, Lorenz A, et al. Reduced efficacy of circulating costimulatory cells after focal cerebral ischemia. Stroke. 2011;42(12):3580–6.
49. Yan J, Read SJ, Henderson RD, Hull R, O'Sullivan JD, McCombe PA, et al. Frequency and function of regulatory T cells after ischaemic stroke in humans. J Neuroimmunol. 2012;243(1–2):89–94.
50. Li Q, Wang Y, Yu F, Wang YM, Zhang C, Hu C, et al. Peripheral Th17/Treg imbalance in patients with atherosclerotic cerebral infarction. Int J Clin Exp Pathol. 2013;6(6):1015–27. PMCID: 3657353.
51. Wigren M, Bjorkbacka H, Andersson L, Ljungcrantz I, Fredrikson GN, Persson M, et al. Low levels of circulating CD4+ FoxP3+ T cells are associated with an increased risk for development of myocardial infarction but not for stroke. Arterioscler Thromb Vasc Biol. 2012;32(8):2000–4.
52. Chamorro A, Meisel A, Planas AM, Urra X, van de Beek D, Veltkamp R. The immunology of acute stroke. Nat Rev Neurol. 2012;8(7):401–10.
53. Hossmann KA. The two pathophysiologies of focal brain ischemia: implications for translational stroke research. J Cereb Blood Flow Metab. 2012;32(7):1310–6. PMCID: 3390813. Epub 2012/01/12. eng.
54. Gauberti M, Martinez de Lizarrondo S, Orset C, Vivien D. Lack of secondary microthrombosis after thrombin-induced stroke in mice and non-human primates. J Thromb Haemost. 2014;12(3):409–14. Epub 2013/12/21. eng.

55. Gauberti M, Vivien D. Letter by Gauberti and Vivien regarding article, “amplification of regulatory T cells using a CD28 superagonist reduces brain damage after ischemic stroke in mice”. Stroke. 2015;46(2):e50–1.
56. Liesz A, Ruger H, Purrucker J, Zorn M, Dalpke A, Mohlenbruch M, et al. Stress mediators and immune dysfunction in patients with acute cerebrovascular diseases. PLoS One. 2013;8(9):e74839. PMCID: 3777986. Epub 2013/09/27. eng.
57. Levings MK, Allan S, d’Hennezel E, Piccirillo CA. Functional dynamics of naturally occurring regulatory T cells in health and autoimmunity. Adv Immunol. 2006;92:119–55. Epub 2006/12/06. eng.
58. Danese S, Rutella S. The Janus face of CD4+ CD25+ regulatory T cells in cancer and autoimmunity. Curr Med Chem. 2007;14(6):649–66. Epub 2007/03/10. eng.
59. Schwartz M, Raposo C. Protective autoimmunity: a unifying model for the immune network involved in CNS repair. Neuroscientist. 2014;20(4):343–58. Epub 2014/01/08. Eng.
60. Walsh JT, Zheng J, Smirnov I, Lorenz U, Tung K, Kipnis J. Regulatory T cells in central nervous system injury: a double-edged sword. J Immunol. 2014;193(10):5013–22. Epub 2014/10/17. eng.
61. Kipnis J, Schwartz M. Controlled autoimmunity in CNS maintenance and repair: naturally occurring CD4+ CD25+ regulatory T-Cells at the crossroads of health and disease. Neuromol Med. 2005;7(3):197–206.
62. Kipnis J, Mizrahi T, Hauben E, Shaked I, Shevach E, Schwartz M. Neuroprotective autoimmunity: naturally occurring CD4+ CD25+ regulatory T cells suppress the ability to withstand injury to the central nervous system. Proc Natl Acad Sci U S A. 2002;99(24):15620–5.
63. Stubbe T, Ebner F, Richter D, Engel O, Klehmet J, Royl G, et al. Regulatory T cells accumulate and proliferate in the ischemic hemisphere for up to 30 days after MCAO. J Cereb Blood Flow Metab. 2013;33(1):37–47. PMCID: 3597367. Epub 2012/09/13. eng.
64. Chen Y, Ruetzler C, Pandipati S, Spatz M, McCarron RM, Becker K, et al. Mucosal tolerance to E-selectin provides cell-mediated protection against ischemic brain injury. Proc Natl Acad Sci U S A. 2003;100(25):15107–12. PMCID: 299916. Epub 2003/12/03. eng.
65. Gee JM, Kalil A, Thullbery M, Becker KJ. Induction of immunologic tolerance to myelin basic protein prevents central nervous system autoimmunity and improves outcome after stroke. Stroke. 2008;39(5):1575–82.
66. Li P, Mao L, Liu X, Gan Y, Zheng J, Thomson AW, et al. Essential role of program death 1-ligand 1 in regulatory T-cell-afforded protection against blood–brain barrier damage after stroke. Stroke. 2014;45(3):857–64. PMCID: 3939692. Epub 2014/02/06. eng.
67. Brea D, Agulla J, Rodriguez-Yanez M, Barral D, Ramos-Cabrer P, Campos F, et al. Regulatory T cells modulate inflammation and reduce infarct volume in experimental brain ischaemia. J Cell Mol Med. 2014;18(8):1571–9. Epub 2014/06/04. eng.

B-Cells in Stroke and Preconditioning-Induced Protection Against Stroke

Uma Maheswari Selvaraj, Katie Poinsatte, and Ann M. Stowe

Abbreviations

APCs	Antigen-presenting cells
BAFF	B-cell-activating factor
BBB	Blood–brain barrier
B cells	B-lymphocytes
BCR	B-cell receptor
BDNF	Brain-derived neurotrophic factor
Bregs	Regulatory B-cells
CCL2	Chemokine (C–C motif) ligand 2
CLN	Cervical lymph node
CNS	Central nervous system
CSF	Cerebrospinal fluid
CSPGs	Chondroitin sulfate proteoglycans
CXCL13	Chemokine (C–X–C motif) ligand 13
DAMPs	Danger-associated molecular pattern molecules
EAE	Experimental autoimmune encephalomyelitis
ICAM-1	Intracellular adhesion molecule
Ig	Immunoglobulin
IL-	Interleukin
IFNγ	Interferon γ
LPS	Lipopolysaccharide
LTP	Long-term potentiation

U.M. Selvaraj • K. Poinsatte • A.M. Stowe (✉)
Department of Neurology & Neurotherapeutics, UT Southwestern Medical Center,
6000 Harry Hines Blvd, MC8813, Dallas, TX 75390, USA
e-mail: Ann.Stowe@utsouthwestern.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_12

MAP2	Microtubule-associated protein 2
MBP	Myelin basic protein
MCAo	Middle cerebral artery occlusion
MHC	Major histocompatibility class
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
$\mu MT^{-/-}$	Transmembrane exon of the IgM heavy chain
MZ B-cells	Marginal zone B-cells
NA	Noradrenaline
nIgM	Natural IgM
NR2A	*N*-methyl-D-aspartate (NMDA) receptor subunit 2A
RA	Rheumatoid Arthritis
RF	Rheumatoid factor
RHP	Repetitive hypoxic preconditioning
ROS	Reactive oxygen species
Sema3a	Semaphorin IIIa
SHP	Single-exposure hypoxic preconditioning
SLE	Systemic lupus erythematosus
TACI	Transmembrane activator and calcium modulator and cyclophilin ligand interactor
TGF-β	Transforming growth factor β
T_h cells	T helper cells
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α

1 Introduction

Within the last decade, research into the role of the immune system following stroke onset has revealed a complex inflammatory response that contributes to both stroke-induced injury, as well as central nervous system (CNS) repair. It is now generally accepted that most leukocyte subsets, including both the innate and adaptive arms of the immune system, are activated within the brain parenchyma following stroke. What is less understood is how the expression, timing, and duration of accumulating leukocytes affect functional recovery in the injured CNS. In fact, it may not be that all inflammation is inherently detrimental to recovery, particularly with regard to regulatory lymphocytes, including T-regulatory cells (Chap. 11). B-lymphocytes, or B-cells, also exhibit a potential impact on recovery mechanisms, particularly with regard to neuroprotective preconditioning treatments. This chapter will begin with an overview of B-cell function, and the role of B-cells in other CNS and systemic inflammatory diseases, with relevance to the clinical stroke populations. The chapter will then focus on the role of B-cells in stroke pathology, as well as preconditioning-induced protection from stroke, including potential mechanisms derived from both animal and clinical preconditioning studies.

2 An Overview of B-Cell Function

2.1 Antigen Processing and Presentation by B-Cells

B-cells require antigen exposure to become fully activated [1–3]. After their initial development in the bone marrow, B-cells migrate to the spleen and differentiate into mature, naïve B-cells. These naïve B-cells enter the bloodstream, circulate, and localize to secondary lymphoid organs, including lymph nodes and spleen. These environments aid B-cells in encountering antigens, which are either soluble and enter through the afferent lymphatics, or large particulate antigens presented on the nearby cell surface of antigen-presenting cells (APCs). This results in an immunological synapse forming between the B-cell and the APC, which can include macrophages (Chap. 10) and follicular dendritic cells. This immunological synapse consists of the B-cell receptor (BCR) on B-cell surface, which recognizes the antigen, surrounded by a ring of adhesion molecules, which improves adherence to the APCs [4, 5]. After the external antigens are recognized and captured by B-cells through their BCR, they undergo processing in the internal compartments of the B-cell for subsequent presentation to $CD4^+$ T-cells (i.e., T helper cells; T_h cells). Specifically, antigen is broken into peptide fragments bound to major histocompatibility class (MHC) II molecules on the B-cell surface, which shifts the role of the B-cell into that of an APC for CD4 T-cell activation [6].

2.2 Activation of B-Cells Through CD4 T-Cell Interactions

Thus, one of the primary functions of B-cells is to directly activate T-cells during an adaptive immune response. After encountering an antigen, B-cells require accessory signals directly from microbial constituents or from an activated CD4 T-cell to form germinal centers for activation and proliferation [7, 8]. This necessity for accessory signals creates a tight control over the differentiation of naïve B-cells into memory, and/or antibody-producing plasma cell populations [9]. The process of antigen recognition is highly complex, and antigens that activate T-cells can be completely distinct from the surface epitopes recognized by B-cells. In fact, T-cells and B-cells need not even necessarily recognize the same protein before directly interacting, which could lead to quick activation following a massive release of antigen, such as occurs following stroke.

B cells interact with CD4 T-cells in "T-cell zones" within the spleen and lymph nodes [10]. When B-cells enter the lymphoid tissue through permissive systemic venules, they migrate through the T-cell zones into the B-cell zone. Here, those B-cells bound to antigens are trapped through activation of various adhesion molecules and engagement of chemokine receptors, thereby optimizing the rate of interaction with an activated CD4 T-cell at the border of T- and B-cell zones. The specific B-cells, activated by CD4 T-cells, clonally expand and both B-cells and T-cells

proliferate for several days [11]. This is effectively the first phase of primary humoral immune response, and has previously been considered to only occur in the CNS during rare pathology [12]. Recent work by Louveau and colleagues [13], however, identified lymphatic vessels within both murine and human brains, which challenges the dogma that the CNS is predominantly immune-privileged and lacks the lymphatic system found in the periphery. Thus, future studies will need to determine if germinal centers develop, either within the parenchyma or adjacent cortical lymphatics, under pathological states that involve antigen presentation and immune activation, as occurs following stroke [14].

2.2.1 B-Cell Differentiation and Relevant Subsets

Activated B-cells in germinal centers, which are continually refined during the entire germinal center response, differentiate into antibody-secreting plasma cells or memory B-cells [15]. B-cells differentiate into plasmablasts secreting high-affinity antibody at rapid rates, but lose MHC II expression and thus the ability to activate CD4 T-cells [16, 17]. Plasma and memory cells exhibit a wide range of lifespan, with some longer-lived plasmablasts accounting for a persistent, long-lasting source of high-affinity antibody [18]. As reviewed below, these long-lasting B-cell populations may have prolonged effects in clinical stroke recovery [19, 20].

2.2.2 Innate-Like B-Cells

Apart from the B-cells described above which stem from the bone marrow (i.e., B-2 cells), there are heterogeneous populations of unconventional B-cells (e.g., B-1 cells, marginal zone (MZ) B-cells) which play major roles in innate sensing and rapid immune response [21, 22]. B-1 cells produce more mature naive cells in peripheral lymphoid tissues, in contrast to conventional B-2 cells, which only divide upon antigen exposure. B-1 cells secrete antibodies which are polyreactive, low-affinity antibodies at steady state with broad reactivity [22]. When they get activated, they can rapidly acquire immune regulatory activities through the secretion of natural immunoglobulin (Ig)M and interleukin (IL)-10. Thus, these innate-like B-cells constitute an important source of IL-10-producing regulatory B-cells (Bregs), which have been shown to play critical roles in autoimmunity, inflammation, and infection following stroke, as described below.

2.2.3 B-Regulatory Cells and IL-10

Multiple murine disease models for both autoimmune [23–25] and inflammatory diseases [26] confirm the potential for B-cells to downregulate pro-inflammatory immune responses. In particular, Bregs exert immunosuppressive activity through

both direct and indirect mechanisms, including production of cytokines (e.g., IL-10, IL-35, and transforming growth factor β (TGF-β) [27, 28], and expression of inhibitory molecules to downregulate the expansion of pathogenic lymphocytes in a cell contact-dependent manner [28–30]. Regulatory T-cells are classically identified by the expression of Foxp3 marker [31, 32]. However, there is no known master transcription factor for Bregs, which are instead defined based on their functional specificities [31, 32]. Similar to regulatory T-cells, Bregs need to be activated before exerting their functions, with different inflammatory environments inducing different types of IL-10-producing regulatory B-cells [31, 33, 34]. Breg populations are highly heterogeneous, though it has been proposed to classify Bregs as "innate type" and "adaptive type" [29].

"Innate" Bregs are derived from innate-like B-cells, and are characterized by the capacity to rapidly produce high amounts of IL-10 and IgM antibodies upon activation [35, 36]. These "innate" Bregs are activated following toll-like receptor (TLR) or microbial stimulation. Some TLR agonists, like lipopolysaccharide (LPS) from gram-negative bacteria, induce IL-10-producing Bregs and reduce disease severity in a murine model of autoimmune disease [37]. Interestingly, systemic LPS preconditioning can induce tolerance to stroke injury [38, 39], though a link between LPS-induced "innate" Bregs and neuroprotection has yet to be established. On the other hand, "adaptive" Bregs demonstrate antigen specificity and are generated via BCR and CD40 responses, and possibly TLR signaling. In the murine autoimmune disease experimental autoimmune encephalomyelitis (EAE) model (described below), B-cells re-stimulated with auto-antigen produced IL-10 through CD40, and loss of Breg induction exacerbated disease [23].

3 Understanding the Contribution of B-Cells to Other Disease States

B-cells are mainly considered to function through antibody production, with effects limited to downstream events including antibodies coating antigen, activation of the complement pathway, and antibody-dependent cellular cytotoxicity [40]. Though antibodies do play a major role in defense against foreign pathogens, antibodies directed to self-antigen (i.e., autoreactive) can directly induce inflammatory conditions resulting in tissue damage. For example, B-cells are present in airway mucosa, and IgE antibodies are involved in allergic respiratory diseases such as asthma [41, 42]. But apart from antibody production, B-cells can also function as antigen-presenting cells, produce inflammatory and regulatory cytokines, as well as anti-inflammatory cytokines. These latter functions, in particular, may contribute to B-cell-mediated protection in the injured CNS. Unfortunately, there is a paucity of B-cell research compared to investigations of post-stroke monocyte, neutrophil, and T-cell populations. As such, it is first necessary to review the role of B-cells established through studies in other inflammatory disease states.

3.1 The Detrimental Role of B-Cells in Autoimmune Disease

The primary function of immune system is to differentiate between self and foreign antigens, and produce effector responses toward the foreign pathogens, whilst avoiding destructive self-targeting [43]. Autoimmune diseases generally involve abnormal recognition of self-antigens, resulting in severe inflammatory conditions and subsequent tissue damage. This occurs due to a combination of both environmental and genetic factors, which leads to a loss of tolerance toward self-antigens. B-cells are usually considered to be detrimental with respect to autoimmune diseases because of the secretion of autoantibodies, which damage tissue and bind receptors, acting as agonists to either activate or inactivate function [44, 45]. Furthermore, B-cells activate the complement system, leading to the release of anaphylatoxins which increase the secretion of pro-inflammatory cytokines and attract other effector cells to the site of injury [46, 47]. Antibody-independent pathogenic functions include providing accessory signals to T-cells to activate an autoreactive T-cell response [48–50]. B-cells secrete pro-inflammatory cytokines like tumor necrosis factor (TNF)α and interferon (IFN)γ [51], and areas of chronic inflammation form ectopic germinal centers [52]. These germinal centers are similar to the germinal centers of the secondary lymphoid organs described above, though the plasma cells in these structures secrete autoantibodies [53]. While at first these diseases may seem irrelevant to stroke, there is a call for the medical community to re-evaluate the role of chronic inflammatory diseases in the development of atherosclerosis, a predominant risk factor for stroke [54].

3.2 Multiple Sclerosis and EAE

Multiple sclerosis (MS) is an inflammatory and demyelinating disease of the human CNS. Most patients with MS exhibit lesions comprised of immune cell infiltrates, demyelination plaques, and axonal damage, with major heterogeneity of the pathological features in patients [55, 56]. EAE is an induced autoimmune disease in mice to model MS, though it is predominantly considered T-cell-mediated, as the adoptive transfer of myelin-specific autoreactive T-cells are sufficient for disease induction [57, 58]. CD4 T-cells specific for CNS antigens, such as myelin oligodendrocyte glycoprotein (MOG), induce inflammation and result in demyelination of the CNS, though IL-10-producing T-cells ameliorate disease [59–61]. The importance of the role of B-cells in MS was first stressed by the reports of a presence of high levels of immunoglobulins in the cerebrospinal fluid (CSF) MS patients [62]. While EAE induction does not require autoantibody production, they can exacerbate the existing inflammatory environment [63, 64]. Studies involving B-cell depletion using anti-IgM antibodies found that B-cell depleted mice were more resistant to the induction of EAE via recombinant MOG protein [64–66]. On the contrary, B-cell depletion during the later parts of the disease resulted in reducing the disease symptoms [57], possibly as B-cell antigen presentation and activation affects the entry of autoreactive T-cells into the CNS [67, 68].

3.3 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a prototypical autoimmune disorder characterized by a significant increase in autoantibodies against a variety of predominantly nuclear self-antigens, (e.g., DNA, ribonucleoproteins, and phospholipids) [69]. SLE patients exhibit diverse symptoms because of the heterogeneous impact of the disease on different tissues and organs, including kidney [70–73] and brain [74], resulting in a challenge to diagnosis the disease. B-cell depletion in SLE mouse models resulted in the absence of nephritis and vasculitis [75], and decreased activation of T-cells [76]. Considering the significance of B-cells in the initiation and progression of SLE, treatments targeting B-cell survival and differentiation for B-cell depletion therapy are being evaluated for clinical applications [77].

3.4 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease which results in inflamed synovial tissue due to infiltration of inflammatory cells into the affected joints [78]. The synovial membrane contains numerous activated CD4 T-cells, which are important for the pathogenesis of RA. B-cells function as APCs to prime autoreactive T-cells in experimental RA [79]. B-cells surround the T-cell aggregate, with activation dependent on the presence of B-cells [78]. Experiments where inflamed tissue samples from RA patients were transplanted into mice lacking lymphocytes confirmed the requirement of B-cells for both disease initiation and direct sustainment of chronic inflammation [80, 81]. B-cells also produce rheumatoid factor (RF) antibody production [82], with the presence of RF antibody correlated with increased mortality and morbidity in RA patients [83].

3.5 Current FDA-Approved B-Cell Therapeutics

CD20 is a B-cell surface marker expressed in different stages of B-cell development. Patients with autoimmune diseases, including MS, SLE, and RA, positively respond to B-cell depletion treatments using an anti-CD20 antibody, commercially known as Rituximab [77, 84–86]. In contrast, B-cell depletion exacerbates disease severity in patients with certain T-cell-mediated autoimmune conditions, including ulcerative colitis and psoriasis [87, 88]. Determination of efficacy of B-cell depletion is limited to changes in B-cell populations in the peripheral circulation. Therefore, the full effect on B-cells population in secondary lymphoid tissues outside of bone marrow, including the CNS, remains unknown [89].

Other B-cell-specific therapies are also under development, including the targeting of B-cell receptors involved in proliferation. A double-blind phase-II study with

Belimumab, a monoclonal antibody affecting the function of the B-cell receptor through the B-cell-activating factor (BAFF) [90]. BAFF is essential for B-cell survival and maturation [91, 92], with Belimumab significantly lowering B-cell counts for active RA patients [90]. Atacicept (i.e., antibodies against transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), another BAFF receptor required for B-cell maintenance and survival) also decreased B-cells in preclinical studies [93], with a Phase-Ib study with patients having moderate to severe RA, demonstrating efficacy [93, 94]. Finally, BR3-Fc, a recombinant protein that blocks BAFF signaling, is under evaluation in clinical trials [95].

Currently, most B-cell therapeutics are focused on B-cell depletion and antibody efficiency [96]. Induction of Bregs, however, also offers a potential therapeutic strategy, though further studies are required to establish the functional stability of Bregs [44]. However, there are some clinical trial results which support this avenue of intervention. Anti-IL-6R (Tocilizumab) treatment for RA increased TGF-β expression in $CD25^{high}$ B-cells, and could potentially function by regulating Bregs [97]. Also Laquinimod, a drug under development for MS and in phase-III clinical trials for SLE, increases the percentage of $CD25^{high}$ B-cells and their IL-10 expression, thereby expanding subpopulations of Bregs [98, 99]. Finally, Copaxone could prove efficacious in upregulating Bregs after stoke, as its effects include promoting the conversion of inflammatory CD4 T-cells to Tregs [100], the upregulation of B-cell-derived IL10 [101, 102], and the neuronal production of growth factors [103], though efficacy in experimental stroke models has been mixed (see Sect. 3.2).

4 B-Cells During Stroke Injury and Recovery

Stroke occurs when there is a loss of blood supply to the brain, either due to a vessel blockage (ischemic stroke) or vessel rupture (hemorrhagic stroke), and is a major cause of death and disability in the global adult population [104–106]. Despite significant improvements in the understanding and identification of factors contributing to post-stroke CNS tissue damage, therapeutic options for stroke are highly limited. Apart from the primary tissue damage due to lack of blood supply, there is a secondary ischemia-reperfusion injury due to the inflammatory cascade. Following the occurrence of either ischemic or hemorrhagic stroke, there is disruption of the blood–brain barrier (BBB), allowing endogenous CNS antigens access to activated lymphocytes [107]. Shortly after experimental stroke onset, endothelial cells within the BBB are activated to recruit peripherally circulating leukocytes to infiltrate into the brain parenchyma. This early CNS sterile inflammation induces an immediate and strong antigen-nonspecific innate immune response against the exposed CNS molecules, resulting in brain tissue damage and functional impairment, which later results in an antigen-specific adaptive immune response [108]. However, not all of the post-stroke immune responses are inherently detrimental to functional recovery. This section will review post-stroke inflammation, with a focus on B-cell-mediated mechanisms of both injury and repair in the CNS.

4.1 Post-Stroke Immune Responses Within the CNS

After stroke, there is a loss of BBB integrity, while reactive oxygen species (ROS) and other inflammatory mediators activate endothelial cells and leukocytes to increase expression of adhesion molecules [109, 110]. This results in the migration and recruitment of peripherally circulating leukocytes to the ischemic cortex, allowing the immune cells to access novel CNS antigens. While this is the first step of the inflammatory cascade, the acute inflammatory response consists of both infiltrating leukocytes and tissue-resident immune cells, including microglia (Chap. 8) [111]. Resident microglia are activated immediately after stroke and secrete pro-inflammatory mediators (e.g., IL-1β, TNF-α). Neutrophils are one of the first leukocytes to infiltrate the ischemic tissue, entering within a few hours after experimental stroke [112, 113]. Neutrophils secrete granules containing pro-inflammatory factors, elastases, myeloperoxidases, and multiple matrix metalloproteinases. The macrophages and mast cells residing in the perivascular space also release multiple pro-inflammatory factors, like TNF-α, IL-8, and IL-1β, as well as vasoactive mediators like histamine and proteases [114–117]. This highly enhances the infiltration of peripheral immune cells into the infarct region in a feed-forward mechanism that collectively constitutes the acute phase of the post-stroke innate immune response.

Lymphocytes, including T-cells and B-cells, enter the brain parenchyma early after onset as well, though T-cells function in the acute phase without any antigen specificity through secretion of cytokines and ROS generation [112, 113]. The dead and damaged cells in the infarct release an array of molecular signals called danger-associated molecular pattern molecules (DAMPs) [118]. These bind to and activate TLRs and scavenger receptors present on APCs to prime them for antigen presentation. This results in the CNS antigens being presented to B-cells and T-cells [14], and the subsequent development of cellular and humoral immunity against the CNS antigens. Data concerning antigen-dependent post-stroke adaptive immune responses are contradictory, as both beneficial and harmful effects to the brain have been shown in experimental stroke models [119–121].

Ortega and colleagues recently characterized the time course of autoreactivity to myelin-derived antigens (e.g., MOG, myelin basic protein (MBP)) and neuronal-derived antigens (e.g., microtubule-associated protein 2 (MAP2), *N*-methyl-D-aspartate (NMDA) receptor subunit, NR2A) [14]. Experimental stroke induced a high autoreactivity to CNS antigen as early as 4 days after onset for both B- and T-cell populations isolated from the spleen and cervical lymph node (CLN). The CLNs are draining lymph nodes for brain, but the rapid response may reflect a previously unstudied APC activation of B- and T-cells occurring in newly identified lymphatic vessels within the CNS [13]. High autoreactive responses to MAP2 and myelin occurred in the mice with the smallest infarct volumes, suggesting autoimmune responses after stroke may not be solely detrimental to recovery. This corroborates a clinical study in stroke patients, wherein Planas and colleagues demonstrated that increased APC presentation of MAP2 and NR2A correlated with smaller infarct volumes and better long-term outcome [122]. This study also found that higher reactivity for MBP correlated with larger infarctions and greater stroke

severity [122], though T-regulatory cells specific for MBP [103], and MBP antigen presentation before stroke [123] improved long-term recovery in animals [14]. Thus, further studies are required to completely elucidate this complex and ambiguous adaptive immune response to understand the mechanisms related to CNS recovery that could be harnessed as potential therapeutic targets.

4.2 B-Cell Potential to Limit Detrimental Acute Post-Stroke Inflammation

As mentioned previously, regulatory T- and B-cells produce anti-inflammatory cytokines that suppress the function of infiltrating pro-inflammatory cells. In fact, IL-10 expression is elevated during major CNS diseases [124], and regulatory lymphocytes play a protective role, limiting the CNS damage due to inflammation in autoimmune diseases such as MS and SLE [44, 61, 125]. Therefore, it is hardly surprising that in the absence of B-cells, there is increased infiltration of multiple leukocyte subsets into the ischemic parenchyma, including neutrophils, monocytes, macrophages, and T-cells, resulting in larger infarct volumes [124]. Confirmatory studies used transgenic mice that have a nonsense mutation introduced into the transmembrane exon of the IgM heavy chain ($\mu MT^{-/-}$), which results in the total deletion of B-cells [126]. These mice developed larger infarct volumes, more severe functional deficits, and had a higher mortality rate following middle cerebral artery occlusion (MCAo), a common experimental model of stroke [127]. The complete absence of B-cells also resulted in higher numbers of activated T-cells, macrophages, microglial cells, and neutrophils in the ischemic brain of $\mu MT^{-/-}$ mice following MCAo [127].

This phenotype was rescued after the adoptive transfer of highly enriched populations of wild-type B-cells to B-cell-deficient $\mu MT^{-/-}$ recipient mice before MCAo [127]. The neuroprotective, anti-inflammatory effect was not observed, however, when IL-10-deficient B-cells were adoptively transferred prior to stroke. Mice receiving B-cells as a neurotherapeutic exhibited higher numbers of regulatory cells in the periphery concomitant with lower numbers of activated inflammatory T-cells [128]. After LPS stimulation, purified B-cells produced significant amounts of IL-10. Adoptive transfer of these cells prior to stroke decreased infarct volumes, confirming the beneficial role of IL-10-producing B-cell subsets on stroke outcome in the acute phase of recovery [124]. These B-cell subsets also increased the regulatory T-cell numbers, co-inhibitory receptor PD-1 expression, and significantly reduced peripheral pro-inflammatory immune cells [124].

As mentioned previously, Copaxone as a therapeutic can induce IL-10 from Bregs [102]. Copaxone treatment (200 μg plus an adjuvant to boost the immune response) decreased infarct volumes in rats 7 days post-MCAo [129]. More recent studies, however, found that Copaxone (70 μg, no adjuvant) administered 30 mins prior to stroke did not reduce infarct volumes at 1 day after stroke [130], nor did Copaxone administration (400 μg, no adjuvant) immediately after MCAo reduce

infarct volumes at 3 and 7 days post-stroke [131]. But the latter study also did not find an upregulation of IL-10, which suggests that the lack of protection in these studies may be confounded by the lack of adjuvant, which increases inflammation, and thus Copaxone uptake, at the site of injection. This may also explain the shortened timeframe given for Copaxone to generate a neuroprotective effect, particularly with regard to the 1-day post-stroke study [130]. Therefore future studies should consider Copaxone as a pharmacologic preconditioning agent (similar to LPS, 4.4.1) when determining if it can induce neuroprotection via Breg upregulation prior to stroke onset.

4.3 Post-Stroke Immune Responses Occurring in the Periphery

Stroke induces systemic immune changes, which affect clinical outcomes for weeks and months following stroke onset [132, 133]. As mentioned above, the initial response to tissue injury following stroke is carried out primarily by the innate immune system, as innate cells are recruited to the ischemic parenchyma and contribute to infarct progression [112]. These inflammatory responses are not confined to the brain, however, but are also concurrent with immunological changes in blood, spleen, bone marrow, and secondary lymphoid organs [134, 135]. Gene expression signatures from the peripheral blood after stroke distinguish different causes of stroke, and this implies a characteristic immune response to CNS injury [136]. Stroke induces systemic immunodepression within days of onset, especially in the cases of large infarct volumes [137]. In fact, most patients with larger strokes have higher levels of circulating IL-6 and IL-10, lower lymphocyte counts, and increased susceptibility to infections (e.g., respiratory, urinary tract) that impede long-term recovery [138, 139]. Liesz et al. used different ischemia models to demonstrate the effect of stroke on systemic immunological and microbiological parameters [140]. They found that post-stroke changes associated with immune system, as well as infectious complications, depended on infarct volume. Only huge infarct volumes induced leucopenia (i.e., reduced numbers of lymphocyte counts) in spleen, lymph nodes, and thymus both during acute (after 24 h) and long-term (after 7 days) recovery.

A majority of these systemic immune changes can be attributed to downstream signaling of noradrenaline (NA) receptors, present on almost all immune cells, including 30–50 % of B-cells [141]. NA receptors are activated by epinephrine released from post-ganglionic sympathetic nerves within hours of experimental stroke onset [142]. Thus, the sympathetic nervous system dramatically alters post-stroke immune responses to increase plasma cortisol, catecholamines, and monocyte-derived IL-10, which in turn decreases the number and activity of circulating immune cells, including B-cells [134, 142]. Transient decreases in IgG levels were reported in post-stroke patients, which was also associated with increased bacterial infections [19]. Stroke injury is itself a strong stress signal that also activates the hypothalamic–pituitary axis, resulting in the production of glucocorticoids

which further induce immunosuppression [142, 143]. Treatment with propanol, a β-adrenergic receptor antagonist, as well as other steroid antagonists rescued lymphocyte apoptosis and reduced susceptibility to infection after stroke in animal models. Clinical studies confirmed that patients on beta-blockers at the time of stroke onset exhibited reduced stroke injury [144], in-hospital mortality [145], and certain infectious co-morbidities [146], but translational to an effective post-stroke therapeutic and the effect on B-cell populations has yet to be established.

4.4 B-Cell Contribution to Neuronal Plasticity

Functional recovery after stroke is limited, as only ~65 % of stroke survivors exhibit full functional recovery even 1 year post-stroke [147]. Unfortunately, many of the long-term sequelae that both inhibit and promote the neuronal plasticity required for functional recovery in the injured CNS are still being investigated. Under physiological conditions, axonal growth and sprouting is thought to be minimized in the adult brain [148], but after CNS injury, there is re-expression of axonal growth factors (e.g., GAP43, CAP23, and MARCKS) within the glial scar bordering the infarct [149–152]. Unfortunately, axonal growth inhibitors are also upregulated, including chondroitin sulfate proteoglycans (CSPGs), NogoA, and semaphorin IIIa (Sema3a), which often impair the permissive environment for post-stroke plasticity [153–158]. Recovery from stroke requires substantial axonal regeneration into the injured brain to connect novel cortical areas, and strengthen relevant connections in an effort to subsume function lost after stroke onset [159–162].

Unfortunately, there are few studies linking leukocyte-derived factors and neuronal plasticity in stroke, though some evidence has been found in experimental models of both autoimmune disease and spinal cord injury, particularly with regard to T-cell-axon interactions [163–167]. But B-cells harbor the potential for direct neuronal effects as well, particularly as T-cell-activated B-cells isolated from germinal centers in the spleen express genes related to axonal growth, differentiation, and repair [168]. Furthermore, antibodies derived from B-cells in the CSF of MS patients show a direct ability to bind demyelinated axons in the CNS, with several distinct axon-binding patterns, though their physiological or pathological function(s) are unclear [169]. B-cells accumulate in lesions following spinal cord injury and produce autoantibodies that cross-react with neuronal antigens, with improved recovery and decreased spinal pathology in the absence of B-cells, with respect to wild-type mice [170, 171].

Of the aforementioned axonal growth-related proteins, T- and B-cell immune responses to Nogo-66 can be both encephalitogenic, increasing the severity of the disease, as well as suppressive, lowering the disease score in EAE [172]. Also BAFF, secreted by B-cells, potentially binds Nogo receptors expressed in neurons and astrocytes, directly affecting the neuronal outgrowth [173]. Bregs also express Sema3A on their surface; hence interaction of B-cells with neurons can potentially be detrimental to post-stroke axon growth [99]. Finally, B-cells produce significant

amounts of brain-derived neurotrophic factor (BDNF), a growth factor widely known to support post-stroke neuronal growth in both in vitro and in vivo studies [174–176]. While many mechanisms exist to support the direct interaction of B-cells on neurons, future studies need to confirm a role specific to post-stroke plasticity.

4.5 B-Cells Mediate Post-Stroke Cognitive Impairment

Stroke patients are highly susceptible to dementia, particularly with regard to vascular dementia [177–179]. In fact, a single stroke increases the risk of developing dementia two-fold when compared to other risk factors like hypertension, hypercholesterolemia, diabetes, and age [20, 178, 180]. However, the underlying mechanism linking stroke and the development of dementia is largely unexplored. Recent work by Doyle and colleagues found that B-cell-mediated post-stroke inflammation induced delayed cognitive deficits at 7 weeks after experimental stroke, which mirrors the clinical risk of developing dementia over the years following stroke onset [20]. Four different experimental stroke models in two mouse strains colocalized B-cells to the infarct region 4–7 weeks after onset, with B-cells clustering and producing IgA and IgG antibodies. Doyle et al. hypothesized that these antibodies activate Fc receptors and complement pathways to induce neuronal damage and subsequent neurological dysfunction, similar to antibody actions during MS [169], with the potential for detrimental antibodies to diffuse into surrounding healthy tissues to further exacerbate recovery. This post-stroke antibody production impaired hippocampal long-term potentiation (LTP), resulting in short-term memory deficits beginning weeks after stroke. Delayed cognitive defects did not occur in $\mu MT^{-/-}$ mice or in mice treated with Rituximab. This role of B-cells in long-term cognitive impairment is in contrast to the role of B-cells in beneficial neuroprotection during the acute stages of stroke [181]. But whether B-cells provide acute neuronal protection, support functional plasticity, or even impede recovery may be dependent on the location, timing, and magnitude of the B-cell response within the injured CNS, which will hopefully be elucidated in future studies.

5 B-Cells During Preconditioning and Ischemic Tolerance to Stroke

5.1 Preconditioning and the Immune System

Preconditioning is the use of sublethal stressors to induce endogenous, global tolerance against subsequent injury [182, 183]. Preconditioning was originally developed to study myocardial ischemia, as Murry et al. observed that four, 5-min coronary branch occlusions, each followed by 5 min of reperfusion, protected against a 40-min

ischemic insult [184]. These brief exposures significantly reduced infarct volume in the myocardium without causing cell death. Since this discovery, preconditioning has been applied to ischemic injury in different organs and tissues, including the liver, kidney, small bowel, skeletal muscle, and retina [185–189]. One promising avenue of research is the use of preconditioning to induce ischemic tolerance in the brain. Preconditioning produces robust neuroprotection in rodent models of stroke, including significant reductions in infarct volumes, strengthened BBB integrity, and improved neurocognitive functioning after stroke [190, 191].

A wide range of noxious stimuli induce neuroprotection, which has led to the development of several preconditioning paradigms (see reviews [183, 192, 193]). One of the classic paradigms of preconditioning is ischemic preconditioning, which includes both brief cerebral ischemia and remote limb ischemia. Other well-studied preconditioning models include oxygen (hypoxia or hyperoxia), temperature (hypothermic or hyperthermic exposures), exercise (voluntary exercise or forced exercise), and various forms of pharmacological preconditioning. Despite the heterogeneity of stimuli used in preconditioning studies, there is a remarkable amount of overlap in the underlying mechanisms responsible for protection. Diverse preconditioning methods are dependent on similar cellular, molecular, and physiological mechanisms involving neurons, astrocytes, and vasculature, suggesting that a complex, multifaceted integration of different systems is necessary for ischemic tolerance [183].

There is increasing evidence that alterations to the immune system are a critical component of preconditioning. During preconditioning, the exposure to a stressful stimulus provokes an inflammatory response within the brain, activating many pathways that play a pathogenic role in stroke [194, 195]. TLRs, cytokines, chemokines, cannabinoids, inducible nitric oxide, and ROS have all been implicated as important mediators of both ischemic injury and preconditioning-induced neuroprotection [196, 197]. Despite their negative role in stroke, these inflammatory molecules appear to be necessary for the induction of endogenous neuroprotection. Studies consistently show that blocking these pro-inflammatory signaling cascades diminishes, if not demolishes, the ischemic tolerance produced by preconditioning [198–200]. The inflammatory response after preconditioning, although not severe enough to cause injury, significantly affects the immune system, “reprogramming” it to respond differently to subsequent injury in favor of a less “pro-inflammatory” response [194, 195].

Despite the growing interest in the role of the immune system in stroke [115, 201], there has been limited exploration of the relationship between adaptive immunity, in particular B-cells, and preconditioning. In the following section, we will discuss how three methods of preconditioning, using the systemic interventions of hypoxia and exercise, as well as the pharmacologic intervention of LPS, are associated with B-cell function, with emphasis on the future translational potential of these findings.

5.1.1 Single-Exposure Hypoxic Preconditioning

Hypoxic preconditioning has been shown to be neuroprotective, both in vivo and in vitro in neonates and adults [192]. However, the efficacy of this protection is dependent on the duration, intensity, and frequency of low-oxygen exposures. Many studies use single-exposure hypoxic preconditioning (SHP), in which an animal or tissue is placed inside an oxygen-controlled chamber filled with 8 % O_2/92 % N_2 gas for several hours. Exposures that are too brief (<3 h) are not protective against subsequent injury, while those that are too long (>5 h) worsen stroke outcome, increasing neuronal death and behavioral deficits [183, 202]. A single moderate exposure to hypoxia (~4 h) is robustly protective, reducing infarct volume, neuronal apoptosis, and neurologic deficits [203]. However, this protection is brief, as a single exposure protects only for 72 h [202, 204].

The ischemic tolerance seen after SHP has been attributed to both neuronal and non-neuronal mechanisms, including increases in antioxidant enzymes, calcium signaling, expression of hypoxia-inducible factors, erythropoietin, and vascular growth factors, activation of opioid receptors, as well as metabolic suppression [202, 204–211]. In addition to these mechanisms, there is increasing evidence that modulation of the immune system by SHP contributes to ischemic tolerance, specifically through activation of pro-inflammatory pathways (Table 1). After SHP, rat cortical neurons increased production of TNF-α and exhibited higher intracellular levels of sphingolipid ceramide, a secondary messenger involved in TNF-α signaling [200]. Inhibiting TNF-α and ceramide attenuated SHP-induced neuroprotection. Additionally, CCL2, a chemokine responsible for the recruitment of pro-inflammatory monocytes, dendritic cells, and memory T-cells, is upregulated in cortical neurons and endothelium after SHP [198]. In the absence of CCL2, the protective effects of SHP are significantly diminished. Additionally, Stowe and colleagues found that independent of CCL2, SHP significantly altered circulating leukocytes prior to injury, decreasing T-cells, monocytes, and granulocytes 12 h after the hypoxic exposure. Interestingly, despite the decline in other immune cell populations, there was a significant increase in the B-cells following SHP, suggesting a potentially unique relationship between SHP-induced neuroprotection and B-cells.

5.1.2 Repetitive Hypoxic Preconditioning

An important limitation of SHP paradigms is the brief time-window of protection [202, 204]. Thus, recent research has begun to investigate the benefits of repeated exposures to hypoxia prior to stroke, or repetitive hypoxic preconditioning (RHP). In a progressive hypoxia model, mice are placed in air-tight jars until gasping occurs, removed for 30 min of recovery, and then returned for a total of four hypoxic exposures, with some evidence that progressive hypoxia can induce neuroprotection [212, 213]. Another RHP method found that 15-h exposures to hypobaric hypoxia

Table 1 Immune system regulation by hypoxic exposures

Paradigm	Stimuli	Animal	Injury model	Duration of protection	Immune-mediated results	Reference
SHP	8 % O_2 for 4 h	Mouse	tMCAo	48 h	Histamine is necessary for hypoxia-induced protection and is necessary for neuroprotective increases in VEGF protein	[207]
	20 min hypoxic exposure	Rat	OGD	24 h	TNF-α and ceramide are necessary for hypoxia-induced protection in vitro	[273]
	8 % O_2 for 4 h	Mouse	tMCAo	48 h	CCL2 is necessary for hypoxia-induced protection	[198]
	8 % O_2 for 4 h	Mouse	tMCAo	24 h	HIF, SphK, S1P, CCL2 are necessary for hypoxia-induced protection	[276]
RHP	Stochastic oxygen exposures	Mouse	tMCAo	8 weeks	RHP reduced infarct volumes	[215]
				4 weeks	RHP reduced leukocyte diapedesis & leukocyte-endothelial adherence	
	Stochastic oxygen exposures	Mouse	tMCAo	2 weeks	RHP increased CXCL13 expression in the cortex and subcortex	[216]
					RHP increased immunosuppressive B-cells while reduced populations of other leukocytes	

tMCAo transient middle cerebral artery occlusion; *OGD* oxygen glucose deprivation; *TNF-α* tumor necrosis factor alpha; *CCL2* chemokine (C–C motif) ligand 2; *HIF* hypoxia-inducible factor; *SphK* sphingosine kinase 2; *S1P* sphingosine-1-phosphate; *CXCL13* chemokine (C–X–C motif) ligand 13

for 4 weeks decreased infarct volumes for up to 1 week following the final exposure [214]. However, the most effective RHP method to date is a preconditioning using stochastic exposures to hypoxia. Over the course of 2 weeks, animals receive hypoxic exposures varying in duration (2 or 4 h) and intensity (8 or 11 % O_2) [215]. This method of RHP produced robust neuroprotection at 2 weeks after the last

exposure, with a 48 % reduction in infarct volume, but included a 32 % reduction in infarct volume still present 8 weeks after RHP, a significant improvement over untreated animals. RHP decreased vascular inflammation, BBB disruption, leukocyte adherence to vessels, as well as overall leukocyte egress into the ischemic hemisphere [190, 215]. The initial paper describing the stochastic RHP methodology did not investigate specific leukocyte populations present in the brain after preconditioning, but subsequent work focused more extensively on the immune mechanisms behind neuroprotection after RHP.

5.1.3 B-Cells in Hypoxic Preconditioning

The potent protection induced by RHP is, in part, due to significant alterations in the post-stroke inflammatory response during ischemic tolerance. RHP reduced the influx of leukocytes into the ischemic brain, including CD4 T-cells, monocytes, and activated macrophages, concurrent with increased B-cell representation compared to untreated controls [216]. RHP maintained B-cell populations at a level equivalent to that of the uninjured, contralateral hemisphere and, similarly, the ratio of B-cells to monocytes was unchanged. This ratio is used to describe pathological immune environments in the CNS, making it a useful prognostic tool in MS [217] and B-cell lymphoma [198, 218]. Overall, RHP modulation of the immune system after stroke was a CNS-specific phenomenon, with minimal changes seen in peripheral immune populations. CNS specificity may be subsequent to an early RHP-mediated increase in CXCL13, a chemokine that recruits B-cells to sites of inflammation [216]. While CXCL13 is widely present throughout the post-stroke brain, it was largely expressed on cortical vessels, and particularly notable on endothelial cells surrounded by astrocytic endfeet at the BBB. Given the established benefits of RHP on BBB integrity [190, 215], it is possible that the recruitment of B-cells into the ischemic hemisphere contributes to improved neurovascular integrity in preconditioned mice.

Prior to stroke onset, RHP exposure altered the immunophenotype of resident splenic B-cells [216]. Microarray analysis found that over 1900 genes were changed 2 weeks following RHP, producing a unique immunosuppressive B-cell phenotype prior to the induction of injury. These genetic alterations suggest that RHP suppresses B–T-cell interactions. In addition to limiting B–T-cell interactions, RHP also inhibited B-cell proliferation, development, and differentiation, contributing to protective immunosuppression. Complementing these findings, ex vivo phenotyping using flow cytometry showed a significant reduction in mature and activated B-cells, as well as a decreased response to stimulation with LPS, suggesting RHP leads to functional impairment and decreased proliferative capacity in B-cells. RHP also induced a higher representation of Bregs, previously implicated in improved outcome after stroke [181]. Indeed, RHP-induced Bregs may directly contribute to the neurovascular protection seen after RHP, although further studies are necessary to confirm a causative role [216].

5.1.4 Clinical Relevance of Hypoxic Preconditioning

Most of the efforts to translate findings from animal models of preconditioning to the clinic have focused on remote ischemic preconditioning, particularly given its success in protecting against myocardial injury [193]. However, there is evidence that hypoxic exposures in moderate doses modulate the immune system in ways that may be protective against stroke. While many studies focus on the innate immune system activation during hypoxia [219], suppression of adaptive immunity is also an important component in the effects of hypoxia on the immune system. After 5 days of extreme hypobaric hypoxia (3200–3800 m), T-cell blastogenesis was impaired in individuals for up to 24 days [220]. Similarly, brief repetitive exposures to normobaric hypoxia have been shown to be anti-inflammatory, significantly decreasing TNF-α in the serum up to 1 week after final exposure [221]. Again, this decrease in TNF-α is consistent with our findings that this cytokine is suppressed through activation of IL-4 pathways after RHP [216]. These clinical studies are limited, though, as they do not look at the effects of hypoxia in the context of illness. However, there is epidemiological evidence that altitude has a positive effect on a wide range of illnesses and injuries such as stroke. A study in Switzerland found that individuals who lived at higher altitudes had decreased risk of coronary heart disease and stroke [222]. But overall, these studies suggest that changes in the immune system due to hypoxia at higher altitudes may lead to a sustained anti-inflammatory phenotype, which may in turn affect susceptibility to injury and illness.

5.1.5 Exercise Preconditioning

Another promising form of preconditioning is exercise preconditioning, which, according to a recent meta-analysis of experimental data, reduces infarct volumes by 42.2 % and decreases neurological deficits by 54.6 % [223]. Two main forms of exercise preconditioning have been used in animal studies: voluntary exercise and forced exercise. During voluntary exercise, rodents are given unrestricted access to running wheels, wherein they usually run in short, rapid spurts [224]. Conversely, forced exercise on a treadmill requires that animals maintain a slower speed for a longer duration [224]. The efficacy of these two methods varies given that preconditioning-induced neuroprotection is contingent on frequency, intensity, and duration of stimuli. Forced exercise produces more potent protection, reducing infarct volumes by 58.5 % as opposed to voluntary exercise, which only led to a 10.4 % reduction [223]. However, rats that underwent voluntary exercise preconditioning had greater motor recovery and higher levels of hippocampal BDNF levels after stroke [225]. Forced exercise also increased corticosterone [226], which can impair the immune system by reducing the functionality of T-cells and reducing the number of circulating B-cells [227].

The benefits of forced and voluntary exercise can be attributed to both neuronal and non-neuronal mechanisms. Exercise increases neurogenesis [228–230], angiogenesis [231, 232], and neuronal metabolism [226, 233], while simultaneously

decreasing BBB disruption [208, 234, 235] and apoptosis [236, 237]. Exercise preconditioning also improves stroke outcome through immune-mediated mechanisms (Table 2). Similar to many other forms of preconditioning, exercise preconditioning relies on pro-inflammatory molecules to induce its neuroprotection. Intracellular adhesion molecule (ICAM)-1, an adhesion molecule that promotes leukocyte rolling, and TNF-α mRNA expression, was greater in rats with 3 weeks of forced exercise [238]. But after stroke, the animals that underwent this exercise preconditioning exhibited decreased expression of the TNF-α receptors TNFR1 and TNFR2 [239], I-CAM expression [238], and subsequent leukocyte infiltration [238], similar to our results in RHP-treated mice [215]. Furthermore, exercised rats had lower levels of TLR4 expression [199], which could decrease the production of pro-inflammatory cytokines and contribute to neuroprotection, though this remains to be confirmed.

5.1.6 B-Cells in Exercise Preconditioning

During acute exercise, epinephrine activates the β2-adgenergic receptors on B-cells, leading to a mobilization of these lymphocytes in the blood [240]. The intensity and duration of exercise modulates this B-cell mobilization, with increased lymphocytosis after intense exercise [240]. Although changes in antibody production in serum appear to be negligible in humans, IgM concentration increases following in vitro mitogen-stimulation [240]. Similarly, rats that had unrestricted access to running wheels showed an increase in natural IgM (nIgM) in their serum and peritoneal cavity [241, 242]. nIgM is beneficial in mounting attacks against bacteria and viruses, as well as activating the complement system, which may be beneficial in post-stroke infection, a common and life-threatening complication in stroke patients described above [242]. Other studies have shown that voluntary and forced exercise increases serum IgG in response to antigens in mice [243–245]. Unfortunately, most studies investigated the effect of exercise on B-cell function within the context of infection, rather than CNS injury. Further studies will need to focus more on the role of immune cells, particularly B-cells, in exercise-induced neuroprotection, in order to determine the extent of their participation in ischemic tolerance.

5.1.7 Clinical Relevance of Exercise Preconditioning

In terms of clinical applications, exercise is one of the most promising forms of preconditioning-induced ischemic tolerance for individuals at a high risk for stroke [246]. Exercise is safe and noninvasive, without the risk of systemic inflammation or immunosuppression, as seen with other forms of preconditioning. It improves overall health, exerting a global anti-inflammatory effect [247]. Clinical studies already show that moderate exercise can reduce the risk of stroke [248, 249], improve vascular function, and diminish other risk factors such as obesity and

Table 2 Effects of exercise on the immune response after stroke

Paradigm	Stimuli	Animal	Injury model	Duration of protection	Immune-mediated results	Reference
Exercise	Forced treadmill running	Rat	tMCAo	48 h	Exercise increased TNF-α and ICAM-1 mRNA levels prior to stroke	[238]
					Exercise decreased leukocyte infiltration and decreased ICAM-1 expression after stroke	
	Forced treadmill running	Rat	tMCAo	48 h	Exercise decreased expression of TNFR1 and TNFR2 receptor expression in the brain	[234]
	Forced treadmill running	Rat	tMCAo	48 h	TNF-α and the ERK 1/2 pathway are necessary for decreased leukocyte infiltration after exercise and exercise-induced protection	[199]
	Forced treadmill running	Rat	tMCAo	48 h	TNF-α and the ERK 1/2 pathway are necessary for exercise-induced reduction of infarct volume, reduction of BBB disruption, reduction of MMP-9 protein, and increased collagen IV	[275]
	Forced treadmill running	Rat	tMCAo	48 h	Exercise reduced TLR-4 mRNA and protein expression in the brain	[274]

TNF-α tumor necrosis factor; *ICAM-1* intracellular adhesion molecule 1; *TNFR1* tumor necrosis factor receptor 1; *TNFR2* tumor necrosis factor receptor 2; *ERK 1/2* extracellular signal-regulated kinases 1 and 2; *MMP-9* matrix metallopeptidase 9; *TLR-4* toll-like receptor 4

high blood pressure [246]. In fact, individuals with active lifestyles prior to stroke have milder strokes, fewer motor deficits, and better functional recovery [250]. Determining the optimal intensity, frequency, and duration of exercise, and determining how exercise can beneficially modulate B-cell phenotypes in individuals, particularly with regard to co-morbidities, requires further research.

5.1.8 LPS Preconditioning

Activation of TLRs and TLR-triggered induction of regulatory factors are critical components of preconditioning, as TLR4-deficient mice were not protected against stroke by ischemic preconditioning [251]. In addition to being necessary for preconditioning-induced ischemic tolerance, systemic administration of ligands for TLR2, TLR4, TLR7, and TLR9 induce neuroprotection in rodent models of stroke [252–255]. Of the endotoxins that have been studied, the benefits of LPS preconditioning have been best characterized [256]. In mice, low systemic doses of LPS (< 1 mg/kg) prior to stroke produces an extended window of preconditioning, reducing infarct volumes for up to 1 week in mice, and 4 days in rats [253, 257]. Neuroprotection of neurons and endothelium significantly contributes to ischemic tolerance after LPS pretreatment. LPS administration 24 h prior to in vitro injury protects cortical neurons and cerebellar granular neurons [257, 258], as well as improves cerebrovascular functioning by reducing vascular permeability and increasing endothelial relaxation and microvascular reperfusion [259–261].

There is strong evidence that the efficacy of LPS preconditioning is largely due to immediate inflammatory responses that reprogram the immune system (Table 3). Within 24 h of LPS administration, there are significant genomic alterations associated with TLR signaling pathways [262]. These alterations have been associated with increased gene expression of anti-inflammatory cytokines and chemokines after stroke in preconditioned animals [263]. The initial LPS-induced increase in TNF-α is followed by a significant reduction at 1 day [257, 264]. Similar to exercise preconditioning, there was also a decrease in TNFR1 after stroke in preconditioned animals [257]. Activation of CCL2, a chemokine previously shown to be important in SHP (see Sect. 4.2.1), induces downstream effectors that reduce inflammation [265] and are required for efficacy of LPS preconditioning [38]. The involvement of TNF-α- and CCL2-mediated pathways in multiple paradigms of preconditioning supports the hypothesis that similar mechanisms may be responsible for preconditioning-induced ischemic tolerance. Finally, LPS preconditioning altered post-stroke leukocyte populations, decreasing neutrophils and monocytes in the brain while increasing circulating lymphocytes [254].

5.1.9 B-Cells in LPS Preconditioning

The role of B-cells in LPS preconditioning has unfortunately not been well-characterized. B-cells were decreased in the plasma after LPS preconditioning, though a similar phenomenon was seen in saline-treated mice, suggesting that loss of circulating B-cells may be subsequent to stroke as opposed to LPS preconditioning [254]. Rosenzweig and colleagues also found that LPS preconditioning did not significantly alter B-cell egress into the ischemic brain, as occurs after RHP [216]. However, this does not exclude the possibility of alterations of B-cell

Table 3 Effects of LPS pre-condtioning in the post-stroke response

Paradigm	Stimuli	Animal	Injury model	Duration of protection	Immune-mediated results	Reference
LPS	0.9 mg/kg, i.v.	Rat	pMCAo	1–4 days	TNF-α is necessary for LPS-induced reduction of infarct volumes	[253]
	0.05 mg/kg i.v.	Rat	pMCAo	24 h	LPS increases PMN infiltration after stroke	[259]
	0.2 mg/kg, i.p.	Mouse	tMCAo	48 h	LPS reduced microglia activation and neutrophil infiltration into the CNS	[254]
					LPS reduced monocytes and neutrophils, increased lymphocytes, and reduced monocyte activation in the blood	
	0.2 mg/kg, i.p.	Mouse	tMCAo	1–7 days	LPS increased pre-stroke TNF-α plasma levels but decreased TNF-α levels after stroke and decreased TNFR1 expression	[257]
			OGD	24 h	TNF-α is necessary for LPS-induced protection in vitro	
	0.2 or 0.8 mg/kg, i.p.	Mouse	tMCAo	72 h	LPS increased TGF-β, CCL5, and IFIT1 gene expression in the brain and IL-10 and CCL5 plasma levels	[263]
	0.2 mg/kg, i.p	Mouse	tMCAo	72 h	LPS alters TLR signaling pathways and cytokine–cytokine receptor interaction pathways	[262]
	0.2 mg/kg, i.p.	Mouse	tMCAo	24 h	LPS increases MCIP1 mRNA and protein levels in the brain and is necessary for LPS-induced reduction of infarct volumes	[38]

PMN polymorphonuclear leukocytes; *tMCAo* transient middle cerebral artery occlusion; *pMCAo* permanent middle cerebral artery occlusion; *TNF-α* tumor necrosis factor; *LPS* lipopolysaccharide; *CCL5* chemokine (C–C motif) ligand 5; *CNS* central nervous system; *OGD* oxygen glucose deprivation; *TGF-β* transforming growth factor β; *TLR* toll-like receptor; *PMN* polymorphonuclear

phenotype, such as the induction of an immunosuppressive B-cell population by LPS or changes in function, including increased IL-10 production. In EAE, TLR stimulation by LPS induces a Breg populations capable of reducing EAE severity [266] with sustained IL-10 production [23]. A proposed two-step model in EAE [266] may describe the role of B-cells in LPS preconditioning, as initial exposure to LPS induces a regulatory B-cell population that maintains an immunosuppressive phenotype via CD40 activation after stroke onset [267, 268], though this has yet to be confirmed.

5.1.10 Clinical Relevance of LPS Preconditioning

Currently, several TLR4 agonists are approved for human use to treat bladder carcinoma and HPV-associated cervical cancer [269]. TLR9 agonists have become increasingly attractive as stroke therapeutic, as they protect from stroke in nonhuman primates and show efficacy in clinical trials as adjuvants to vaccines to treat cancer, HIV, and allergies [270, 271]. Despite the promise of these trials, there are still significant concerns about the use of LPS in stroke patients. Clinical LPS administration is often used as a model of endotoxin tolerance because of the resultant acute inflammatory response followed by extended immunosuppression [272], a potential confounder that may not benefit long-term stroke recovery, considering prophylactic treatments with even low doses of LPS may induce endotoxin tolerance in stroke patients, worsening immunosuppression and increasing risk of infection [195].

6 Summary

This chapter reviews the current literature regarding the role of B-cells in stroke recovery, as well during ischemic tolerance to stroke induced by several preconditioning paradigms. B-cells predominantly mediate pathology in several autoimmune diseases, yet early evidence in stroke suggests that B-cells mediate both acute neuroprotection, as well as long-term pathology, in the injured CNS. What is most apparent from this review is that there is a lack of investigative studies linking B-cells directly to autoimmunity, neuronal plasticity, and overall functional recovery following stroke—in either experimental or clinical studies. While it is useful to review data from other CNS autoimmune diseases to predict potential mechanisms for B-cells during pathology, future studies should use the immunologic techniques established in MS, SLE, and RA models to determine how B-cells, particularly during an autoimmune response, shape the microenvironment within the ischemic CNS to support ischemic brain repair.

References

1. Reth M, Wienands J. Initiation and processing of signals from the B cell antigen receptor. Annu Rev Immunol. 1997;15:453–79 [Research Support, Non-U.S. Gov't Review].
2. Carrasco YR, Batista FD. B cell recognition of membrane-bound antigen: an exquisite way of sensing ligands. Curr Opin Immunol. 2006;18(3):286–91 [Research Support, Non-U.S. Gov't Review].
3. Carrasco YR, Batista FD. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. Immunity. 2007;27(1):160–71 [Research Support, Non-U.S. Gov't].
4. Yuseff MI, Lankar D, Lennon-Dumenil AM. Dynamics of membrane trafficking downstream of B and T cell receptor engagement: impact on immune synapses. Traffic. 2009;10(6):629–36 [Research Support, Non-U.S. Gov't Review].
5. Carrasco YR, Fleire SJ, Cameron T, Dustin ML, Batista FD. LFA-1/ICAM-1 interaction lowers the threshold of B cell activation by facilitating B cell adhesion and synapse formation. Immunity. 2004;20(5):589–99 [Research Support, Non-U.S. Gov't].
6. Mitchison NA. Landmark—T-cell-B-cell cooperation. Nat Rev Immunol. 2004;4(4):308–12.
7. Andersson J, Melchers F. T-cell-dependent activation of resting B-cells—requirement for both nonspecific unrestricted and antigen-specific Ia-restricted soluble factors. Proc Natl Acad Sci U S A. 1981;78(4):2497–501.
8. Liebson HJ, Marrack P, Kappler J. B cell helper factors. II. Synergy among three helper factors in the response of T cell- and macrophage-depleted B cells. J Immunol. 1982;129(4):1398–402.
9. Niiro H, Clark EA. Regulation of B-cell fate by antigen-receptor signals. Nat Rev Immunol. 2002;2(12):945–56 [Research Support, U.S. Gov't, P.H.S. Review].
10. Okada T, Miller MJ, Parker I, Krummel MF, Neighbors M, Hartley SB, et al. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. PLoS Biol. 2005;3(6):e150 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].
11. Garside P, Ingulli E, Merica RR, Johnson JG, Noelle RJ, Jenkins MK. Visualization of specific B and T lymphocyte interactions in the lymph node. Science. 1998;281(5373):96–9 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].
12. Lee FY, Wen MC, Wang J. A 28 year old lady with a pituitary mass. Brain Pathol. 2012;22(4):563–6.
13. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Rouhani SJ, Peske JD, et al. Structural and functional features of central nervous system lymphatic vessels. Nature. 2015;523(7560):337–41.
14. Ortega SB, Noorbhai I, Poinsatte K, Kong X, Anderson A, Monson NL, et al. Stroke induces a rapid adaptive autoimmune response to novel neuronal antigens. Discov Med. 2015;19(106):381–92.
15. Liu YJ, Joshua DE, Williams GT, Smith CA, Gordon J, MacLennan IC. Mechanism of antigen-driven selection in germinal centres. Nature. 1989;342(6252):929–31 [Research Support, Non-U.S. Gov't].
16. Sartoris S, Tosi G, De Lerma BA, Cestari T, Accolla RS. Active suppression of the class II transactivator-encoding AIR-1 locus is responsible for the lack of major histocompatibility complex class II gene expression observed during differentiation from B cells to plasma cells. Eur J Immunol. 1996;26(10):2456–60 [Research Support, Non-U.S. Gov't].
17. Latron F, Jotterand-Bellomo M, Maffei A, Scarpellino L, Bernard M, Strominger JL, et al. Active suppression of major histocompatibility complex class II gene expression during differentiation from B cells to plasma cells. Proc Natl Acad Sci U S A. 1988;85(7):2229–33.
18. Slifka MK, Ahmed R. Long-lived plasma cells: a mechanism for maintaining persistent antibody production. Curr Opin Immunol. 1998;10(3):252–8 [Review].

19. Liesz A, Roth S, Zorn M, Sun L, Hofmann K, Veltkamp R. Acquired immunoglobulin G deficiency in stroke patients and experimental brain ischemia. Exp Neurol. 2015;271:46–52.
20. Doyle KP, Quach LN, Sole M, Axtell RC, Nguyen TV, Soler-Llavina GJ, et al. B-lymphocyte-mediated delayed cognitive impairment following stroke. J Neurosci. 2015;35(5):2133–45.
21. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity. 2001;14(5):617–29 [Research Support, U.S. Gov't, P.H.S.].
22. Bendelac A, Bonneville M, Kearney JF. Autoreactivity by design: innate B and T lymphocytes. Nat Rev Immunol. 2001;1(3):177–86 [Review].
23. Fillatreau S, Sweenie CH, McGeachy MJ, Gray D, Anderton SM. B cells regulate autoimmunity by provision of IL-10. Nat Immunol. 2002;3(10):944–50 [Research Support, Non-U.S. Gov't].
24. Matsushita T, Horikawa M, Iwata Y, Tedder TF. Regulatory B cells (B10 cells) and regulatory T cells have independent roles in controlling experimental autoimmune encephalomyelitis initiation and late-phase immunopathogenesis. J Immunol. 2010;185(4):2240–52 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
25. Ray A, Mann MK, Basu S, Dittel BN. A case for regulatory B cells in controlling the severity of autoimmune-mediated inflammation in experimental autoimmune encephalomyelitis and multiple sclerosis. J Neuroimmunol. 2011;230(1–2):1–9 [Review].
26. Shimomura Y, Mizoguchi E, Sugimoto K, Kibe R, Benno Y, Mizoguchi A, et al. Regulatory role of B-1 B cells in chronic colitis. Int Immunol. 2008;20(6):729–37 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
27. Rosser EC, Mauri C. Regulatory B cells: origin, phenotype, and function. Immunity. 2015;42(4):607–12 [Research Support, Non-U.S. Gov't Review].
28. Gray D, Gray M. What are regulatory B cells? Eur J Immunol. 2010;40(10):2677–9.
29. Mizoguchi A, Bhan AK. A case for regulatory B cells. J Immunol. 2006;176(2):705–10 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review].
30. Lundy SK. Killer B lymphocytes: the evidence and the potential. Inflamm Res. 2009;58(7):345–57 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review].
31. Zhang XM. Regulatory functions of innate-like B cells. Cell Mol Immunol. 2013;10(2): 113–21.
32. Rudensky AY. Regulatory T cells and Foxp3. Immunol Rev. 2011;241:260–8.
33. Mauri C, Bosma A. Immune regulatory function of B cells. Annu Rev Immunol. 2012;30:221–41.
34. DiLillo DJ, Matsushita T, Tedder TF. B10 cells and regulatory B cells balance immune responses during inflammation, autoimmunity, and cancer. Ann N Y Acad Sci. 2010;1183: 38–57.
35. O'Garra A, Chang R, Go N, Hastings R, Haughton G, Howard M. Ly-1 B (B-1) cells are the main source of B cell-derived interleukin 10. Eur J Immunol. 1992;22(3):711–7 [Research Support, Non-U.S. Gov't].
36. Spencer NF, Daynes RA. IL-12 directly stimulates expression of IL-10 by CD5+ B cells and IL-6 by both CD5+ and CD5- B cells: possible involvement in age-associated cytokine dysregulation. Int Immunol. 1997;9(5):745–54 [Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.].
37. Lampropoulou V, Hoehlig K, Roch T, Neves P, Calderon Gomez E, Sweenie CH, et al. TLR-activated B cells suppress T cell-mediated autoimmunity. J Immunol. 2008;180(7):4763–73 [Research Support, Non-U.S. Gov't].
38. Liang J, Wang J, Saad Y, Warble L, Becerra E, Kolattukudy PE. Participation of MCP-induced protein 1 in lipopolysaccharide preconditioning-induced ischemic stroke tolerance by regulating the expression of proinflammatory cytokines. J Neuroinflammation. 2011;8:182.
39. Kunz A, Park L, Abe T, Gallo EF, Anrather J, Zhou P, et al. Neurovascular protection by ischemic tolerance: role of nitric oxide and reactive oxygen species. J Neurosci. 2007;27(27): 7083–93.

40. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. Blood. 2008;112(5): 1570–80.
41. Takhar P, Smurthwaite L, Coker HA, Fear DJ, Banfield GK, Carr VA, et al. Allergen drives class switching to IgE in the nasal mucosa in allergic rhinitis. J Immunol. 2005;174(8): 5024–32.
42. KleinJan A, Vinke JG, Severijnen LWFM, Fokkens WJ. Local production and detection of (specific) IgE in nasal B-cells and plasma cells of allergic rhinitis patients. Eur Respir J. 2000;15(3):491–7.
43. Sinha AA, Lopez MT, Mcdevitt HO. Autoimmune-diseases—the failure of self tolerance. Science. 1990;248(4961):1380–8.
44. Yang M, Rui K, Wang S, Lu L. Regulatory B cells in autoimmune diseases. Cell Mol Immunol. 2013;10(2):122–32 [Research Support, Non-U.S. Gov't Review].
45. Edwards JCW, Cambridge G, Abrahams VM. Do self-perpetuating B lymphocytes drive human autoimmune disease? Immunology. 1999;97(2):188–96.
46. Bindon CI, Hale G, Bruggemann M, Waldmann H. Human monoclonal IgG isotypes differ in complement activating function at the level of C4 as well as C1q. J Exp Med. 1988;168(1):127–42 [Research Support, Non-U.S. Gov't].
47. Tom BH, Raffel S. Complement-dependent anaphylactic reactions. Infect Immun. 1975;11(6):1284–90 [Research Support, U.S. Gov't, P.H.S.].
48. Shlomchik MJ, Craft JE, Mamula MJ. From T to B and back again: positive feedback in systemic autoimmune disease. Nat Rev Immunol. 2001;1(2):147–53.
49. Rodriguez-Pinto D, Moreno J. B cells can prime naive CD4(+) T cells in vivo in the absence of other professional antigen-presenting cells in a CD154-CD40-dependent manner. Eur J Immunol. 2005;35(4):1097–105.
50. Yan J, Harvey BP, Gee RJ, Shlomchik MJ, Mamula MJ. B cells drive early T cell autoimmunity in vivo prior to dendritic cell-mediated autoantigen presentation. J Immunol. 2006;177(7):4481–7.
51. Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, et al. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol. 2000;1(6): 475–82.
52. Martin F, Chan AC. B cell immunobiology in disease: evolving concepts from the clinic. Annu Rev Immunol. 2006;24:467–96.
53. Manzo A, Paoletti S, Carulli M, Blades MC, Barone F, Yanni G, et al. Systematic microanatomical analysis of CXCL13 and CCL21 in situ production and progressive lymphoid organization in rheumatoid synovitis. Eur J Immunol. 2005;35(5):1347–59 [Research Support, Non-U.S. Gov't].
54. Kucharz EJ. Chronic inflammation-enhanced atherosclerosis: can we consider it as a new clinical syndrome? Med Hypotheses. 2012;78(3):396–7.
55. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple sclerosis. N Engl J Med. 2000;343(13):938–52 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review].
56. Pierson ER, Stromnes IM, Goverman JM. B cells promote induction of experimental autoimmune encephalomyelitis by facilitating reactivation of T cells in the central nervous system. J Immunol. 2014;192(3):929–39 [Research Support, N.I.H., Extramural].
57. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. J Clin Invest. 2008;118(10):3420–30.
58. Pettinelli CB, Mcfarlin DE. Adoptive transfer of experimental allergic encephalomyelitis in Sjl-J mice after invitro activation of lymph-node cells by myelin basic-protein—requirement for Lyt 1+ 2- lymphocytes-T. J Immunol. 1981;127(4):1420–3.
59. Ando DG, Clayton J, Kono D, Urban JL, Sercarz EE. Encephalitogenic T cells in the B10.PL model of experimental allergic encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. Cell Immunol. 1989;124(1):132–43.

60. O'Connor RA, Prendergast CT, Sabatos CA, Lau CW, Leech MD, Wraith DC, et al. Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. J Immunol. 2008;181(6):3750–4 [Comparative Study Research Support, Non-U.S. Gov't].
61. Koutrolos M, Berer K, Kawakami N, Wekerle H, Krishnamoorthy G. Treg cells mediate recovery from EAE by controlling effector T cell proliferation and motility in the CNS. Acta Neuropathol Commun. 2014;2:163 [Research Support, Non-U.S. Gov't].
62. Kabat EA, Glusman M, Knaub V. Quantitative estimation of the albumin and gamma-globulin in normal and pathologic cerebrospinal fluid by immunochemical methods. Am J Med. 1948;4(5):653–62.
63. Linington C, Bradl M, Lassmann H, Brunner C, Vass K. Augmentation of demyelination in rat acute allergic encephalomyelitis by circulating mouse monoclonal antibodies directed against a myelin/oligodendrocyte glycoprotein. Am J Pathol. 1988;130(3):443–54 [Research Support, Non-U.S. Gov't].
64. Lyons JA, San M, Happ MP, Cross AH. B cells are critical to induction of experimental allergic encephalomyelitis by protein but not by a short encephalitogenic peptide. Eur J Immunol. 1999;29(11):3432–9 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].
65. Gausas J, Paterson PY, Day ED, Dalcanto MC. Intact B-cell activity is essential for complete expression of experimental allergic encephalomyelitis in Lewis rats. Cell Immunol. 1982;72(2):360–6.
66. Willenborg DO, Sjollema P, Danta G. Immunoglobulin deficient rats as donors and recipients of effector-cells of allergic encephalomyelitis. J Neuroimmunol. 1986;11(2):93–103.
67. Bettelli E, Baeten D, Jager A, Sobel RA, Kuchroo VK. Myelin oligodendrocyte glycoprotein-specific T and B cells cooperate to induce a Devic-like disease in mice. J Clin Invest. 2006;116(9):2393–402 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
68. Krishnamoorthy G, Lassmann H, Wekerle H, Holz A. Spontaneous opticospinal encephalomyelitis in a double-transgenic mouse model of autoimmune T cell/B cell cooperation. J Clin Invest. 2006;116(9):2385–92 [Research Support, Non-U.S. Gov't].
69. Mok CC, Lau CS. Pathogenesis of systemic lupus erythematosus. J Clin Pathol. 2003;56(7): 481–90.
70. Alba P, Bento L, Cuadrado MJ, Karim Y, Tungekar MF, Abbs I, et al. Anti-dsDNA, anti-Sm antibodies, and the lupus anticoagulant: significant factors associated with lupus nephritis. Ann Rheum Dis. 2003;62(6):556–60.
71. Forger F, Matthias T, Oppermann M, Becker H, Helmke K. Clinical significance of anti-dsDNA antibody isotypes: IgG/IgM ratio of anti-dsDNA antibodies as a prognostic marker for lupus nephritis. Lupus. 2004;13(1):36–44.
72. Cortes-Hernandez J, Ordi-Ros J, Labrador M, Bujan S, Balada E, Segarra A, et al. Antihistone and anti-double-stranded deoxyribonucleic acid antibodies are associated with renal disease in systemic lupus erythematosus. Am J Med. 2004;116(3):165–73.
73. Chan VSF, Tsang HHL, Tam RCY, Lu LW, Lau CS. B-cell-targeted therapies in systemic lupus erythematosus. Cell Mol Immunol. 2013;10(2):133–42.
74. Reichlin M. Ribosomal P, antibodies and CNS lupus. Lupus. 2003;12(12):916–8.
75. Shlomchik MJ, Madaio MP, Ni D, Trounstein M, Huszar D. The role of B-cells in Lpr/Lpr-induced autoimmunity. J Cell Biochem. 1995;10:155.
76. Chan O, Shlomchik MJ. A new role for B cells in systemic autoimmunity: B cells promote spontaneous T cell activation in MRL-lpr/lpr mice. J Immunol. 1998;160(1):51–9 [Research Support, U.S. Gov't, P.H.S.].
77. Anolik JH, Barnard J, Cappione A, Pugh-Bernard AE, Felgar RE, Looney RJ, et al. Rituximab improves peripheral B cell abnormalities in human systemic lupus erythematosus. Arthritis Rheum. 2004;50(11):3580–90 [Clinical Trial, Clinical Trial, Phase I, Clinical Trial, Phase II, Controlled Clinical Trial, Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].

78. Kim HJ, Berek C. B cells in rheumatoid arthritis. Arthritis Res. 2000;2(2):126–31 [Research Support, Non-U.S. Gov't Review].
79. O'Neill SK, Shlomchik MJ, Glant TT, Cao YX, Doodes PD, Finnegan A. Antigen-specific B cells are required as APCs and autoantibody-producing cells for induction of severe autoimmune arthritis. J Immunol. 2005;174(6):3781–8.
80. Takemura S, Klimiuk PA, Braun A, Goronzy JJ, Weyand CM. T cell activation in rheumatoid synovium is B cell dependent. J Immunol. 2001;167(8):4710–8.
81. Panayi GS, Corrigall VM, Pitzalis C. Pathogenesis of rheumatoid arthritis—the role of T cells and other beasts. Rheum Dis ClinNorth Am. 2001;27(2):317–34.
82. Zhang Z, Bridges Jr SL. Pathogenesis of rheumatoid arthritis. Role of B lymphocytes. Rheum Dis Clin North Am. 2001;27(2):335–53 [Review].
83. Carson DA, Chen PP, Kipps TJ. New roles for rheumatoid factor. J Clin Invest. 1991;87(2):379–83 [Research Support, U.S. Gov't, P.H.S. Review].
84. Edwards JC, Cambridge G. Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes. Rheumatology. 2001;40(2):205–11 [Clinical Trial Research Support, Non-U.S. Gov't].
85. Edwards JC, Cambridge G. Prospects for B-cell-targeted therapy in autoimmune disease. Rheumatology. 2005;44(2):151–6 [Research Support, Non-U.S. Gov't Review].
86. Hauser SL, Waubant E, Arnold DL, Vollmer T, Antel J, Fox RJ, et al. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. N Engl J Med. 2008;358(7):676–88 [Clinical Trial, Phase II Multicenter Study Randomized Controlled Trial Research Support, Non-U.S. Gov't].
87. Goetz M, Atreya R, Ghalibafian M, Galle PR, Neurath MF. Exacerbation of ulcerative colitis after rituximab salvage therapy. Inflamm Bowel Dis. 2007;13(11):1365–8 [Case Reports].
88. Dass S, Vital EM, Emery P. Development of psoriasis after B cell depletion with rituximab. Arthritis Rheum. 2007;56(8):2715–8 [Case Reports].
89. Westermann J, Pabst R. Lymphocyte subsets in the blood: a diagnostic window on the lymphoid system? Immunol Today. 1990;11(11):406–10 [Research Support, Non-U.S. Gov't Review].
90. McKay J, Chwalinska-Sadowska H, Boling E, Valente R, Limanni A, Racewicz A, et al. Belimumab (BmAb), a fully human monoclonal antibody to B-lymphocyte stimulator (BLyS), combined with standard of care therapy reduces the signs and symptoms of rheumatoid arthritis in a heterogenous subject population. Arthritis Rheum. 2005;52(9):S710–1.
91. Mackay F, Schneider P, Rennert P, Browning J. BAFF and APRIL: a tutorial on B cell survival. Annu Rev Immunol. 2003;21:231–64.
92. Ng LG, Sutherland APR, Newton R, Qian F, Cachero TG, Scott ML, et al. B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. J Immunol. 2004;173(2):807–17.
93. Carbonatto M, Yu P, Bertolino M, Vigna E, Steidler S, Fava L, et al. Nonclinical safety, pharmacokinetics, and pharmacodynamics of atacicept. Toxicol Sci. 2008;105(1):200–10 [Research Support, Non-U.S. Gov't].
94. Tak PP, Thurlings RM, Dimic A, Mircetic V, Rischmueller M, Nasanov E, et al. A phase Ib study to investigate atacicept (TACI-Ig) in patients with rheumatoid arthritis. Ann Rheum Dis. 2007;66:A10.
95. Fleischmann R, Wei N, Shaw M, Birbara C, Anand B, Gujrathi S, et al. BR3-Fc phase I study: safety, pharmacokinetics (PK) and pharmacodynamic (PD) effects of a novel BR3-Fc fusion protein in patients with rheumatoid arthritis. Arthritis Rheum. 2006;54(9):S229–30.
96. Desjarlais JR, Lazar GA, Zhukovsky EA, Chu SY. Optimizing engagement of the immune system by anti-tumor antibodies: an engineer's perspective. Drug Discov Today. 2007;12(21–22):898–910 [Review].
97. Snir A, Kessel A, Haj T, Rosner I, Slobodin G, Toubi E. Anti-IL-6 receptor antibody (tocilizumab): a B cell targeting therapy. Clin Exp Rheumatol. 2011;29(4):697–700.
98. Toubi E, Nussbaum S, Staun-Ram E, Snir A, Melamed D, Hayardeny L, et al. Laquinimod modulates B cells and their regulatory effects on T cells in multiple sclerosis. J Neuroimmunol. 2012;251(1–2):45–54.

99. Vadasz Z, Haj T, Kessel A, Toubi E. B-regulatory cells in autoimmunity and immune mediated inflammation. FEBS Lett. 2013;587(13):2074–8.
100. Tennakoon DK, Mehta RS, Ortega SB, Bhoj V, Racke MK, Karandikar NJ. Therapeutic induction of regulatory, cytotoxic CD8+ T cells in multiple sclerosis. J Immunol. 2006; 176(11):7119–29.
101. Lalive PH, Neuhaus O, Benkhoucha M, Burger D, Hohlfeld R, Zamvil SS, et al. Glatiramer acetate in the treatment of multiple sclerosis: emerging concepts regarding its mechanism of action. CNS Drugs. 2011;25(5):401–14.
102. Putheti P, Soderstrom M, Link H, Huang YM. Effect of glatiramer acetate (Copaxone) on CD4+CD25high T regulatory cells and their IL-10 production in multiple sclerosis. J Neuroimmunol. 2003;144(1–2):125–31.
103. Kala M, Miravalle A, Vollmer T. Recent insights into the mechanism of action of glatiramer acetate. J Neuroimmunol. 2011;235(1–2):9–17.
104. Towfighi A, Saver JL. Stroke declines from third to fourth leading cause of death in the United States historical perspective and challenges ahead. Stroke. 2011;42(8):2351–5.
105. Donnan GA, Fisher M, Macleod M, Davis SM. Stroke. Lancet. 2008;371(9624):1612–23.
106. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, et al. Executive summary: heart disease and stroke statistics-2014 update a report from the American Heart Association. Circulation. 2014;129(3):399–410.
107. Schroeter M, Jander S, Witte OW, Stoll G. Local immune-responses in the rat cerebral-cortex after middle cerebral-artery occlusion. J Neuroimmunol. 1994;55(2):195–203.
108. del Zoppo GJ, Becker KJ, Hallenbeck JM. Inflammation after stroke—is it harmful? Arch Neurol. 2001;58(4):669–72.
109. Lagrange P, Romero IA, Minn A, Revest PA. Transendothelial permeability changes induced by free radicals in an in vitro model of the blood–brain barrier. Free Radic Biol Med. 1999;27(5–6):667–72.
110. Heo JH, Han SW, Lee SK. Free radicals as triggers of brain edema formation after stroke. Free Radic Biol Med. 2005;39(1):51–70.
111. Aktas O, Ullrich O, Infante-Duarte C, Nitsch R, Zipp F. Neuronal damage in brain inflammation. Arch Neurol. 2007;64(2):185–9 [Research Support, Non-U.S. Gov't Review].
112. Jin R, Yang GJ, Li GH. Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. J Leukoc Biol. 2010;87(5):779–89.
113. Kim JY, Kawabori M, Yenari MA. Innate inflammatory responses in stroke: mechanisms and potential therapeutic targets. Curr Med Chem. 2014;21(18):2076–97.
114. Konsman JP, Drukarch B, Van Dam AM. (Peri)vascular production and action of pro-inflammatory cytokines in brain pathology. Clin Sci. 2007;112(1):1–25 [Review].
115. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. Nat Med. 2011;17(7):796–808 [Research Support, N.I.H., Extramural Review].
116. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. Immunity. 2005;23(4):344–6 [Comment Research Support, Non-U.S. Gov't Review].
117. Yilmaz G, Granger DN. Leukocyte recruitment and ischemic brain injury. Neuromol Med. 2010;12(2):193–204 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
118. Liesz A, Dalpke A, Mracsko E, Antoine DJ, Roth S, Zhou W, et al. DAMP signaling is a key pathway inducing immune modulation after brain injury. J Neurosci. 2015;35(2):583–98.
119. Hallenbeck J. Adaptive immunity—introduction. Stroke. 2007;38(2):768–9.
120. Schwartz M, Kipnis J. Autoimmunity on alert: naturally occurring regulatory CD4(+) CD25(+) T cells as part of the evolutionary compromise between a 'need' and a 'risk'. Trends Immunol. 2002;23(11):530–4.
121. Gee JM, Kalil A, Shea C, Becker KJ. Lymphocytes—potential mediators of postischemic injury and neuroprotection. Stroke. 2007;38(2):783–8.
122. Planas AM, Gomez-Choco M, Urra X, Gorina R, Caballero M, Chamorro A. Brain-derived antigens in lymphoid tissue of patients with acute stroke. J Immunol. 2012;188(5):2156–63 [Research Support, Non-U.S. Gov't].

123. Becker KJ, McCarron RM, Ruetzler C, Laban O, Sternberg E, Flanders KC, et al. Immunologic tolerance to myelin basic protein decreases stroke size after transient focal cerebral ischemia. Proc Natl Acad Sci U S A. 1997;94(20):10873–8.
124. Bodhankar S, Chen Y, Vandenbark AA, Murphy SJ, Offner H. IL-10-producing B-cells limit CNS inflammation and infarct volume in experimental stroke. Metab Brain Dis. 2013;28(3):375–86 [Research Support, N.I.H., Extramural Research Support, U.S. Gov't, Non-P.H.S.].
125. Chavele KM, Ehrenstein MR. Regulatory T-cells in systemic lupus erythematosus and rheumatoid arthritis. FEBS Lett. 2011;585(23):3603–10 [Review].
126. Kitamura D, Roes J, Kuhn R, Rajewsky K. A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. Nature. 1991;350(6317):423–6 [Research Support, Non-U.S. Gov't].
127. Ren XF, Akiyoshi K, Dziennis S, Vandenbark AA, Herson PS, Hurn PD, et al. Regulatory B cells limit CNS inflammation and neurologic deficits in murine experimental stroke. J Neurosci. 2011;31(23):8556–63.
128. Bodhankar S, Chen Y, Vandenbark AA, Murphy SJ, Offner H. Treatment of experimental stroke with IL-10-producing B-cells reduces infarct size and peripheral and CNS inflammation in wild-type B-cell-sufficient mice. Metab Brain Dis. 2014;29(1):59–73 [Evaluation Studies Research Support, N.I.H., Extramural Research Support, U.S. Gov't, Non-P.H.S.].
129. Ibarra A, Avendano H, Cruz Y. Copolymer-1 (Cop-1) improves neurological recovery after middle cerebral artery occlusion in rats. Neurosci Lett. 2007;425(2):110–3.
130. Kraft P, Gobel K, Meuth SG, Kleinschnitz C. Glatiramer acetate does not protect from acute ischemic stroke in mice. Exp Transl Stroke Med. 2014;6(1):4.
131. Poittevin M, Deroide N, Azibani F, Delcayre C, Giannesini C, Levy BI, et al. Glatiramer Acetate administration does not reduce damage after cerebral ischemia in mice. J Neuroimmunol. 2013;254(1–2):55–62.
132. McColl BW, Rothwell NJ, Allan SM. Systemic inflammatory stimulus potentiates the acute phase and CXC chemokine responses to experimental stroke and exacerbates brain damage via interleukin-1- and neutrophil-dependent mechanisms. J Neurosci. 2007;27(16):4403–12.
133. Elkind MS, Cheng J, Rundek T, Boden-Albala B, Sacco RL. Leukocyte count predicts outcome after ischemic stroke: the Northern Manhattan Stroke Study. J Stroke Cerebrovasc Dis. 2004;13(5):220–7.
134. Meisel C, Schwab JM, Prass K, Meisel A, Dirnag U. Central nervous system injury-induced immune deficiency syndrome. Nat Rev Neurosci. 2005;6(10):775–86.
135. Denes A, McColl BW, Leow-Dyke SF, Chapman KZ, Humphreys NE, Grencis RK, et al. Experimental stroke-induced changes in the bone marrow reveal complex regulation of leukocyte responses. J Cereb Blood Flow Metab. 2011;31(4):1036–50.
136. Jickling GC, Xu HC, Stamova B, Ander BP, Zhan XH, Tian YF, et al. Signatures of cardioembolic and large-vessel ischemic stroke. Ann Neurol. 2010;68(5):681–92.
137. Harms H, Reimnitz P, Bohner G, Werich T, Klingebiel R, Meisel C, et al. Influence of stroke localization on autonomic activation, immunodepression, and post-stroke infection. Cerebrovasc Dis. 2011;32(6):552–60 [Clinical Trial, Phase II, Randomized Controlled Trial Research Support, Non-U.S. Gov't].
138. Chamorro A, Amaro S, Vargas M, Obach V, Cervera A, Torres F, et al. Interleukin 10, monocytes and increased risk of early infection in ischaemic stroke. J Neurol Neurosurg Psychiatry. 2006;77(11):1279–81 [Research Support, Non-U.S. Gov't].
139. Waje-Andreassen U, Krakenes J, Ulvestad E, Thomassen L, Myhr KM, Aarseth J, et al. IL-6: an early marker for outcome in acute ischemic stroke. Acta Neurol Scand. 2005;111(6):360–5 [Research Support, Non-U.S. Gov't].
140. Liesz A, Hagmann S, Zschoche C, Adamek J, Zhou W, Sun L, et al. The spectrum of systemic immune alterations after murine focal ischemia: immunodepression versus immunomodulation. Stroke. 2009;40(8):2849–58 [Comparative Study Research Support, Non-U.S. Gov't].

141. Kohm AP, Sanders VM. Norepinephrine and beta 2-adrenergic receptor stimulation regulate CD4+ T and B lymphocyte function in vitro and in vivo. Pharmacol Rev. 2001;53(4): 487–525.
142. Mracsko E, Liesz A, Karcher S, Zorn M, Bari F, Veltkamp R. Differential effects of sympathetic nervous system and hypothalamic-pituitary–adrenal axis on systemic immune cells after severe experimental stroke. Brain Behav Immun. 2014;41:200–9.
143. Elenkov IJ, Chrousos GP. Stress system—organization, physiology and immunoregulation. Neuroimmunomodulation. 2006;13(5–6):257–67 [Research Support, Non-U.S. Gov't Review].
144. Barer DH, Cruickshank JM, Ebrahim SB, Mitchell JR. Low dose beta blockade in acute stroke ("BEST" trial): an evaluation. Br Med J (Clin Res Ed). 1988;296(6624):737–41.
145. Phelan C, Alaigh V, Fortunato G, Staff I, Sansing L. Effect of beta-adrenergic antagonists on in-hospital mortality after ischemic stroke. J Stroke Cerebrovasc Dis. 2015;8:1998–2004.
146. Maier IL, Karch A, Mikolajczyk R, Bahr M, Liman J. Effect of beta-blocker therapy on the risk of infections and death after acute stroke—a historical cohort study. PLoS One. 2015;10(2):e0116836.
147. Wolfe CDA. The impact of stroke. Br Med Bull. 2000;56(2):275–86.
148. Silver J, Miller JH. Regeneration beyond the glial scar. Nat Rev Neurosci. 2004;5(2): 146–56.
149. Stroemer RP, Kent TA, Hulsebosch CE. Neocortical neural sprouting, synaptogenesis, and behavioral recovery after neocortical infarction in rats. Stroke. 1995;26(11):2135–44.
150. Carmichael ST, Archibeque I, Luke L, Nolan T, Momiy J, Li SL. Growth-associated gene expression after stroke: evidence for a growth-promoting region in peri-infarct cortex. Exp Neurol. 2005;193(2):291–311.
151. Li Y, Jiang N, Powers C, Chopp M. Neuronal damage and plasticity identified by microtubule-associated protein 2, growth-associated protein 43, and cyclin D1 immunoreactivity after focal cerebral ischemia in rats. Stroke. 1998;29(9):1972–80.
152. Lu AG, Tang Y, Ran RQ, Clark JF, Aronow BJ, Sharp FR. Genomics of the periinfarction cortex after focal cerebral ischemia. J Cereb Blood Flow Metab. 2003;23(7):786–810.
153. De Winter F, Oudega M, Lankhorst AJ, Hamers FP, Blits B, Ruitenberg MJ, et al. Injury-induced class 3 semaphorin expression in the rat spinal cord. Exp Neurol. 2002;175(1): 61–75.
154. Bundesen LQ, Scheel TA, Bregman BS, Kromer LF. Ephrin-B2 and EphB2 regulation of astrocyte-meningeal fibroblast interactions in response to spinal cord lesions in adult rats. J Neurosci. 2003;23(21):7789–800.
155. Hunt D, Coffin RS, Prinjha RK, Campbell G, Anderson PN. Nogo-A expression in the intact and injured nervous system. Mol Cell Neurosci. 2003;24(4):1083–102.
156. Jones LL, Margolis RU, Tuszynski MH. The chondroitin sulfate proteoglycans neurocan, brevican, phosphacan, and versican are differentially regulated following spinal cord injury. Exp Neurol. 2003;182(2):399–411.
157. Benson MD, Romero MI, Lush ME, Lu QR, Henkemeyer M, Parada LF. Ephrin-B3 is a myelin-based inhibitor of neurite outgrowth. Proc Natl Acad Sci U S A. 2005;102(30): 10694–9.
158. Morgenstern DA, Asher RA, Fawcett JW. Chondroitin sulphate proteoglycans in the CNS injury response. Prog Brain Res. 2002;137:313–32.
159. Napieralski JA, Butler AK, Chesselet MF. Anatomical and functional evidence for lesion-specific sprouting of corticostriatal input in the adult rat. J Comp Neurol. 1996;373(4): 484–97.
160. Carmichael ST, Wei L, Rovainen CM, Woolsey TA. New patterns of intracortical projections after focal cortical stroke. Neurobiol Dis. 2001;8(5):910–22 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].
161. Dancause N, Barbay S, Frost SB, Plautz EJ, Chen D, Zoubina EV, et al. Extensive cortical rewiring after brain injury. J Neurosci. 2005;25(44):10167–79 [Comparative Study Research

Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].
162. Carmichael ST, Chesselet MF. Synchronous neuronal activity is a signal for axonal sprouting after cortical lesions in the adult. J Neurosci. 2002;22(14):6062–70.
163. Ishii H, Kubo T, Kumanogoh A, Yamashita T. Th1 cells promote neurite outgrowth from cortical neurons via a mechanism dependent on semaphorins. Biochem Biophys Res Commun. 2010;402(1):168–72.
164. Wang HJ, Hu JG, Shen L, Wang R, Wang QY, Zhang C, et al. Passive immunization with myelin basic protein activated T cells suppresses axonal dieback but does not promote axonal regeneration following spinal cord hemisection in adult rats. Int J Neurosci. 2012;122(8): 458–65.
165. Al-Izki S, Pryce G, Hankey DJ, Lidster K, von Kutzleben SM, Browne L, et al. Lesional-targeting of neuroprotection to the inflammatory penumbra in experimental multiple sclerosis. Brain. 2014;137(Pt 1):92–108.
166. Reick C, Ellrichmann G, Thone J, Scannevin RH, Saft C, Linker RA, et al. Neuroprotective dimethyl fumarate synergizes with immunomodulatory interferon beta to provide enhanced axon protection in autoimmune neuroinflammation. Exp Neurol. 2014;257:50–6.
167. Tanabe S, Yamashita T. Repulsive guidance molecule-a is involved in Th17-cell-induced neurodegeneration in autoimmune encephalomyelitis. Cell Rep. 2014;9(4):1459–70.
168. Yu D, Cook MC, Shin DM, Silva DG, Marshall J, Toellner KM, et al. Axon growth and guidance genes identify T-dependent germinal centre B cells. Immunol Cell Biol. 2008;86(1): 3–14.
169. Zhang Y, Da RR, Guo W, Ren HM, Hilgenberg LG, Sobel RA, et al. Axon reactive B cells clonally expanded in the cerebrospinal fluid of patients with multiple sclerosis. J Clin Immunol. 2005;25(3):254–64.
170. Ankeny DP, Popovich PG. B cells and autoantibodies: complex roles in CNS injury. Trends Immunol. 2010;31(9):332–8.
171. Ankeny DP, Lucin KM, Sanders VM, McGaughy VM, Popovich PG. Spinal cord injury triggers systemic autoimmunity: evidence for chronic B lymphocyte activation and lupus-like autoantibody synthesis. J Neurochem. 2006;99(4):1073–87.
172. Fontoura P, Ho PP, DeVoss J, Zheng B, Lee BJ, Kidd BA, et al. Immunity to the extracellular domain of Nogo-A modulates experimental autoimmune encephalomyelitis. J Immunol. 2004;173(11):6981–92 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].
173. Vincent FB, Morand EF, Schneider P, Mackay F. The BAFF/APRIL system in SLE pathogenesis. Nat Rev Rheumatol. 2014;10(6):365–73 [Review].
174. Edling AE, Nanavati T, Johnson JM, Tuohy VK. Human and murine lymphocyte neurotrophin expression is confined to B cells. J Neurosci Res. 2004;77(5):709–17.
175. Fauchais AL, Lalloue F, Lise MC, Boumediene A, Preud'homme JL, Vidal E, et al. Role of endogenous brain-derived neurotrophic factor and sortilin in B cell survival. J Immunol. 2008;181(5):3027–38.
176. Begum-Haque S, Christy M, Ochoa-Reparaz J, Nowak EC, Mielcarz D, Hague A, et al. Augmentation of regulatory B cell activity in experimental allergic encephalomyelitis by glatiramer acetate. J Neuroimmunol. 2011;232(1–2):136–44.
177. Barba R, Martinez-Espinosa S, Rodriguez-Garcia E, Pondal M, Vivancos J, Del Ser T. Poststroke dementia: clinical features and risk factors. Stroke. 2000;31(7):1494–501.
178. Leys D, Henon H, Mackowiak-Cordoliani MA, Pasquier F. Poststroke dementia. Lancet Neurol. 2005;4(11):752–9.
179. Bejot Y, Aboa-Eboule C, Durier J, Rouaud O, Jacquin A, Ponavoy E, et al. Prevalence of early dementia after first-ever stroke: a 24-year population-based study. Stroke. 2011;42(3): 607–12.
180. Savva GM, Stephan BC. Alzheimer's Society Vascular Dementia Systematic Review G. Epidemiological studies of the effect of stroke on incident dementia: a systematic review. Stroke. 2010;41(1):e41–6.

181. Offner H, Hurn PD. A novel hypothesis: regulatory B lymphocytes shape outcome from experimental stroke. Transl Stroke Res. 2012;3(3):324–30.
182. Gidday JM. Pharmacologic preconditioning: translating the promise. Transl Stroke Res. 2010;1(1):19–30.
183. Gidday JM. Cerebral preconditioning and ischaemic tolerance. Nat Rev Neurosci. 2006;7(6):437–48.
184. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation. 1986;74(5):1124–36. [Research Support, U.S. Gov't, P.H.S.
185. Serafin A, Fernandez-Zabalegui L, Prats N, Wu ZY, Rosello-Catafau J, Peralta C. Ischemic preconditioning: tolerance to hepatic ischemia-reperfusion injury. Histol Histopathol. 2004;19(1):281–9 [Research Support, Non-U.S. Gov't Review].
186. Yoon YE, Lee KS, Choi KH, Kim KH, Yang SC, Han WK. Preconditioning strategies for kidney ischemia reperfusion injury: implications of the "time-window" in remote ischemic preconditioning. PLoS One. 2015;10(4):e0124130 [Research Support, Non-U.S. Gov't].
187. Erling Junior N, Montero EF, Sannomiya P, Poli-de-Figueiredo LF. Local and remote ischemic preconditioning protect against intestinal ischemic/reperfusion injury after supraceliac aortic clamping. Clinics. 2013;68(12):1548–54 [Research Support, Non-U.S. Gov't].
188. Kocman EA, Ozatik O, Sahin A, Guney T, Kose AA, Dag I, et al. Effects of ischemic preconditioning protocols on skeletal muscle ischemia-reperfusion injury. J Surg Res. 2015; 193(2):942–52 [Comparative Study Evaluation Studies Research Support, Non-U.S. Gov't].
189. Roth S, Li B, Rosenbaum PS, Gupta H, Goldstein IM, Maxwell KM, et al. Preconditioning provides complete protection against retinal ischemic injury in rats. Invest Ophthalmol Vis Sci. 1998;39(5):777–85 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].
190. Poinsatte K, Selvaraj UM, Ortega SB, Plautz EJ, Kong X, Gidday JM, et al. Quantification of neurovascular protection following repetitive hypoxic preconditioning and transient middle cerebral artery occlusion in mice. J Vis Exp. 2015;99:e52675.
191. Hu X, Lu Y, Zhang Y, Li Y, Jiang L. Remote ischemic preconditioning improves spatial learning and memory ability after focal cerebral ischemia-reperfusion in rats. Perfusion. 2013;28(6):546–51.
192. Stetler RA, Leak RK, Gan Y, Li P, Zhang F, Hu X, et al. Preconditioning provides neuroprotection in models of CNS disease: paradigms and clinical significance. Prog Neurobiol. 2014;114:58–83 [Research Suppo[Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S. Review].
193. Dirnagl U, Becker K, Meisel A. Preconditioning and tolerance against cerebral ischaemia: from experimental strategies to clinical use. Lancet Neurol. 2009;8(4):398–412 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review].
194. Kariko K, Weissman D, Welsh FA. Inhibition of toll-like receptor and cytokine signaling—a unifying theme in ischemic tolerance. J Cereb Blood Flow Metab. 2004;24(11):1288–304 [Research Support, U.S. Gov't, P.H.S. Review].
195. Garcia-Bonilla L, Benakis C, Moore J, Iadecola C, Anrather J. Immune mechanisms in cerebral ischemic tolerance. Front Neurosci. 2014;8:44 [Review].
196. Liu XQ, Sheng R, Qin ZH. The neuroprotective mechanism of brain ischemic preconditioning. Acta Pharmacol Sin. 2009;30(8):1071–80 [Research Support, Non-U.S. Gov't Review].
197. Marsh BJ, Williams-Karnesky RL, Stenzel-Poore MP. Toll-like receptor signaling in endogenous neuroprotection and stroke. Neuroscience. 2009;158(3):1007–20 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review].
198. Stowe AM, Wacker BK, Cravens PD, Perfater JL, Li MK, Hu R, et al. CCL2 upregulation triggers hypoxic preconditioning-induced protection from stroke. J Neuroinflammation. 2012;9:33 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
199. Curry A, Guo M, Patel R, Liebelt B, Sprague S, Lai Q, et al. Exercise pre-conditioning reduces brain inflammation in stroke via tumor necrosis factor-alpha, extracellular signal-regulated

kinase 1/2 and matrix metalloproteinase-9 activity. Neurol Res. 2010;32(7):756–62 [Research Support, Non-U.S. Gov't].
200. Endres M, Fan G, Hirt L, Fujii M, Matsushita K, Liu X, et al. Ischemic brain damage in mice after selectively modifying BDNF or NT4 gene expression. J Cereb Blood Flow Metab. 2000;20(1):139–44 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].
201. Chamorro A, Meisel A, Planas AM, Urra X, van de Beek D, Veltkamp R. The immunology of acute stroke. Nat Rev Neurol. 2012;8(7):401–10 [Research Support, Non-U.S. Gov't Review].
202. Prass K, Scharff A, Ruscher K, Lowl D, Muselmann C, Victorov I, et al. Hypoxia-induced stroke tolerance in the mouse is mediated by erythropoietin. Stroke. 2003;34(8):1981–6 [Research Support, Non-U.S. Gov't].
203. Wacker BK, Park TS, Gidday JM. Hypoxic preconditioning-induced cerebral ischemic tolerance: role of microvascular sphingosine kinase 2. Stroke. 2009;40(10):3342–8 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].
204. Bernaudin M, Nedelec AS, Divoux D, MacKenzie ET, Petit E, Schumann-Bard P. Normobaric hypoxia induces tolerance to focal permanent cerebral ischemia in association with an increased expression of hypoxia-inducible factor-1 and its target genes, erythropoietin and VEGF, in the adult mouse brain. J Cereb Blood Flow Metab. 2002;22(4):393–403.
205. Gao CJ, Niu L, Ren PC, Wang W, Zhu C, Li YQ, et al. Hypoxic preconditioning attenuates global cerebral ischemic injury following asphyxial cardiac arrest through regulation of delta opioid receptor system. Neuroscience. 2012;202:352–62 [Research Support, Non-U.S. Gov't].
206. Zhang J, Qian H, Zhao P, Hong SS, Xia Y. Rapid hypoxia preconditioning protects cortical neurons from glutamate toxicity through delta-opioid receptor. Stroke. 2006;37(4):1094–9 [Research Support, N.I.H., Extramural].
207. Fan YY, Hu WW, Dai HB, Zhang JX, Zhang LY, He P, et al. Activation of the central histaminergic system is involved in hypoxia-induced stroke tolerance in adult mice. J Cereb Blood Flow Metab. 2011;31(1):305–14 [Research Support, Non-U.S. Gov't].
208. Yu S, Zhao T, Guo M, Fang H, Ma J, Ding A, et al. Hypoxic preconditioning up-regulates glucose transport activity and glucose transporter (GLUT1 and GLUT3) gene expression after acute anoxic exposure in the cultured rat hippocampal neurons and astrocytes. Brain Res. 2008;1211:22–9 [Research Support, Non-U.S. Gov't].
209. Arthur PG, Lim SC, Meloni BP, Munns SE, Chan A, Knuckey NW. The protective effect of hypoxic preconditioning on cortical neuronal cultures is associated with increases in the activity of several antioxidant enzymes. Brain Res. 2004;1017(1–2):146–54 [Comparative Study Research Support, Non-U.S. Gov't].
210. Bickler PE, Fahlman CS, Gray J, McKleroy W. Inositol 1,4,5-triphosphate receptors and NAD(P)H mediate Ca2+ signaling required for hypoxic preconditioning of hippocampal neurons. Neuroscience. 2009;160(1):51–60 [Research Support, N.I.H., Extramural].
211. Liu J, Narasimhan P, Yu F, Chan PH. Neuroprotection by hypoxic preconditioning involves oxidative stress-mediated expression of hypoxia-inducible factor and erythropoietin. Stroke. 2005;36(6):1264–9 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].
212. Bu X, Zhang N, Yang X, Liu Y, Du J, Liang J, et al. Proteomic analysis of cPKCbetaII-interacting proteins involved in HPC-induced neuroprotection against cerebral ischemia of mice. J Neurochem. 2011;117(2):346–56 [Research Support, Non-U.S. Gov't].
213. Zhang N, Yin Y, Han S, Jiang J, Yang W, Bu X, et al. Hypoxic preconditioning induced neuroprotection against cerebral ischemic injuries and its cPKCgamma-mediated molecular mechanism. Neurochem Int. 2011;58(6):684–92 [Research Support, Non-U.S. Gov't].
214. Lin AM, Dung SW, Chen CF, Chen WH, Ho LT. Hypoxic preconditioning prevents cortical infarction by transient focal ischemia-reperfusion. Ann N Y Acad Sci. 2003;993:168–78 [discussion 95-6], [Research Support, Non-U.S. Gov't].
215. Stowe AM, Altay T, Freie AB, Gidday JM. Repetitive hypoxia extends endogenous neurovascular protection for stroke. Ann Neurol. 2011;69(6):975–85 [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't].

216. Monson NL, Ortega SB, Ireland SJ, Meeuwissen AJ, Chen D, Plautz EJ, et al. Repetitive hypoxic preconditioning induces an immunosuppressed B cell phenotype during endogenous protection from stroke. J Neuroinflammation. 2014;11:22.
217. Cepok S, Jacobsen M, Schock S, Omer B, Jaekel S, Boddeker I, et al. Patterns of cerebrospinal fluid pathology correlate with disease progression in multiple sclerosis. Brain. 2001;124(Pt 11):2169–76 [Comparative Study Research Support, Non-U.S. Gov't].
218. Tadmor T, Bari A, Sacchi S, Marcheselli L, Liardo EV, Avivi I, et al. Monocyte count at diagnosis is a prognostic parameter in diffuse large B-cell lymphoma: results from a large multicenter study involving 1191 patients in the pre- and post-rituximab era. Haematologica. 2014;99(1):125–30 [Multicenter Study Research Support, Non-U.S. Gov't].
219. Nizet V, Johnson RS. Interdependence of hypoxic and innate immune responses. Nat Rev Immunol. 2009;9(9):609–17 [Research Support, N.I.H., Extramural Research Support, Non--U.S. Gov't Review].
220. Kitaev MI, Tokhtabaev AG. T-and B-lymphocytes in adaptation to high altitude. Kosm Biol Aviakosm Med. 1981;15(3):87–9.
221. Serebrovskaya TV, Nikolsky IS, Nikolska VV, Mallet RT, Ishchuk VA. Intermittent hypoxia mobilizes hematopoietic progenitors and augments cellular and humoral elements of innate immunity in adult men. High Alt Med Biol. 2011;12(3):243–52 [Research Support, Non-U.S. Gov't].
222. Faeh D, Gutzwiller F, Bopp M. Swiss National Cohort Study G. Lower mortality from coronary heart disease and stroke at higher altitudes in Switzerland. Circulation. 2009;120(6):495–501 [Research Support, Non-U.S. Gov't].
223. Egan KJ, Janssen H, Sena ES, Longley L, Speare S, Howells DW, et al. Exercise reduces infarct volume and facilitates neurobehavioral recovery: results from a systematic review and meta-analysis of exercise in experimental models of focal ischemia. Neurorehabil Neural Repair. 2014;28(8):800–12 [Meta-Analysis Research Support, Non-U.S. Gov't Review].
224. Noble EG, Moraska A, Mazzeo RS, Roth DA, Olsson MC, Moore RL, et al. Differential expression of stress proteins in rat myocardium after free wheel or treadmill run training. J Appl Physiol. 1999;86(5):1696–701 [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].
225. Ke Z, Yip SP, Li L, Zheng XX, Tong KY. The effects of voluntary, involuntary, and forced exercises on brain-derived neurotrophic factor and motor function recovery: a rat brain ischemia model. PLoS One. 2011;6(2):e16643 [Research Support, Non-U.S. Gov't].
226. Hayes K, Sprague S, Guo M, Davis W, Friedman A, Kumar A, et al. Forced, not voluntary, exercise effectively induces neuroprotection in stroke. Acta Neuropathol. 2008;115(3):289–96 [Research Support, Non-U.S. Gov't].
227. Stevenson JR, Kreiling JA, Taylor R. Effects of corticosterone on responses of murine splenic B and T cells to phytohemagglutinin, concanavalin A, and lipopolysaccharide. Immunol Invest. 1989;18(8):951–60.
228. Kim SE, Ko IG, Kim BK, Shin MS, Cho S, Kim CJ, et al. Treadmill exercise prevents aging-induced failure of memory through an increase in neurogenesis and suppression of apoptosis in rat hippocampus. Exp Gerontol. 2010;45(5):357–65 [Research Support, Non-U.S. Gov't].
229. Leasure JL, Jones M. Forced and voluntary exercise differentially affect brain and behavior. Neuroscience. 2008;156(3):456–65 [Research Support, Non-U.S. Gov't].
230. van Praag H, Shubert T, Zhao C, Gage FH. Exercise enhances learning and hippocampal neurogenesis in aged mice. J Neurosci. 2005;25(38):8680–5 [Comparative Study Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.].
231. Ding Y, Li J, Luan X, Ding YH, Lai Q, Rafols JA, et al. Exercise pre-conditioning reduces brain damage in ischemic rats that may be associated with regional angiogenesis and cellular overexpression of neurotrophin. Neuroscience. 2004;124(3):583–91 [Research Support, Non-U.S. Gov't].
232. Zhang F, Wu Y, Jia J. Exercise preconditioning and brain ischemic tolerance. Neuroscience. 2011;177:170–6 [Research Support, Non-U.S. Gov't Review].

233. Kinni H, Guo M, Ding JY, Konakondla S, Dornbos 3rd D, Tran R, et al. Cerebral metabolism after forced or voluntary physical exercise. Brain Res. 2011;1388:48–55.
234. Ding YH, Ding Y, Li J, Bessert DA, Rafols JA. Exercise pre-conditioning strengthens brain microvascular integrity in a rat stroke model. Neurol Res. 2006;28(2):184–9 [Research Support, Non-U.S. Gov't].
235. Ding YH, Li J, Yao WX, Rafols JA, Clark JC, Ding Y. Exercise preconditioning upregulates cerebral integrins and enhances cerebrovascular integrity in ischemic rats. Acta Neuropathol. 2006;112(1):74–84 [Research Support, Non-U.S. Gov't].
236. Liebelt B, Papapetrou P, Ali A, Guo M, Ji X, Peng C, et al. Exercise preconditioning reduces neuronal apoptosis in stroke by up-regulating heat shock protein-70 (heat shock protein-72) and extracellular-signal-regulated-kinase 1/2. Neuroscience. 2010;166(4):1091–100 [Research Support, Non-U.S. Gov't].
237. Chaudhry K, Rogers R, Guo M, Lai Q, Goel G, Liebelt B, et al. Matrix metalloproteinase-9 (MMP-9) expression and extracellular signal-regulated kinase 1 and 2 (ERK1/2) activation in exercise-reduced neuronal apoptosis after stroke. Neurosci Lett. 2010;474(2):109–14.
238. Ding YH, Young CN, Luan X, Li J, Rafols JA, Clark JC, et al. Exercise preconditioning ameliorates inflammatory injury in ischemic rats during reperfusion. Acta Neuropathol. 2005;109(3):237–46 [Comparative Study Research Support, Non-U.S. Gov't].
239. Ding YH, Mrizek M, Lai Q, Wu Y, Reyes Jr R, Li J, et al. Exercise preconditioning reduces brain damage and inhibits TNF-alpha receptor expression after hypoxia/reoxygenation: an in vivo and in vitro study. Curr Neurovasc Res. 2006;3(4):263–71 [Research Support, Non-U.S. Gov't].
240. Walsh NP, Gleeson M, Shephard RJ, Gleeson M, Woods JA, Bishop NC, et al. Position statement. Part one: immune function and exercise. Exerc Immunol Rev. 2011;17:6–63 [Review].
241. Elphick GF, Greenwood BN, Campisi J, Fleshner M. Increased serum nIgM in voluntarily physically active rats: a potential role for B-1 cells. J Appl Physiol. 2003;94(2):660–7 [Research Support, U.S. Gov't, P.H.S.].
242. Elphick GF, Wieseler-Frank J, Greenwood BN, Campisi J, Fleshner M. B-1 cell (CD5+/CD11b+) numbers and nIgM levels are elevated in physically active vs. sedentary rats. J Appl Physiol. 2003;95(1):199–206.
243. Douglass JH. The effects of physical tracing on the immunological response in mice. J Sports Med Phys Fitness. 1974;14(1):48–54.
244. Liu YG, Wang SY. The enhancing effect of exercise on the production of antibody to Salmonella typhi in mice. Immunol Lett. 1987;14(2):117–20 [Comparative Study].
245. Suzuki K, Tagami K. Voluntary wheel-running exercise enhances antigen-specific antibody-producing splenic B cell response and prolongs IgG half-life in the blood. Eur J Appl Physiol. 2005;94(5–6):514–9 [Research Support, Non-U.S. Gov't].
246. Alevizos A, Lentzas J, Kokkoris S, Mariolis A, Korantzopoulos P. Physical activity and stroke risk. Int J Clin Pract. 2005;59(8):922–30 [Review].
247. Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease. Nat Rev Immunol. 2011;11(9):607–15.
248. Diep L, Kwagyan J, Kurantsin-Mills J, Weir R, Jayam-Trouth A. Association of physical activity level and stroke outcomes in men and women: a meta-analysis. J Womens Health. 2010;19(10):1815–22 [Comparative Study Meta-Analysis Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review].
249. Lee CD, Folsom AR, Blair SN. Physical activity and stroke risk: a meta-analysis. Stroke. 2003;34(10):2475–81 [Meta-Analysis Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.].
250. Stroud N, Mazwi TM, Case LD, Brown Jr RD, Brott TG, Worrall BB, et al. Prestroke physical activity and early functional status after stroke. J Neurol Neurosurg Psychiatry. 2009;80(9):1019–22 [Multicenter Study Research Support, N.I.H., Extramural].
251. Pradillo JM, Fernandez-Lopez D, Garcia-Yebenes I, Sobrado M, Hurtado O, Moro MA, et al. Toll-like receptor 4 is involved in neuroprotection afforded by ischemic preconditioning. J Neurochem. 2009;109(1):287–94.

252. Leung PY, Stevens SL, Packard AE, Lessov NS, Yang T, Conrad VK, et al. Toll-like receptor 7 preconditioning induces robust neuroprotection against stroke by a novel type I interferon-mediated mechanism. Stroke. 2012;43(5):1383–9.
253. Tasaki K, Ruetzler CA, Ohtsuki T, Martin D, Nawashiro H, Hallenbeck JM. Lipopolysaccharide pre-treatment induces resistance against subsequent focal cerebral ischemic damage in spontaneously hypertensive rats. Brain Res. 1997;748(1–2):267–70.
254. Rosenzweig HL, Lessov NS, Henshall DC, Minami M, Simon RP, Stenzel-Poore MP. Endotoxin preconditioning prevents cellular inflammatory response during ischemic neuroprotection in mice. Stroke. 2004;35(11):2576–81.
255. Stevens SL, Ciesielski TM, Marsh BJ, Yang T, Homen DS, Boule JL, et al. Toll-like receptor 9: a new target of ischemic preconditioning in the brain. J Cereb Blood Flow Metab. 2008;28(5):1040–7.
256. Stevens SL, Vartanian KB, Stenzel-Poore MP. Reprogramming the response to stroke by preconditioning. Stroke. 2014;45(8):2527–31.
257. Rosenzweig HL, Minami M, Lessov NS, Coste SC, Stevens SL, Henshall DC, et al. Endotoxin preconditioning protects against the cytotoxic effects of TNFalpha after stroke: a novel role for TNFalpha in LPS-ischemic tolerance. J Cereb Blood Flow Metab. 2007;27(10):1663–74.
258. Lastres-Becker I, Cartmell T, Molina-Holgado F. Endotoxin preconditioning protects neurones from in vitro ischemia: role of endogenous IL-1beta and TNF-alpha. J Neuroimmunol. 2006;173(1–2):108–16.
259. Ahmed SH, He YY, Nassief A, Xu J, Xu XM, Hsu CY, et al. Effects of lipopolysaccharide priming on acute ischemic brain injury. Stroke. 2000;31(1):193–9.
260. Bastide M, Gele P, Petrault O, Pu Q, Caliez A, Robin E, et al. Delayed cerebrovascular protective effect of lipopolysaccharide in parallel to brain ischemic tolerance. J Cereb Blood Flow Metab. 2003;23(4):399–405.
261. Dawson DA, Furuya K, Gotoh J, Nakao Y, Hallenbeck JM. Cerebrovascular hemodynamics and ischemic tolerance: lipopolysaccharide-induced resistance to focal cerebral ischemia is not due to changes in severity of the initial ischemic insult, but is associated with preservation of microvascular perfusion. J Cereb Blood Flow Metab. 1999;19(6):616–23.
262. Stevens SL, Leung PY, Vartanian KB, Gopalan B, Yang T, Simon RP, et al. Multiple preconditioning paradigms converge on interferon regulatory factor-dependent signaling to promote tolerance to ischemic brain injury. J Neurosci. 2011;31(23):8456–63 [Research Support, N.I.H., Extramural].
263. Vartanian KB, Stevens SL, Marsh BJ, Williams-Karnesky R, Lessov NS, Stenzel-Poore MP. LPS preconditioning redirects TLR signaling following stroke: TRIF-IRF3 plays a seminal role in mediating tolerance to ischemic injury. J Neuroinflammation. 2011;8:140 [Research Support, N.I.H., Extramural].
264. Zimmermann C, Ginis I, Furuya K, Klimanis D, Ruetzler C, Spatz M, et al. Lipopolysaccharide-induced ischemic tolerance is associated with increased levels of ceramide in brain and in plasma. Brain Res. 2001;895(1–2):59–65.
265. Yao H, Ma R, Yang L, Hu G, Chen X, Duan M, et al. MiR-9 promotes microglial activation by targeting MCPIP1. Nat Commun. 2014;5:4386.
266. Lampropoulou V, Calderon-Gomez E, Roch T, Neves P, Shen P, Stervbo U, et al. Suppressive functions of activated B cells in autoimmune diseases reveal the dual roles of Toll-like receptors in immunity. Immunol Rev. 2010;233(1):146–61.
267. Ishikawa M, Vowinkel T, Stokes KY, Arumugam TV, Yilmaz G, Nanda A, et al. CD40/CD40 ligand signaling in mouse cerebral microvasculature after focal ischemia/reperfusion. Circulation. 2005;111(13):1690–6.
268. Garlichs CD, Kozina S, Fateh-Moghadam S, Handschu R, Tomandl B, Stumpf C, et al. Upregulation of CD40-CD40 ligand (CD154) in patients with acute cerebral ischemia. Stroke. 2003;34(6):1412–8.
269. Galluzzi L, Vacchelli E, Eggermont A, Fridman WH, Galon J, Sautes-Fridman C, et al. Trial Watch: experimental toll-like receptor agonists for cancer therapy. Oncoimmunology. 2012;1(5):699–716.

270. Bahjat FR, Williams-Karnesky RL, Kohama SG, West GA, Doyle KP, Spector MD, et al. Proof of concept: pharmacological preconditioning with a toll-like receptor agonist protects against cerebrovascular injury in a primate model of stroke. J Cereb Blood Flow Metab. 2011;31(5):1229–42.
271. Krieg AM. Therapeutic potential of toll-like receptor 9 activation. Nat Rev Drug Discov. 2006;5(6):471–84.
272. Lopez-Collazo E, del Fresno C. Pathophysiology of endotoxin tolerance: mechanisms and clinical consequences. Crit Care. 2013;17(6):242.
273. Liu, Jie, et al. Hypoxic preconditioning protects cultured neurons against hypoxic stress via TNF-α and ceramide. American Journal of Physiology-Cell Physiology 278.1 (2000): C144–C153.
274. Zwagerman, Nathan, et al. Toll-like receptor-4 and cytokine cascade in stroke after exercise. Neurological research 32.2 (2010): 123–126.
275. Guo, Miao, et al. Preischemic induction of TNF-α by physical exercise reduces blood–brain barrier dysfunction in stroke. Journal of Cerebral Blood Flow & Metabolism 28.8 (2008): 1422–1430.
276. Wacker, Bradley K., Jennifer L. Perfater, and Jeffrey M. Gidday. Hypoxic preconditioning induces stroke tolerance in mice via a cascading HIF, sphingosine kinase, and CCL2 signaling pathway. Journal of neurochemistry123.6 (2012): 954–962.

Mast Cell as an Early Responder in Ischemic Brain Injury

Perttu J. Lindsberg, Olli S. Mattila, and Daniel Strbian

Abbreviations

BBB	blood–brain barrier
eNOS	endothelial nitric oxide synthase
IL	interleukin
LPS	lipopolysaccharide
MC	mast cell
MMP	matrix metalloproteinase
MCAO	middle cerebral artery occlusion
NVU	neurovascular unit
PAF	platelet-activating factor
PAI-1	plasminogen activator inhibitor-1
PAR	proteinase activated receptor 2
SCF	stem cell factor
TIMP	tissue inhibitor of metalloproteinases
tPA	tissue plasminogen activator
TNFα	tumor necrosis factor-α
WPBs	Weibel–Palade bodies
VWF	von Willebrand factor

P.J. Lindsberg, M.D., Ph.D. (✉) • O.S. Mattila, M.D. • D. Strbian, M.D., Ph.D.
Neurology, Clinical Neurosciences, Helsinki University Hospital,
Haartmaninkatu 8, 00290 Helsinki, Finland

Molecular Neurology, Research Programs Unit, Biomedicum Helsinki,
University of Helsinki, Helsinki, Finland
e-mail: perttu.lindsberg@hus.fi

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_13

1 Introduction

Mast cells (MCs) are bone marrow derived, tissue-homing mononuclear white blood cells (leukocytes) that undergo differentiation upon entry into target organs. Committed bone marrow mast cell progenitors are released into the circulation from where they subsequently migrate into peripheral tissues, undergo maturation, and become terminally differentiated by the influence of cytokines within the surrounding milieu [1]. The progression of these cells to fully mature MCs is dependent on KIT activation which occurs as a consequence of stem cell factor (SCF)-induced KIT dimerization and autophosphorylation [1]. Central to this process is stem cell factor (SCF), the ligand for the c-kit tyrosine kinase receptor expressed on the surface of mast cells. KIT activation significantly regulates many aspects of MC differentiation, proliferation, activation, and survival [1, 2]. Mutant mice lacking either c-kit or SCF are MC deficient. MCs characteristically express a high-affinity receptor (FcεRI) for the Fc region of IgE, the least-abundant member of the antibodies. This receptor is of high affinity so that binding of IgE molecules is in essence irreversible.

MCs reside within or in the proximity to tissues that form a barrier to diverse noxious environmental challenges, i.e., in epithelium, mucosa, and the perivascular space. They are most commonly regarded as key effectors in the pathogenesis of allergic diseases and anaphylaxis [2]. However, the meticulous study of MC biology has established that they can generate or release various cytokines such as TNFα, potent proteases such as chymase and tryptase, vasoactive substances such as histamine and eicosanoids, and regulators of hemostasis such as heparin and tPA [3]. They also produce and release numerous interstitial matrix-degrading enzymes such as matrix metalloproteases (e.g., MMP-9). This armamentarium of mediators equips MCs to take part in a wide array of biological processes. This chapter describes recent evidence strongly supporting a role for MCs in the pathophysiology of cerebral ischemia.

2 Mast Cells in Health and Disease

MCs have gained a notorious reputation as mediators of anaphylaxis and allergic, atopic disorders. The spectrum of mast cell mediators, however, indicate a key role in diverse pathophysiological processes, such as chronic inflammatory processes, wound healing, angiogenesis, fibrosis, and tumors. Recent research has also demonstrated an important role in cardiac diseases, cardiac failure, cardiomyopathy, coronary syndromes, and atherosclerosis. Terminally differentiated, tissue-resident MCs are long lived [4], which coheres with a role in modulating local immunopathogenetic effects in chronic vascular disease. At least in the human the longevity of MCs is dependent upon the continued presence of SCF [1]. Clearly, MCs perform a critical role in protecting the host from a variety of environmental hazards [4, 5], but in the long-lived human, the cellular functions of MCs become involved in many

disorders with a strong autoimmune component. The role of MCs has been established in the pathophysiology of atopic dermatitis, psoriasis, asthma, and interstitial cystitis, and is highly probable in multiple sclerosis, coronary artery disease, irritable bowel disorder, and arthritis. The sphere of potential diseases where MCs and their mediators may play a role is, however, extensive and we must refer to other comprehensive reviews for more detailed scrutiny [6, 7].

3 Mechanisms of Action as an Inflammatory Cell

MCs are most efficiently activated via the classical IgE-mediated pathway but they can also be activated by a host of substances such as lipopolysaccharide, cytokines, hormones, immunoglobulins, neuropeptides (such as substance P), endothelin I, and activated complement components [8]. Functional activation of MCs leads to rapid degranulation of their mediators, many of which are preformed and stored in secretory granules. Piecemeal degranulation is an alternative form of mediator discharge permitting the selective secretion of certain mediators (and not the whole content) of the secretory granules. The preformed mediators include tryptase, histamine, serotonin (5-hydroxytryptamine), serine proteases, proteoglycans and cytokines such as tumor necrosis factor α (TNFα) and neutrophil chemokines CXCL1 and CXCL2 [9].

MC activation also leads to *de novo* synthesis of chemokines, cytokines, and eicosanoids that requires time for synthesis. For example, arachidonic acid metabolites (prostaglandins and leukotrienes), platelet activating factor (PAF), and several chemokines (e.g., CXCL1 and CXCL2) and cytokines (Il-1β and IL-6) can be synthesized *de novo*. The balance of engaging inhibitory and activatory cell-surface receptors on MCs determines whether the cell becomes active on encountering a challenge. Once activated, MC's response is further regulated by the balance of both positive and negative intracellular molecular events that extend well afar from the conventional role of kinases and phosphatases [10, 11]. MCs are equipped with particular antigen presenting capacity and phagocytosis of microbes and antigens [2, 12], although their main mode of immunologic function is to act as local effector cells and attract other immune cells to the site of inflammation.

4 Cerebrocranial Mast Cells

The presence and activity of MCs within the cerebrum, already recognized by Nobel-prize winning immunologist Paul Ehrlich in the late nineteenth century, has received little attention up until now. MCs are not easily visible in standard histopathological stainings such as hematoxylin-eosin. They can be clearly detected with a handful of specialized staining procedures, many of which utilize visualization of the abundant heparin sulfate contained in the cytoplasmic MC granules. These can be demonstrated by metachromatic dyes like toluidine blue (Fig. 1a), azures A

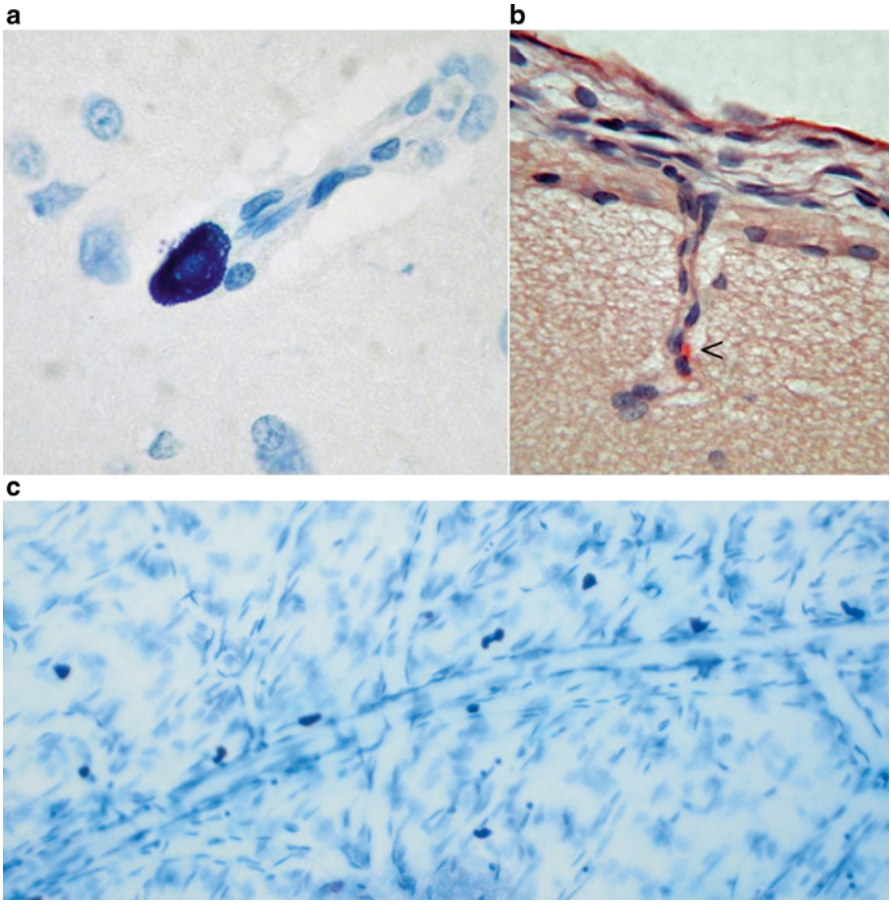

Fig. 1 Mast cells in cerebral and meningeal tissues. (**a**) Toluidine blue staining of a rat brain section demonstrating a densely packed granule-containing MC in association to a small blood vessel. A few solitary granules are also visible surrounding the outer cell membrane. (**b**) Demonstration of a rat brain MC with naphtol AS-D chloroacetate esterase (Leder) staining. A typical position close to a cortical penetrating arteriole can be observed. (**c**) Toluidine blue staining of mouse dura, showing the closely set localisation of MC in meningeal tissues

and B, thionin and methylene blue, which induce purple or violet staining of the granules. Toluidine blue and Giemsa are the most routinely used stainings. Egg white avidin attaches to heparin as well and has been used in a fluorophore-conjugated staining regimen. Tissue MCs can also be detected by enzyme cytochemistry by demonstration of Naphtol AS-D chloroacetate esterase activity (Leder staining) (Fig. 1b), by immunocytochemistry against tryptase, and by visualization of histamine fluorescence after *o*-phthaldialdehyde exposure [13].

Using these techniques, the presence of MCs has long been observed in substantial numbers in various craniocerebral structures. In the mammalian brain, MCs are found abundantly in the meninges, choroid plexus, hypothalamus, thalamus, olfactory bulb, and the midbrain [14–17]. MCs are also observed in the cerebral cortex

[14, 15, 18], characteristically at the branching points of cortical penetrating arterioles (Fig. 1b). A typical position for MCs is the Virchow–Robin perivascular space. They are situated in the brain parenchymal side of basal lamina, nesting between glial processes [19]. MCs are very abundant also in the dural meninges (Fig. 1c). From these locations, MCs can influence not only the blood vessels but interact also with neurons, glial cells, microglia, and extracellular matrix components. Intramuscular injection of compound 48/80 in several species has triggered striking Evans blue extravasation in MC-rich brain areas but not in MC-devoid regions equipped with fenestrated capillaries [20]. A tremendous challenge for research is that the type of MCs is to a large extent species-dependent, and the cell population fluctuates dynamically during development as well as in the course of behavioral and physiological events, and various kinds of stress.

The role of craniocerebral MCs remains elusive, but can be reflected upon an evolutionary perspective. MCs might have transformed from a cell which was directly involved in the compartmentalization and killing of pathogens into a cell type which orchestrates complex reactions leading to segregation and clearing of invading microbes. From a simple effector cell, the MC has become a coordinating cell which operates not only through innate mechanisms but also with the contribution of adaptive immune mechanisms [21]. It is of fundamental importance for the pathogenic mechanisms within the CNS, that of the resident cells in the brain, only MCs are able to acutely respond by releasing massive amounts of preformed mediators (reviewed in [22, 23]). The impact of MC activation is not uniformly proinflammatory, but can also attenuate the response of circulating leukocytes as in the mouse hippocampal brain trauma model [24].

In the brain, MCs have been ascribed a wide spectrum of physiological functions serving not only host response and innate immunity but also endocrine regulation [20]. MCs have been traditionally held as a first line of host defense against pathogens, allergens, toxins, and tissue injury, but with evolutionary accumulation of various membrane-bound receptors and diverse granule-contained mediators, MCs have engaged in quite diverse regulating functions also in many normal physiological and homeostatic functions along with the evolution of CNS.

5 Diversity of MCs

MCs of different organotypic origins show a substantial heterogeneity regarding their granule content and certain outer membrane epitopes. The organotypic difference has been used to classify MCs, which traditionally have been divided into mucosal or connective tissue type serosal (peritoneal) types. One key diversity is that of the main protease content: in humans, MCs can be distinguished based upon whether they contain tryptase (MCT) or tryptase and chymase (MCTC) [25]. Another example of diversity is the lack of c-kit receptor for SCF reported in some cerebral MCs [26]. Interestingly, blood transfusion results in MCs of donor origin settling in tissues of WT mice including the brain, and this relocation is restricted to

regions bearing host MCs, but transfusion in KitW-sh/W-sh MC-deficient mice strain results in MCs in the pinna of the ear, not in the brain [27]. Another brain tissue-specific MC characteristic is the low expression of the IgE-binding, high-affinity receptor FcεRI. The molecular and anatomical heterogeneity of the brain MCs has recently been reviewed in detail [20].

However, experimental studies have indicated that, in keeping with the perivascular position of MCs in the brain, the released vasoactive mediators furnish them with a capacity to regulate the permeability of BBB [28, 29].

6 MCs in the Priming of Inflammatory Cell Response

MCs are best known for their potent effector functions in allergic and hypersensitivity disorders. These phlogistic responses have long been known to be biphasic, the immediate phase occurring within minutes, and the late phase within several hours. The former is caused by the release of MC granule contents (histamine, TNFα, chymase, tryptase, heparin, cathepsin-G, and assorted chemokines). The second phase is characterized by *de novo* synthesis of MC mediators, such as leukotrienes, cytokines, prostaglandins, and additional chemotactic factors. However, MCs recently have been demonstrated to be involved in a vast array of considerably more complex immune functions that go well beyond allergies and contain the development of autoimmune disorders and peripheral tolerance, as well as the initiation and maintenance of adaptive and innate host responses [30].

In the development of local inflammatory tissue response, MCs have been implicated in recruiting neutrophils that are frequently the first immune cells to enter an inflamed or infected tissue site [31, 32]. In a mouse model of lipopolysaccharide (LPS)-induced peritonitis, MC granules containing preformed chemokines CXCL1 and CXCL2 were shown to be released by MCs within 15 min of in vivo stimulation, constituting an ideal mechanism to stimulate local neutrophil entry from the circulation at an early stage following the inflammatory signal [33]. Earlier work has emphasized the role of MC-granule dependent TNFα in mediating the initial phase of neutrophil recruitment following immune-complex mediated peritonitis in mice [34]. In addition, MCs, like macrophages, also were demonstrated to newly synthesize CXCL1 and CXCL2, making detectable amounts within 1 h of LPS treatment. Neutrophil extravasation was diminished in mice devoid of MCs [33].

Another facet of the local inflammatory response is the increase of vasopermeability and tissue edema. A well-established view is that several MC mediators, especially the vasoactive amines histamine, serotonin, and vascular endothelial growth factor A (VEGF-A), increase capillary leakage and can rapidly trigger tissue edema [35], but numerous other MC mediators such as PAF and leukotrienes participate in this response. Histamine receptor antagonists are therapeutically used to treat edema formation and allergic reactions associated with aberrant MC activity e.g., in pulmonary asthma, which can be also treated by the classic MC-stabilizing medicament, sodium cromoglycate. MCs can also trigger vascular permeability by paracrine mechanisms such as heparin-initiated bradykinin formation [36].

Although sparsely scattered in tissues, MCs are strategically positioned in close proximity to blood vessels and thus in a position to directly influence circulating leukocytes [33]. The same principle seems to be active in mounting local inflammation also in the various inflammatory diseases of the brain [6]. It is noteworthy that the described physiological and pathophysiological roles of MCs are likely to be strongly species-specific, because the immunological key characteristic such as the palette of granule constituents, membrane receptors, and activation modes have significant variability across different animal species.

7 Mast Cells as a Member of the Neurovascular Unit

The co-orchestrated roles of endothelial cells, neurons, astrocytes, pericytes, and extracellular matrix in the pathobiology of cerebral blood vessels have been encompassed under the term "neurovascular unit" (NVU). We and others have viewed MCs in the context of the NVU responding to cerebral ischemia [23, 37]. In the micromilieu of the brain parenchyma resident MCs lie in close proximity to other cell types of the NVU (Fig. 2). In addition to their role as initiators of acute inflammatory and vasoactive events, functional, unstressed MCs are certain to interact with these surrounding cell types, with bidirectional effects. Modes of interaction are likely to include direct cellular connections and released mediators, but interestingly, may also include cell signaling via release of exosomes, small membrane vesicles packed with RNA, capable of altering function of surrounding cell types [38]. For now, most research on cerebral MCs has focused on the pathophysiology of different neurological disorders, and more detailed investigation of MC interaction with other NVU cell types has been scarce, mostly relying on in vitro cell culture methods. Therefore the participation of MCs in the various physiological functions of the NVU is still to be characterized.

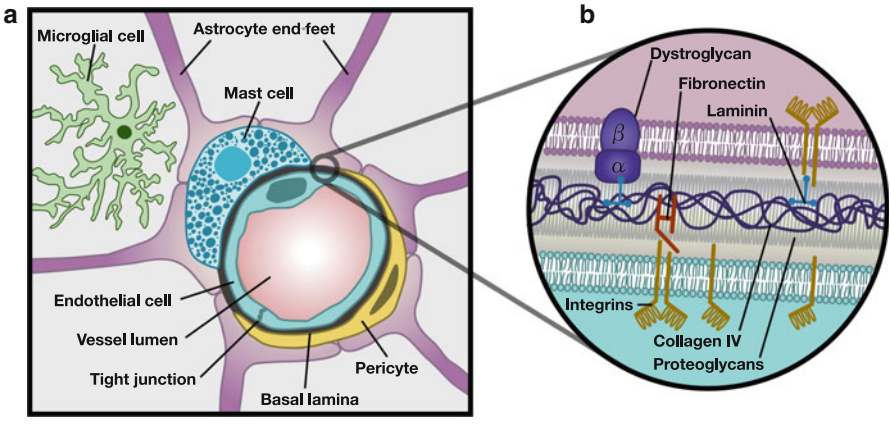

Fig. 2 Illustration of MCs at the NVU (**a**) and components of the basal lamina (**b**)

8 Evidence for a Role of MCs in Cerebral Ischemia

8.1 Mast Cell Activation in Experimental Models of Cerebral Ischemia

Initial observations of MC activation in experimental focal cerebral ischemia were made 15 years ago in our laboratory [39, 40]. In these experiments, degranulated cerebral MCs were observed in perivascular locations often associated with adjacent perivascular edema, based on which we hypothesized MCs to be involved in the pathophysiology of cerebral ischemia. Later on, MC activation and subsequent mediator release were reported in an in vitro model of oxygen-glucose deprivation [41]. In a separate study we further showed a dose-dependent activation of MCs (as measured by histamine release) in vitro as a reaction to tissue plasminogen activator (tPA), used for treatment of focal cerebral ischemia [42]. MC activation has also been described in neonatal rat stroke models. Biran et al. showed that ischemia induced histamine accumulation in the infarct core at 6–12 h after ischemia induction, whereas it was located in the penumbra at 24–48 h [43]. This study also showed that histamine accumulated in neuronal cells before they degenerated, which was accompanied by an increase in the MC count at 12 and 48 h. Activation of MCs after experimental hypoxic-ischemia injury in the neonate brain has later been replicated by additional studies [44].

8.2 The Effects of MCs in Experimental Focal Ischemic Brain Damage

Investigation on the role of MCs in focal cerebral ischemia began with experiments using a transient middle cerebral artery occlusion (MCAO) model in rats. Applying pharmacological activation and inhibition of MCs, we found MCs to play a central part in the development of space occupying brain edema, BBB disruption, and neutrophil infiltration 3 h after transient ischemia and reperfusion [18]. For example, MC stabilization with intraventricularly applied cromoglycate led to a 39 % reduction in hemispheric expansion, 51 % reduction in BBB leakage, and a 37 % reduction in postischemic neutrophil infiltration. Involvement of MCs was subsequently studied using the same model together with intravenous tPA, simulating thrombolytic treatment [42]. MC stabilization showed reduction not only of edema, BBB disruption, and neutrophil infiltration, but also of perilous hemorrhage formation, neurological deterioration, and mortality. In both of these proof-of-concept studies, experiments were repeated using gene-manipulated MC-devoid rats lacking the c-kit ligand necessary for stem cell-dependent MC differentiation, demonstrating an even stronger protective effect with MC deficiency [18, 42].

More recently, our initial experimental findings in rats were replicated by two groups using a mouse MCAO model of transient ischemia. Studying later timepoints at 3 days and 2 weeks, Arac et al. showed significant reductions in brain edema, granulocyte infiltration, and infarct size in two different MC-deficient mouse strains [45]. Importantly, the study demonstrated the central participation of the meningeal MC population mediated via interleukin (IL)-6 and, to a lesser extent, chemokine ligand 7. Another study with pharmacological stabilization of MCs and genetically MC-deficient mice supported the involvement MCs in the pathophysiology of ischemia-mediated edema and inflammation, and suggested a role for endoglin, endothelin-1, and MMP-9, but not for TNFα [46]. Interestingly, this study also reported an increase of MC count by 50 % in the infarcted hemisphere 4 h after transient MCAO. Together, these observations provide solid evidence to support direct involvement of MCs in the pathophysiology of focal cerebral ischemia.

8.3 MCs and Neuroprotection

The initial in vivo stroke model experiments were not designed to study late-stage neuroprotection, and did not notice any effect regarding the lesion volume in association with MC stabilization or genotypic MC deficiency [18]. However, another study with longer follow-up of 24-h revealed a clear effect on functional recovery both after pharmacological stabilization of mast cells and in rats with mast cell deficiency [42], although no significant differences in infarct sizes were seen. More recent studies have also showed a reduction of infarct volume after MC stabilization in adult wild-type mice [45, 46]. Again, additional supporting evidence of MC involvement in tissue injury comes from neonate models. One set of experiments showed MC involvement in hypoxic-ischemic brain damage in the immature rat [47] and later observations showed that MC stabilization translated into reduced neuronal loss and brain atrophy [44]. The authors suggested the possibility of MC-derived IL-9 to be involved in the detrimental effect, which was supported by others [48]. Immunohistochemical co-localization studies of histamine and microtubule-associated protein 2 revealed accumulation in neuronal cells prior to their degeneration, and increased MC counts in the corresponding regions [43]. Histamine immunoreactivity was detected in MCs at 2, 6, and 12 h after ischemia, but disappeared at 24 h along with a concomitant observation of MC degranulation. Another study showed an effect of cyclosporin A in protecting against mild ischemic injury in neonatal rat brain [49]. The observed effect in reduction of histamine release from MCs is, however, IgE-dependent, and may not be of major importance in this setting. These data support a role for MCs in experimental ischemia-induced neuronal death, at least in neonatal cerebral ischemia.

9 Mast Cells and BBB in Ischemia–Reperfusion Injury

As the neurovascular unit responds to the sudden insult of ischemia and subsequent reperfusion, MCs are ideally located to initiate and aggravate known pathways of BBB disruption [23]. As discussed earlier, the activation of MCs is known to occur in a biphasic manner [3], starting with acute release of potent preformed granule contents that quickly spread to interact with the abluminal side of endothelial cells, the surrounding basal lamina, and other cell types of the neurovascular unit (Fig. 3). The second, later phase of MC activation is characterized by *de novo* production and release of mediators to support and prolong the initiated inflammatory response [3].

Very early on hypoxia, acidosis, formation of reactive oxygen species, and changes in blood flow act to disrupt cellular homeostasis of the neurovascular unit [37]. These reactions, together with intravascular blood coagulation, complement activation, and activation of the sympathetic nervous system, are likely initiators of MC activation next to parenchymal microvessels and within meningeal tissues [23].

The most imminent MC effects are amplified through the adjacent endothelium, which is the initial site of BBB leakage and failure early after reperfusion [50]. Histamine, an abundant and highly soluble MC mediator, acts through endothelial histamine receptors to activate calcium influx and convert the cells into a proinflammatory state [51]. The carefully characterized effects of histamine include increased endothelial permeability [51, 52], activation of endothelial nitric oxide synthase (eNOS) [53], and acute release of Weibel–Palade bodies (WPBs) [54], the main storage site for von Willebrand Factor (VWF) and P-selectin that act to support acute leukocyte infiltration [55, 56]. The MC protease tryptase may further activate endothelium through cleavage of the proteinase activated receptor 2 (PAR-2), with similar

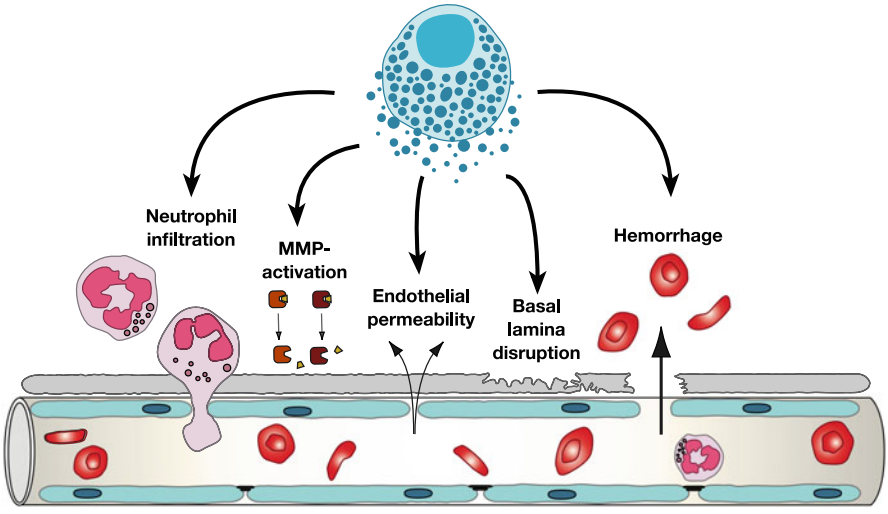

Fig. 3 Main pathways of MC-mediated blood–brain barrier disruption

effects to histamine [57]. A third MC mediator, Cathepsin G, has also been shown to induce endothelial permeability and influx of calcium into endothelial cells [58, 59].

As reperfusion injury advances, a storm of proteolytic enzymes is activated within the structures of the cerebral microvasculature [60, 61]. Ultrastructural evidence suggests that an early disruption of basal lamina [62], endothelial tight junctions [63], and other cellular connections ensues [64], and may locally progress to cause structural failure of the vascular wall, leading to edema and eventually hemorrhage [65]. Experimental evidence indicates that the wide armamentarium of MC mediators may have a central role in enhancing this proteolytic cascade [23].

The family of matrix metalloproteinase (MMP) enzymes, especially the gelatinases MMP-2 and -9, are thought to be a central proteolytic pathway, minutely characterized in experimental stroke models [61, 66–68]. For example, in human stroke patients plasma levels of MMP-9 are correlated with the incidence of significant hemorrhagic transformation [69, 70]. Using a rat MCAO stroke model, we found that both genetic MC deficiency and pharmacological MC stabilization with intracerebroventricular cromoglycate were able to significantly reduce the percentage of microvessels with high gelatinase activity in the ischemic hemisphere as early as 3 h after reperfusion (−64 % and −36 %, respectively) [71]. This finding is likely a sum of several MC-mediated effects on the MMP-cascade.

MCs have been shown to release both MMP-2 and -9 [72], which fits with the gelatinolytic activity we observed in the granules of activated cerebral MCs [71]. The MC protease chymase is capable of activating proMMP-1, proMMP-2, and proMMP-9 [72–74] and degrades tissue inhibitor of metalloproteinases (TIMP) -1, an important endogenous MMP-inhibitor [75]. MC tryptase has also been shown to activate proMMP-2 and proMMP-3 [76, 77]. Further on, in vitro studies have shown that histamine induces MMP-2 production in endothelial cells [78] and MMP-9 production in astrocytes [79]. MC proteases can also degrade components of the basal lamina directly: chymase is capable of degrading fibronectin [80] and cathepsin G, found in a subset of MCs, is able to degrade fibronectin and laminin [81, 82].

Progression of postischemic BBB disruption is accompanied by unrestrained granulocyte infiltration, beginning hours after reperfusion [83], which acts to further drive inflammation, increase proteolysis and barrier permeability [84], and disrupt microvascular flow [85]. MCs seem to have a central role in activating leukocyte recruitment, as data from three individual laboratories show that MC inhibition significantly reduces both early and late granulocyte infiltration after transient MCAO (3–6 h and 3 days postreperfusion, respectively) [18, 42, 45, 46].

MCs secrete a wide range of mediators that can augment granulocyte infiltration. In addition to the endothelial dependent effects of histamine and tryptase described above, MCs are capable of releasing preformed TNFα, which further increases endothelial permeability, endothelial adhesion molecule expression, and neutrophil infiltration [86–88]. Moreover, chymase is thought to have direct chemotactic effects on neutrophils [89]. As MC activation endures, *de novo* production of mediators continues to support infiltration of granulocytes. IL-1 is capable of increasing both endothelial barrier permeability and neutrophil infiltration [90, 91]. Further, in a recent report, Arac et al. demonstrated that IL-6 is central for MC-dependent neutrophil infiltration

in a later phase, 3 days postreperfusion. In these experiments reconstitution of MC-deficient mice with wild-type MCs returned typical neutrophil infiltration and brain swelling, while reconstitution with IL-6-deficient MCs did not [45].

To sum up, the effects of MCs in experimental models of ischemic stroke are well in line with the known effects of the wide armamentarium of MC mediators. However, the individual contribution of these mediators is still unknown, and will require further experimental work, preferably by reconstituting MC-deficient mice with MCs deficient for the studied mediator [45]. Although meningeal MCs appear to be central at later timepoints after reperfusion (3 days and 2 weeks) [45], the relative contribution of different MC populations in the ultra-acute and early phases (0–48 h) of ischemic stroke is still unknown.

10 Mast Cells, Blood Coagulation, and Fibrinolysis

In addition to their potent vasoactive, proteolytic, and chemotactic effects, MCs are known as a profibrinolytic, anticoagulant, and antithrombotic cell type, with several effects on thrombotic pathways [23, 92]. As reperfusion injury advances to break down structures of the vascular wall, the unphysiologically strong activation of MCs may partake in initiating dangerous intraparenchymal hemorrhage, one of the most feared complications of acute stroke treatment. Supporting this hypothesis, in experiments using a rat stroke model with 90 min of transient MCAO combined with intravenous tPA, both genetic and pharmacological MC inhibition led to almost total abrogation of intraparenchymal hemorrhage [42]. Again, a spectrum of MC mediators have effects on hemostasis.

Heparin is the central anticoagulant mediator released from MCs, a negatively charged glycosaminoglycan which catalyzes antithrombin III-mediated inactivation of coagulation factors, most importantly activated factor X and thrombin [93]. Heparin can inhibit binding of platelets onto collagen IV [94], revealed upon disruption of the vascular wall. Further, heparin also releases tissue factor pathway inhibitor (TFPI) from the surface of endothelial cells, a mediator which can inhibit arterial thrombosis [95]. Lastly, heparin has recently been shown to activate the plasma contact system, inducing rapid generation of bradykinin without activation of blood coagulation [36], which may act to further increase endothelial permeability and leukocyte infiltration.

MCs also have more direct effects on fibrinogen and fibrin. MC tryptase has been shown to degrade fibrinogen, preventing normal fibrin formation [96, 97]. Tryptase also activates pro-urokinase [98], an important plasminogen activator, initiating plasmin-mediated breakdown of fibrin. Moreover, MCs have been shown to directly secrete tissue plasminogen activator (tPA), another central plasminogen activator, without accompanying secretion of plasminogen activator inhibitors, like plasminogen activator inhibitor-1 (PAI-1) [99]. Importantly, a wide collection of experimental evidence has shown that plasminogen activators, especially tPA, have important effects on proteolysis and inflammation at the BBB, in addition to direct

pro-excitotoxic effects [100]. Of note, a positive feedback loop may exist between fibrinolysis and further MC activation, as certain fibrinolytic breakdown products of fibrinogen have been shown to activate MCs [101].

The endothelial effects of MC mediators contribute an additional pathway for modification of hemostasis. In patients with anaphylaxis, extremely strong MC activation and subsequent release of endothelial WPBs have been shown to induce a rapid increase in circulating levels of both vWF and tPA, and induce systemic plasminogen activation [102]. The significance of this pathway during localized MC activation is still to be uncovered, but may have both fibrinolytic and proaggregatory effects.

In the setting of acute inflammation, the physiological purpose of these described anticoagulant, fibrinolytic, and antithrombotic MC effects may be in regulating thrombosis activated by inflammatory pathways, to ensure adequate blood flow to the inflamed tissue area, and counteract the inhibitory effects of fibrin formation on leukocyte recruitment [103]. More generally, in the resting state, MCs have been suggested to protect the brain microvasculature against thrombotic challenges [104]. In line with these hypotheses, several products of the coagulation cascade have been shown to activate MCs, including bradykinin, thrombin, and activated factor X [105–107].

11 Conclusions

To conclude, recent experimental research suggests that MCs and their nominal responses increasing the permeability of the vascular wall play a significant, deleterious role following acute cerebral ischemia. MCs should be regarded as a potent inflammatory cell type that can interact via a multitude of mediators and signalosomes with its neighboring cells within the NVU, and may also have more distant effects within the CNS. MCs are a unique resident inflammatory cell type, settled in the proximity of the vascular wall already at the outset of ischemia, capable of quickly degranulating, leading to degradation of the basal membrane, BBB damage, brain edema, and hemorrhage.

Future goals of MC research include examination of whether MC mediators are released early after cerebral ischemia in man. In view of the significant species differences in the immunological characteristics and mediator contents of MCs, more evidence is needed on the magnitude of MC-dependent chemokine release, neutrophil targeting, and other secondary effects on postischemic tissue integrity. The potential involvement of the meningeal MC population is an attractive area of future research, as these MCs may be more readily influenced with pharmacological means due to their localization outside the BBB. MCs are candidate cells to become novel pharmacologic targets at the NVU to limit ischemic and hemorrhagic brain damage associated with reperfusion therapies.

Acknowledgments Academic stroke research in Helsinki is supported by financial resources from governmental and nonprofit foundations, the Helsinki University Central Hospital governmental subsidiary funds for clinical research (DS, PJL), the Finnish Medical Foundation (DS), the Sigrid Jusélius Foundation (PJL), the Maire Taponen Foundation (PJL), and the Paavo Nurmi Foundation (PJL).

References

1. Gilfillan AM, Austin SJ, Metcalfe DD. Mast cell biology: introduction and overview. Adv Exp Med Biol. 2011;716:2–12.
2. Galli SJ, Tsai M. Mast cells in allergy and infection: versatile effector and regulatory cells in innate and adaptive immunity. Eur J Immunol. 2010;40(7):1843–51.
3. Kunder CA, St John AL, Abraham SN. Mast cell modulation of the vascular and lymphatic endothelium. Blood. 2011;118(20):5383–93.
4. Voehringer D. Protective and pathological roles of mast cells and basophils. Nat Rev Immunol. 2013;13(5):362–75.
5. Akahoshi M, Song CH, Piliponsky AM, Metz M, Guzzetta A, Abrink M, et al. Mast cell chymase reduces the toxicity of Gila monster venom, scorpion venom, and vasoactive intestinal polypeptide in mice. J Clin Invest. 2011;121(10):4180–91.
6. Theoharides TC, Alysandratos K-D, Angelidou A, Delivanis D-A, Sismanopoulos N, Zhang B, et al. Mast cells and inflammation. Biochim Biophys Acta. 2012;1822(1):21–33.
7. Pejler G, Rönnberg E, Waern I, Wernersson S. Mast cell proteases: multifaceted regulators of inflammatory disease. Blood. 2010;115(24):4981–90.
8. Abraham SN, St John AL. Mast cell-orchestrated immunity to pathogens. Nat Rev Immunol. 2010;10(6):440–52.
9. Wernersson S, Pejler G. Mast cell secretory granules: armed for battle. Nat Rev Immunol. 2014;14(7):478–94.
10. Rivera J, Gilfillan AM. Molecular regulation of mast cell activation. J Allergy Clin Immunol. 2006;117(6):1214–25. quiz1226.
11. Gilfillan AM, Rivera J. The tyrosine kinase network regulating mast cell activation. Immunol Rev. 2009;228(1):149–69.
12. Hofmann AM, Abraham SN. New roles for mast cells in modulating allergic reactions and immunity against pathogens. Curr Opin Immunol. 2009;21(6):679–86.
13. Edvinsson L, Cervoś-Navarro J, Larsson LI, Owman C, Rönnberg AL. Regional distribution of mast cells containing histamine, dopamine, or 5-hydroxytryptamine in the mammalian brain. Neurology. 1977;27(9):878–83.
14. Theoharides TC. Mast cells: the immune gate to the brain. Life Sci. 1990;46(9):607–17.
15. Silver R, Silverman AJ, Vitković L, Lederhendler II. Mast cells in the brain: evidence and functional significance. Trends Neurosci. 1996;19(1):25–31.
16. Goldschmidt RC, Hough LB, Glick SD, Padawer J. Mast cells in rat thalamus: nuclear localization, sex difference and left–right asymmetry. Brain Res. 1984;323(2):209–17.
17. Dropp JJ. Mast cells in mammalian brain. Acta Anat. 1976;94(1):1–21.
18. Strbian D, Karjalainen-Lindsberg M-L, Tatlisumak T, Lindsberg PJ. Cerebral mast cells regulate early ischemic brain swelling and neutrophil accumulation. J Cereb Blood Flow Metab. 2006;26(5):605–12.
19. Silverman AJ, Sutherland AK, Wilhelm M, Silver R. Mast cells migrate from blood to brain. J Neurosci. 2000;20(1):401–8.
20. Silver R, Curley JP. Mast cells on the mind: new insights and opportunities. Trends Neurosci. 2013;36(9):513–21.
21. Crivellato E, Ribatti D. The mast cell: an evolutionary perspective. Biol Rev Camb Philos Soc. 2010;85(2):347–60.

22. Strbian D, Kovanen PT, Karjalainen-Lindsberg M-L, Tatlisumak T, Lindsberg PJ. An emerging role of mast cells in cerebral ischemia and hemorrhage. Ann Med. 2009;41(6):438–50.
23. Lindsberg PJ, Strbian D, Karjalainen-Lindsberg M-L. Mast cells as early responders in the regulation of acute blood–brain barrier changes after cerebral ischemia and hemorrhage. J Cereb Blood Flow Metab. 2010;30(4):689–702.
24. Hendrix S, Kramer P, Pehl D, Warnke K, Boato F, Nelissen S, et al. Mast cells protect from post-traumatic brain inflammation by the mast cell-specific chymase mouse mast cell protease-4. FASEB J. 2013;27(3):920–9.
25. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils, and eosinophils. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S73–80.
26. Shanas U, Bhasin R, Sutherland AK, Silverman AJ, Silver R. Brain mast cells lack the c-kit receptor: immunocytochemical evidence. J Neuroimmunol. 1998;90(2):207–11.
27. Nautiyal KM, Liu C, Dong X, Silver R. Blood-borne donor mast cell precursors migrate to mast cell-rich brain regions in the adult mouse. J Neuroimmunol. 2011;240–241:142–6.
28. Zhuang X, Silverman AJ, Silver R. Brain mast cell degranulation regulates blood–brain barrier. J Neurobiol. 1996;31(4):393–403.
29. Esposito P, Gheorghe D, Kandere K, Pang X, Connolly R, Jacobson S, et al. Acute stress increases permeability of the blood–brain-barrier through activation of brain mast cells. Brain Res. 2001;888(1):117–27.
30. Metz M, Maurer M. Mast cells—key effector cells in immune responses. Trends Immunol. 2007;28(5):234–41.
31. Borregaard N. Neutrophils, from marrow to microbes. Immunity. 2010;33(5):657–70.
32. Nathan C. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol. 2006;6(3):173–82.
33. De Filippo K, Dudeck A, Hasenberg M, Nye E, van Rooijen N, Hartmann K, et al. Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. Blood. 2013;121(24):4930–7.
34. Zhang Y, Ramos BF, Jakschik BA. Neutrophil recruitment by tumor necrosis factor from mast cells in immune complex peritonitis. Science. 1992;258(5090):1957–9.
35. Nagy JA, Benjamin L, Zeng H, Dvorak AM, Dvorak HF. Vascular permeability, vascular hyperpermeability and angiogenesis. Angiogenesis. 2008;11(2):109–19.
36. Oschatz C, Maas C, Lecher B, Jansen T, Björkqvist J, Tradler T, et al. Mast cells increase vascular permeability by heparin-initiated bradykinin formation in vivo. Immunity. 2011;34(2):258–68.
37. Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. Nat Med. 2011;17(7):796–808.
38. Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6):654–9.
39. Karjalainen-Lindsberg ML, Tatlisumak T, Lindsberg PJ. Mast cells in ischemic rat brain. Conference Proceeding. San Diego: Society for Neuroscience; 2001. Abstract No: 330.11
40. Strbian D, Tatlisumak T, Karjalainen-Lindsberg ML, Lindsberg PJ. Mast cells regulate ischemic brain edema. Conference Proceeding. J Cereb Blood Flow Metab. 2003; 23(Suppl 1):166
41. Hu W, Shen Y, Fu Q, Dai H, Tu H, Wei E, et al. Effect of oxygen-glucose deprivation on degranulation and histamine release of mast cells. Cell Tissue Res. 2005;322(3):437–41.
42. Strbian D, Karjalainen-Lindsberg M-L, Kovanen PT, Tatlisumak T, Lindsberg PJ. Mast cell stabilization reduces hemorrhage formation and mortality after administration of thrombolytics in experimental ischemic stroke. Circulation. 2007;116(4):411–8.
43. Biran V, Cochois V, Karroubi A, Arrang JM, Charriaut-Marlangue C, Héron A. Stroke induces histamine accumulation and mast cell degranulation in the neonatal rat brain. Brain Pathol. 2008;18(1):1–9.
44. Jin Y, Silverman AJ, Vannucci SJ. Mast cells are early responders after hypoxia-ischemia in immature rat brain. Stroke. 2009;40(9):3107–12.

45. Arac A, Grimbaldeston MA, Nepomuceno ARB, Olayiwola O, Pereira MP, Nishiyama Y, et al. Evidence that meningeal mast cells can worsen stroke pathology in mice. Am J Pathol. 2014;184(9):2493–504.
46. McKittrick CM, Lawrence CE, Carswell HVO. Mast cells promote blood–brain barrier breakdown and neutrophil infiltration in a mouse model of focal cerebral ischemia. J Cereb Blood Flow Metab. 2015;35(4):638–47.
47. Jin Y, Silverman A-J, Vannucci SJ. Mast cell stabilization limits hypoxic-ischemic brain damage in the immature rat. Dev Neurosci. 2007;29(4–5):373–84.
48. Patkai J, Mesples B, Dommergues MA, Fromont G, Thornton EM, Renauld JC, et al. Deleterious effects of IL-9-activated mast cells and neuroprotection by antihistamine drugs in the developing mouse brain. Pediatr Res. 2001;50(2):222–30.
49. Leger P-L, De Paulis D, Branco S, Bonnin P, Couture-Lepetit E, Baud O, et al. Evaluation of cyclosporine A in a stroke model in the immature rat brain. Exp Neurol. 2011;230(1):58–66.
50. Kuntz M, Mysiorek C, Pétrault O, Pétrault M, Uzbekov R, Bordet R, et al. Stroke-induced brain parenchymal injury drives blood–brain barrier early leakage kinetics: a combined in vivo/in vitro study. J Cereb Blood Flow Metab. 2014;34(1):95–107.
51. Tiruppathi C, Minshall RD, Paria BC, Vogel SM, Malik AB. Role of Ca2+ signaling in the regulation of endothelial permeability. Vascul Pharmacol. 2002;39(4–5):173–85.
52. Winter MC, Shasby SS, Ries DR, Shasby DM. Histamine selectively interrupts VE-cadherin adhesion independently of capacitive calcium entry. Am J Physiol Lung Cell Mol Physiol. 2004;287(4):L816–23.
53. Li H, Burkhardt C, Heinrich U-R, Brausch I, Xia N, Förstermann U. Histamine upregulates gene expression of endothelial nitric oxide synthase in human vascular endothelial cells. Circulation. 2003;107(18):2348–54.
54. van Mourik JA, Romani de Wit T, Voorberg J. Biogenesis and exocytosis of Weibel-Palade bodies. Histochem Cell Biol. 2002;117(2):113–22.
55. Jones DA, Abbassi O, McIntire LV, McEver RP, Smith CW. P-selectin mediates neutrophil rolling on histamine-stimulated endothelial cells. Biophys J. 1993;65(4):1560–9.
56. Pendu R, Terraube V, Christophe OD, Gahmberg CG, de Groot PG, Lenting PJ, et al. P-selectin glycoprotein ligand 1 and beta2-integrins cooperate in the adhesion of leukocytes to von Willebrand factor. Blood. 2006;108(12):3746–52.
57. Meyer MC, Creer MH, McHowat J. Potential role for mast cell tryptase in recruitment of inflammatory cells to endothelium. Am J Physiol Cell Physiol. 2005;289(6):C1485–91.
58. Schechter NM, Irani AM, Sprows JL, Abernethy J, Wintroub B, Schwartz LB. Identification of a cathepsin G-like proteinase in the MCTC type of human mast cell. J Immunol. 1990;145(8):2652–61.
59. Peterson MW, Gruenhaupt D, Shasby DM. Neutrophil cathepsin G increases calcium flux and inositol polyphosphate production in cultured endothelial cells. J Immunol. 1989;143(2): 609–16.
60. Fukuda S, Fini CA, Mabuchi T, Koziol JA, Eggleston LL, del Zoppo GJ. Focal cerebral ischemia induces active proteases that degrade microvascular matrix. Stroke. 2004;35(4):998–1004.
61. Yang Y, Rosenberg GA. Blood–brain barrier breakdown in acute and chronic cerebrovascular disease. Stroke. 2011;42(11):3323–8.
62. Hamann GF, Okada Y, Fitridge R, del Zoppo GJ. Microvascular basal lamina antigens disappear during cerebral ischemia and reperfusion. Stroke. 1995;26(11):2120–6.
63. Yang Y, Estrada EY, Thompson JF, Liu W, Rosenberg GA. Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. J Cereb Blood Flow Metab. 2007;27(4):697–709.
64. Wagner S, Tagaya M, Koziol JA, Quaranta V, del Zoppo GJ. Rapid disruption of an astrocyte interaction with the extracellular matrix mediated by integrin alpha 6 beta 4 during focal cerebral ischemia/reperfusion. Stroke. 1997;28(4):858–65.
65. Hamann GF, Okada Y, del Zoppo GJ. Hemorrhagic transformation and microvascular integrity during focal cerebral ischemia/reperfusion. J Cereb Blood Flow Metab. 1996;16(6): 1373–8.

66. Amantea D, Corasaniti MT, Mercuri NB, Bernardi G, Bagetta G. Brain regional and cellular localization of gelatinase activity in rat that have undergone transient middle cerebral artery occlusion. Neuroscience. 2008;152(1):8–17.
67. Gu Z, Cui J, Brown S, Fridman R, Mobashery S, Strongin AY, et al. A highly specific inhibitor of matrix metalloproteinase-9 rescues laminin from proteolysis and neurons from apoptosis in transient focal cerebral ischemia. J Neurosci. 2005;25(27):6401–8.
68. Rosenberg GA. Matrix metalloproteinases in neuroinflammation. Glia. 2002;39(3):279–91.
69. Castellanos M, Leira R, Serena J, Pumar JM, Lizasoain I, Castillo J, et al. Plasma metalloproteinase-9 concentration predicts hemorrhagic transformation in acute ischemic stroke. Stroke. 2003;34(1):40–6.
70. Rosell A, Ortega-Aznar A, Alvarez-Sabín J, Fernández-Cadenas I, Ribó M, Molina CA, et al. Increased brain expression of matrix metalloproteinase-9 after ischemic and hemorrhagic human stroke. Stroke. 2006;37(6):1399–406.
71. Mattila OS, Strbian D, Saksi J, Pikkarainen TO, Rantanen V, Tatlisumak T, et al. Cerebral mast cells mediate blood–brain barrier disruption in acute experimental ischemic stroke through perivascular gelatinase activation. Stroke. 2011;42(12):3600–5.
72. Fang KC, Wolters PJ, Steinhoff M, Bidgol A, Blount JL, Caughey GH. Mast cell expression of gelatinases A and B is regulated by kit ligand and TGF-beta. J Immunol. 1999;162(9): 5528–35.
73. Tchougounova E, Lundequist A, Fajardo I, Winberg J-O, Abrink M, Pejler G. A key role for mast cell chymase in the activation of pro-matrix metalloprotease-9 and pro-matrix metalloprotease-2. J Biol Chem. 2005;280(10):9291–6.
74. Saarinen J, Kalkkinen N, Welgus HG, Kovanen PT. Activation of human interstitial procollagenase through direct cleavage of the Leu83-Thr84 bond by mast cell chymase. J Biol Chem. 1994;269(27):18134–40.
75. Frank BT, Rossall JC, Caughey GH, Fang KC. Mast cell tissue inhibitor of metalloproteinase-1 is cleaved and inactivated extracellularly by alpha-chymase. J Immunol. 2001;166(4):2783–92.
76. Lohi J, Harvima I, Keski-Oja J. Pericellular substrates of human mast cell tryptase: 72,000 dalton gelatinase and fibronectin. J Cell Biochem. 1992;50(4):337–49.
77. Gruber BL, Marchese MJ, Suzuki K, Schwartz LB, Okada Y, Nagase H, et al. Synovial procollagenase activation by human mast cell tryptase dependence upon matrix metalloproteinase 3 activation. J Clin Invest. 1989;84(5):1657–62.
78. Doyle JL, Haas TL. Differential role of beta-catenin in VEGF and histamine-induced MMP-2 production in microvascular endothelial cells. J Cell Biochem. 2009;107(2):272–83.
79. Patel A, Vasanthan V, Fu W, Fahlman RP, MacTavish D, Jhamandas JH. Histamine induces the production of matrix metalloproteinase-9 in human astrocytic cultures via H1-receptor subtype. Brain Struct Funct. 2015:1–16
80. Okumura K, Takai S, Muramatsu M, Katayama S, Sakaguchi M, Kishi K, et al. Human chymase degrades human fibronectin. Clin Chim Acta. 2004;347(1–2):223–5.
81. Vartio T, Seppä H, Vaheri A. Susceptibility of soluble and matrix fibronectins to degradation by tissue proteinases, mast cell chymase and cathepsin G. J Biol Chem. 1981;256(1):471–7.
82. Heck LW, Blackburn WD, Irwin MH, Abrahamson DR. Degradation of basement membrane laminin by human neutrophil elastase and cathepsin G. Am J Pathol. 1990;136(6):1267–74.
83. Lindsberg PJ, Carpén O, Paetau A, Karjalainen-Lindsberg M-L, Kaste M. Endothelial ICAM-1 expression associated with inflammatory cell response in human ischemic stroke. Circulation. 1996;94(5):939–45.
84. Rosell A, Cuadrado E, Ortega-Aznar A, Hernández-Guillamon M, Lo EH, Montaner J. MMP-9-positive neutrophil infiltration is associated to blood–brain barrier breakdown and basal lamina type IV collagen degradation during hemorrhagic transformation after human ischemic stroke. Stroke. 2008;39(4):1121–6.
85. del Zoppo GJ, Schmid-Schönbein GW, Mori E, Copeland BR, Chang CM. Polymorphonuclear leukocytes occlude capillaries following middle cerebral artery occlusion and reperfusion in baboons. Stroke. 1991;22(10):1276–83.
86. Barone FC, Arvin B, White RF, Miller A, Webb CL, Willette RN, et al. Tumor necrosis factor-alpha. A mediator of focal ischemic brain injury. Stroke. 1997;28(6):1233–44.

87. Gotsch U, Jäger U, Dominis M, Vestweber D. Expression of P-selectin on endothelial cells is upregulated by LPS and TNF-alpha in vivo. Cell Adhes Commun. 1994;2(1):7–14.
88. Wong D, Prameya R, Dorovini-Zis K. Adhesion and migration of polymorphonuclear leukocytes across human brain microvessel endothelial cells are differentially regulated by endothelial cell adhesion molecules and modulate monolayer permeability. J Neuroimmunol. 2007;184(1–2):136–48.
89. Tani K, Ogushi F, Kido H, Kawano T, Kunori Y, Kamimura T, et al. Chymase is a potent chemoattractant for human monocytes and neutrophils. J Leukoc Biol. 2000;67(4):585–9.
90. Yamasaki Y, Matsuura N, Shozuhara H, Onodera H, Itoyama Y, Kogure K. Interleukin-1 as a pathogenetic mediator of ischemic brain damage in rats. Stroke. 1995;26(4):676–80. discussion 681.
91. Hara H, Fink K, Endres M, Friedlander RM, Gagliardini V, Yuan J, et al. Attenuation of transient focal cerebral ischemic injury in transgenic mice expressing a mutant ICE inhibitory protein. J Cereb Blood Flow Metab. 1997;17(4):370–5.
92. Valent P. New aspects in thrombosis research: possible role of mast cells as profibrinolytic and antithrombotic cells. Thromb Haemost. 2002;87(5):786–90.
93. Hirsh J. Heparin. N Engl J Med. 1991;324(22):1565–74.
94. Lassila R, Lindstedt K, Kovanen PT. Native macromolecular heparin proteoglycans exocytosed from stimulated rat serosal mast cells strongly inhibit platelet–collagen interactions. Arterioscler Thromb Vasc Biol. 1997;17(12):3578–87.
95. White TA, Johnson T, Zarzhevsky N, Tom C, Delacroix S, Holroyd EW, et al. Endothelial-derived tissue factor pathway inhibitor regulates arterial thrombosis but is not required for development or hemostasis. Blood. 2010;116(10):1787–94.
96. Schwartz LB, Bradford TR, Littman BH, Wintroub BU. The fibrinogenolytic activity of purified tryptase from human lung mast cells. J Immunol. 1985;135(4):2762–7.
97. Thomas VA, Wheeless CJ, Stack MS, Johnson DA. Human mast cell tryptase fibrinogenolysis: kinetics, anticoagulation mechanism, and cell adhesion disruption. Biochemistry. 1998;37(8):2291–8.
98. Stack MS, Johnson DA. Human mast cell tryptase activates single-chain urinary-type plasminogen activator (pro-urokinase). J Biol Chem. 1994;269(13):9416–9.
99. Sillaber C. The mast cell as site of tissue-type plasminogen activator expression and fibrinolysis. J Immunol. 1999;162(2):1032–41.
100. Vivien D, Gauberti M, Montagne A, Defer G, Touzé E. Impact of tissue plasminogen activator on the neurovascular unit: from clinical data to experimental evidence. J Cereb Blood Flow Metab. 2011;31(11):2119–34.
101. Basheer M, Schwalb H, Nesher M, Gilon D, Shefler I, Mekori YA, et al. Mast cell activation by fibrinogen-related homologous c-terminal peptides (haptides) modulates systemic blood pressure. J Allergy Clin Immunol. 2010;126(5):1041–8.
102. van der Linden PW, Hack CE, Struyvenberg A, Roem D, Brouwer MC, de Boer JP, et al. Controlled insect-sting challenge in 55 patients: correlation between activation of plasminogen and the development of anaphylactic shock. Blood. 1993;82(6):1740–8.
103. Kaplan ZS, Zarpellon A, Alwis I, Yuan Y, McFadyen J, Ghasemzadeh M, et al. Thrombin-dependent intravascular leukocyte trafficking regulated by fibrin and the platelet receptors GPIb and PAR4. Nat Commun. 2015;6:7835.
104. Kitamura Y, Taguchi T, Yokoyama M, Inoue M, Yamatodani A, Asano H, et al. Higher susceptibility of mast-cell-deficient W/WV mutant mice to brain thromboembolism and mortality caused by intravenous injection of India ink. Am J Pathol. 1986;122(3):469.
105. Bueb JL, Mousli M, Landry Y, Regoli D. Structure–activity studies of bradykinin analogues on rat mast cell histamine release. Peptides. 1993;14(4):685–9.
106. Razin E, Marx G. Thrombin-induced degranulation of cultured bone marrow-derived mast cells. J Immunol. 1984;133(6):3282–5.
107. Cirino G, Cicala C, Bucci M, Sorrentino L, Ambrosini G, DeDominicis G, et al. Factor Xa as an interface between coagulation and inflammation. Molecular mimicry of factor Xa association with effector cell protease receptor-1 induces acute inflammation in vivo. J Clin Invest. 1997;99(10):2446–51.

Roles of Neutrophils in Stroke

Glen C. Jickling and Frank R. Sharp

1 Introduction

Neutrophils are among the first immune cells to respond to ischemic brain, and contribute to processes central to the pathogenesis of ischemic stroke including clot formation and atherosclerosis. Neutrophils have been implicated in worsening outcomes in several diseases including ischemic stroke, myocardial infarction, diabetic retinopathy, sickle cell disease, transfusion related acute lung injury, acute respiratory distress syndrome, and renal microvasculopathy [1].

Many of the neutrophil functions designed to kill pathogens also influence a variety of processes that can initiate or promote cerebral infarction. During the process of phagocytosis neutrophils produce Reactive Oxygen Species (ROS) such as superoxide and hypochlorous acid via NADPH oxidase and Myeloperoxidase (MPO), respectively, [2] which can directly injure the Blood Brain Barrier (BBB) and possibly brain cells. During the process of degranulation, normally designed to kill pathogens, neutrophils release proteases including MMPs (-1, -2, -8, -9, -13), elastase, cathepsin G, and Proteinase 3 that can degrade extracellular matrix and damage the BBB and brain cells. Neutrophils can also release or respond to pro-inflammatory cytokines (e.g., IL-1β, IL6, IL-8, TNF-α) and chemokines (MCP-1/CCL2, MIP-1alpha/CCL3,CCL5/CCR5) that could worsen ischemic brain injury [3]. A variety of neutrophil receptors interact with damaged endothelium and with platelets to promote clotting/thrombosis [4, 5] and promote atherosclerosis [5, 6] (Fig. 1). The current status of research in these areas will be summarized here.

G.C. Jickling (✉) • F.R. Sharp
Department of Neurology, MIND Institute Wet Labs Room 2415, University of California at Davis Medical Center, 2805 50th Street, Sacramento, CA 95817, USA
e-mail: gcjickling@ucdavis.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_14

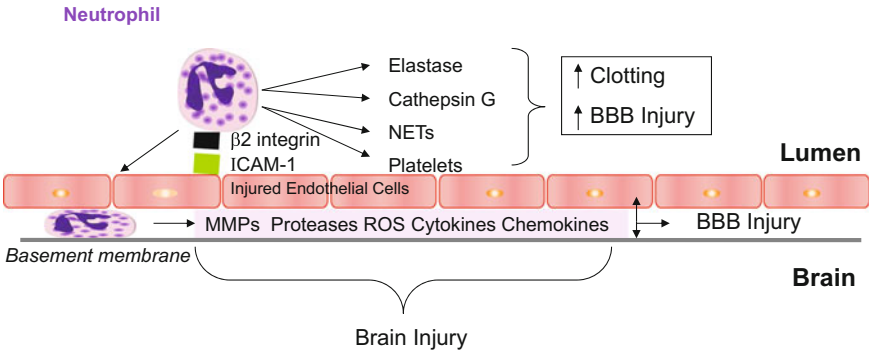

Fig. 1 Neutrophil activation and adhesion in acute ischemic stroke. Following ischemic stroke cytokines and damage associated molecular patterns are released. These promote neutrophil recruitment and activation, including the release of reactive oxygen species, proteases, cytokines. Neutrophils adhere to activated endothelium through adhesion molecules that promote neutrophil–endothelial interactions and neutrophil migration with resulting effects on the blood brain barrier and brain parenchyma (Abbreviations: *CCL*, CC-chemokine ligand; *CXCL*, CXC-chemokine ligand; *CCR*, CC-chemokine receptor; *CXCR*, CXC chemokine receptor; *DAMPs*, damage associated molecular patterns; *IL*, interleukin; *ILR*, interleukin receptor; *FPR*, formyl peptide receptor; *HMGB1*, high mobility group box 1; *HSP72*, heat shock protein 72; *ICAM-1*, intracellular adhesion molecule-1; *MAC-1*, macrophage 1 antigen; *MMP-9*, matrix metalloproteinase 9; *PSGL-1*, P-selectin glycoprotein ligand-1; *TLR*, toll-like receptor; *TNFa*, tumor necrosis factor alpha)

In assessing the role of neutrophils in acute ischemic stroke, this review focuses on the first few days following stroke. This is in part because most human and animal infarct volumes are stable by 24 h and do not change greatly thereafter for many days. In addition, the majority of the animal studies have focused on this early time period presumably for similar reasons. This focus, however, is not meant to say neutrophils do not have an impact on the long-term outcomes after stroke. As discussed shortly, human studies point to neutrophil numbers being extremely important in all types of cardiovascular and cerebrovascular disease in so far as risk of disease, severity of disease, and prognosis/outcomes. In addition, neutrophils have important roles prior to stroke since they promote clotting and atherosclerosis as noted below.

It is notable that several large clinical trials aimed at interfering with leukocyte/neutrophil function have failed in human stroke [7–9]. This is an important reminder that the roles of neutrophils in animal stroke models may or may not recapitulate the role of neutrophils in human stroke. Indeed, 50–70 % of leukocytes in humans are neutrophils, whereas only 10–25 % of rodent leukocytes are neutrophils [2]. Humans are more susceptible to infection than rodents, and infection clearly worsens stroke outcomes [10, 11]. Thus, suppression of neutrophil function could be more problematic in human than rodent stroke. Thus, identifying neutrophil targets most likely to benefit stroke outcome without affecting antimicrobial functions of neutrophils is likely important and is discussed where information is available.

The failure of anti-neutrophil therapies in humans has also raised the question of whether leukocytes and neutrophils in particular are a pathological variable in

ischemic stroke or simply a bystander reaction to damaged tissue not actually causing damage [12]. Many experimental studies do not demonstrate a causal role for neutrophils in brain injury following ischemic stroke [12]. For instance, demonstrating a change in infiltration of neutrophils or neutrophil markers with a given treatment does not demonstrate the neutrophils contributed to the injury. Even when a causal role is demonstrated, it is often not clear whether this would be relevant for one or all three major causes of stroke in humans: large vessel, cardioembolic, and small vessel/lacunar strokes. In addition, neutrophils appear to play a lesser role in stroke models where there is little or no reperfusion which may complicate translation to humans, and might limit treatments where there is probably little or no reperfusion in ischemic stroke. This chapter will consider some of these issues, and attempt to determine if there are any specific functions or molecules ascribed to neutrophils that might be causative in human stroke, not adversely affect anti-pathogen roles of neutrophils, and represent reasonable treatment targets.

2 Brief Biology of Pathogen Function of Neutrophils

Neutrophils are terminally differentiated cells that develop in the bone marrow [2, 5]). Once released into the circulation neutrophils seek signs of infection and inflammation via local cytokines, chemokines or damage associated molecular patterns (DAMPs) or PAMPs. Infectious or inflammatory activation of neutrophils leads to adhesion to vessel walls and migration inside tissue to the site of infection or inflammation. Notably, blood-derived neutrophils found in ischemic brain often show that they have just proliferated (are BrdU positive) [13].

When an infectious particle is encountered by neutrophils, it is phagocytosed. NADPH oxidase and MPO generate ROS, and proteolytic contents of neutrophil intracellular granules are released into the phagosomes to kill and digest the microorganisms [5]. Neutrophils have been found to form DNA based neutrophil extracellular traps (NETs) (described below) that immobilize pathogens to prevent spread and to also directly kill pathogens [2]. The neutrophils generally die via apoptosis within a day or two, though infection and inflammation can increase neutrophil survival. Once neutrophils die, macrophage removal of dead neutrophils is initiated [5] via a IL-23, IL-17, G-CSF pathway [2]. Besides this traditional role in infection, neutrophils also play a role in noninfectious inflammatory processes including ischemic stroke and heart disease. Evidence for this in humans has come in part from neutrophil responses in peripheral blood of patients with stroke.

3 Neutrophil Response in Ischemic Stroke

In patients with ischemic stroke, the number of circulating neutrophils rises within the first few hours of stroke onset [14] (Figs. 1 and 2). This increase is associated with stroke severity [15], infarct volume [16], and worse functional outcomes [17].

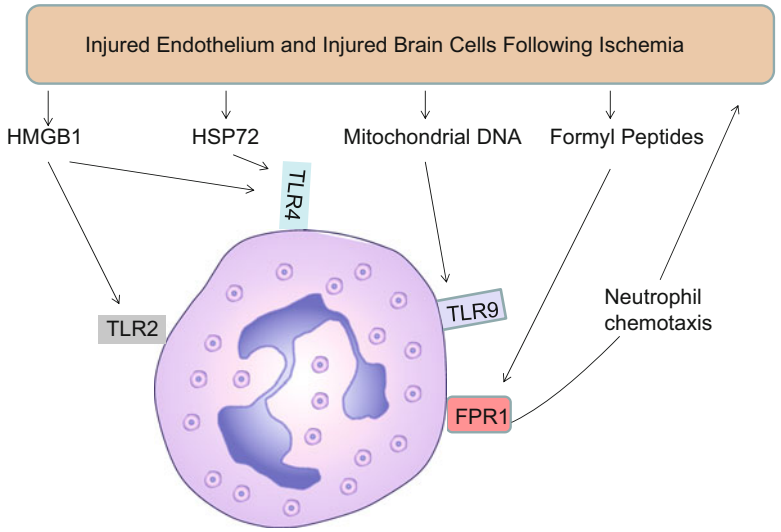

Fig. 2 Ischemic brain injury results in the release in a number of factors that activate circulating neutrophils. These include HMGB1, HSP72, mitochondrial DNA, and formyl peptides. Activated neutrophils are recruited to site of tissue injury

In contrast to neutrophils, lymphocytes decrease following ischemic stroke. Thus, the neutrophil to lymphocytes ratio is increased following stroke, and is associated with mortality and infarct size [18].

The rise in neutrophils following stroke occurs as a result of enhanced production, increased release from the bone marrow and spleen, and possibly from a reduction in neutrophil apoptosis [19]. Neutrophils express several endothelial adhesion molecules (PSGL-1, ESL-1, CD44, LFA-1, MAC-1) within 15 min of ischemia (Fig. 3). By 2 h, neutrophil rolling and adhesion is present in the pial vessels of the brain [20–22]. After 6–8 h, neutrophils have surrounded cerebral vessels and infiltration has begun [23, 24]. By 24–48 h of ischemic stroke, neutrophil infiltration into brain has peaked [22, 25]. The increase in neutrophils following ischemic stroke is associated with increased expression of adhesion molecules, cytokines/chemokines, proteases, and ROS.

A preponderance of data to date suggest neutrophil pro-inflammatory activation post stroke is associated with increased infarct size, increased BBB disruption, hemorrhagic transformation, and worse neurological outcomes. These studies are summarized below. However, often studies of neutrophils in stroke have used markers that are not specific to neutrophils and can be found on other immune cells. For example, MPO is often interpreted as a marker of neutrophils, however is also expressed by monocytes/macrophages and microglia. Thus when evaluating the role of neutrophils in stroke it is important to be cognizant that reported associations of neutrophils may also reflect a potential contribution from other immune cells.

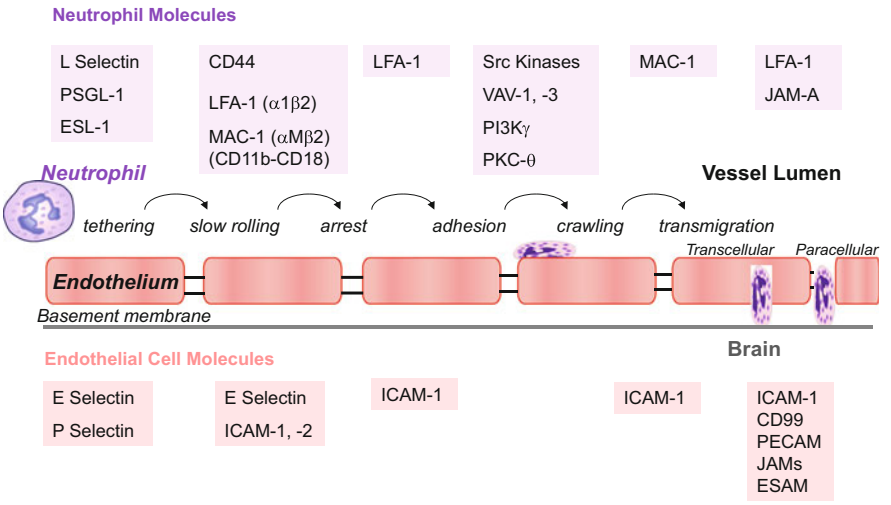

Fig. 3 Neutrophil recruitment, adhesion, and transmigration. Neutrophils express specific adhesion molecules that bind activated endothelium to promote adhesion and migration. In ischemic stroke the adhesion molecules expressed on neutrophils and endothelium involved in neutrophil tethering, rolling, arrest, adhesion, crawling, and transmigration remain poorly defined and may differ from the peripheral vasculature. Further studies are required to delineate the molecules involved in the neutrophil recruitment cascade following ischemic stroke and determine the extent of transmigration. (Abbreviations: "?" indicates unclear role in ischemic stroke; *ICAM-1*, intracellular adhesion molecule-1; *LFA-1*, lymphocyte function-associated antigen 1; *MAC-1*, macrophage 1 antigen; *PSGL-1*, P-selectin glycoprotein ligand-1)

4 Neutrophils and Thrombosis

Neutrophils are important contributors to clot formation (Fig. 4). When neutrophils are depleted, thrombus formation is reduced [26]. A neutrophil promotes thrombosis through several mechanisms including formation of tissue factor, interactions with platelets, release of NETs, and release of proteases that act on coagulation factors [26, 27].

Tissue factor interacts with coagulation factor VIIa to initiate activation of the extrinsic coagulation pathway and promote thrombus formation. Activated neutrophils are an important source of tissue factor [26, 27]. Whether neutrophil-derived tissue factor promotes thrombosis in ischemic stroke requires further study. Tissue factor is increased in rheumatoid arthritis, systemic lupus erythematosus, and Crohn's disease, each of which is associated with ischemic stroke. Neutrophil-derived tissue factor may contribute to this increased stroke risk.

Neutrophils have a number of interactions with platelets that result in enhanced platelet aggregation and clot formation [28]. Ligands expressed by neutrophils include PSGL-1 that binds platelet P-selectin, MAC-1 (CD11b-CD18, $\alpha_M\beta_2$) that binds platelet GPIbα, and binds a fibrin-glycoprotein IIbIIa (gp IIbIIIa, $\alpha_{IIb}\beta_3$ integrin)

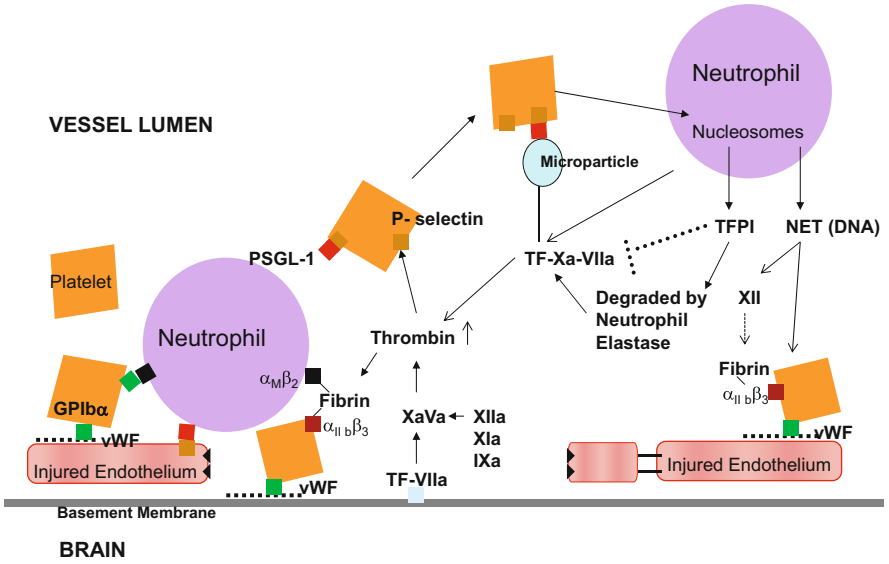

Fig. 4 Role of neutrophils in thrombus formation. Neutrophils promote thrombosis through interactions with platelets, proteolytic cleavage of clotting factors (TFPI, coagulation factor X), and release of prothrombotic molecules (neutrophil extracellular traps, tissue factor) (Abbreviations: *ICAM-1*, intracellular adhesion molecule-1; *MAC-1*, macrophage 1 antigen; *NET*, neutrophil extracellular traps; *PSGL-1*, P-selectin glycoprotein ligand-1; *TF*, tissue factor; *TFPI*, tissue factor pathway inhibitor; *vWF*, von Willebrand factor)

complex (Fig. 4). Platelets also release soluble CD40L that stimulate neutrophil expression of MAC-1. In ischemic stroke, neutrophil–platelet interactions may be important to thrombus formation and vessel occlusion. In patients with recent ischemic stroke, neutrophil–platelet complexes are increased [29]. The stroke prevention therapies dipyridamole and candesartan both inhibit neutrophil expression of adhesion molecules which prevent thrombus formation [30]. Abciximab and eptifibatide act on the gpIIbIIa receptor, which mediates neutrophil–platelet interactions. Clopidogrel blocks platelet activation and resultant confirmation change in gpIIbIIIa, and thus also prevents neutrophil–platelet interactions [31]. In sickle cell disease, ischemic stroke may result from altered neutrophil–platelet interactions and enhanced neutrophil-dependent platelet aggregation [32].

Neutrophil-derived proteases contribute to thrombus formation. Cathepsin G and elastase act on coagulation factor X to promote coagulation [33]. Elastase also degrades tissue factor pathway inhibitor alpha (TFPIα), which increases levels of tissue factor that promote clot formation [33]. Neutrophil cathepsin G plays a role in neutrophil–platelet interactions [34]. Inhibiting cathepsin G reduces bleeding time and has greater antithrombotic effect than aspirin. In rodent stroke, inhibiting cathepsin G improves cerebral blood flow, reduces brain injury, and improves behavioral outcomes [34]. Neutrophils also release the protease ADAMTS13, which cleaves hyperactive ultra-large von Willebrand factor (ULVWF) and affects

thrombosis in ischemic stroke [35]. Thus, targeting neutrophil proteases may have potential as novel antithrombotic therapies to prevent ischemic stroke [34].

NETs may also contribute to thrombus formation in ischemic stroke [36]. NETs are derived from neutrophils and composed primarily of DNA. Though they typically bind pathogens, NETs can trigger platelet activation and promote thrombus formation [37]. Blocking NET formation with DNase reduces clot formation [38]. NETs have been implicated in deep venous thrombosis but as yet not in ischemic stroke [36]. Neutrophils also release DNA–histone complexes that not only trap and eliminate pathogens but also promote thrombus formation. The thrombogenic potential of NETs is supported by the finding that DNase inhibits thrombus formation related to DNA–histone complexes [39].

5 Neutrophils in Atherosclerosis

Atherosclerosis is a major cause of ischemic stroke occurring in both the extracranial and intracranial vasculature that supplies the brain. Neutrophils contribute both to the formation of atherosclerosis and to the rupture of plaque that causes thrombosis and brain ischemia [40] (Fig. 5).

In atherosclerosis, neutrophils are recruited early to sites of endothelial injury through cytokines, chemokines, and adhesion molecules (Fig. 5). In mice deficient in the adhesion molecules P-selectin (platelets) [41], CD18 (neutrophils, monocytes), or ICAM-1 (endothelial cells), formation of atherosclerotic plaque is reduced [42]. P-selectin is important for platelet deposition in plaques. Platelets secrete CCL5 which acts on neutrophil CCR5 receptor to promote neutrophil recruitment to injured endothelium [43]. Neutrophils adhere to endothelium through CD18 and ICAM-1. In turn, recruited neutrophils promote monocyte recruitment via CRAMP (cathelicidin antimicrobial peptide) [5]. In mice, deletion of CRAMP or depletion of neutrophils reduces atherosclerosis [44, 45].

Chemokines and cytokines may be potential targets to reduce atherosclerosis in stroke (Fig. 5). Evasin-3 is a CXC chemokine-binding protein that inhibits neutrophil activation. When administered in rodent models, carotid atherosclerosis is decreased, intraplaque neutrophil content is reduced, and matrix metalloproteinase-9 (MMP-9) activity is diminished [46]. The beneficial effects of increased HDL (high-density lipoprotein) on atherosclerosis may be mediated in part through neutrophils. Increasing HDL decreases neutrophil activation by proinflammatory cytokines (TNF-α, IL-1, and IL-8) [6].

Neutrophils also contribute to atherosclerosis by promoting the formation of oxidized lipids (Fig. 5). Neutrophil NADPH oxidase and myeloperoxidase (MPO) are a major source of ROS that promote formation of oxidized low density lipoproteins (oxLDL). oxLDL are taken up by scavenger macrophages to form foam cells in atherosclerotic plaques (Fig. 5). Neutrophil-derived ROS also contribute to vessel injury and endothelial dysfunction in atherosclerosis, cause vascular smooth muscle proliferation, and activate MMPs which contribute to plaque rupture [6, 47].

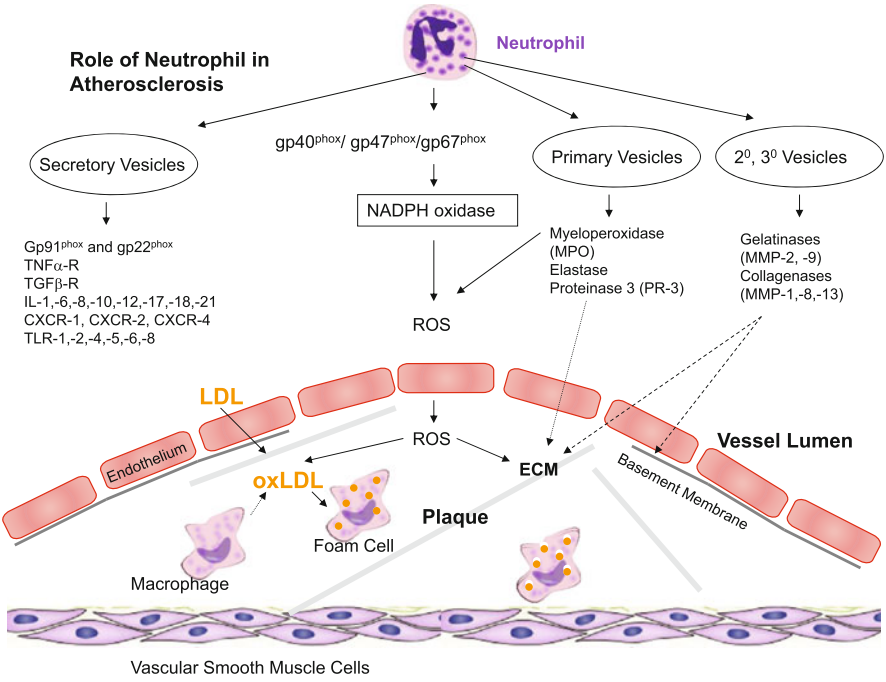

Fig. 5 Role of neutrophils in atherosclerosis. Neutrophils promote the formation of atherosclerosis though interactions with platelets and by enhancing monocyte infiltration into damaged endothelium. Neutrophils promote atherosclerotic plaque progression and rupture via the release of cytokines, reactive oxygen species that activated macrophage foam cells, producing oxidized-LDL, and proteolytic degradation of the fibrous cap (Abbreviations: *BM*, basement membrane; *CCL*, CC-chemokine ligand; *CRAMP*, cathelicidin antimicrobial peptide; *ECM*, extracellular matrix; *FPR*, formyl peptic receptor; *ICAM-1*, intracellular adhesion molecule-1; *IL*, interleukin; *INF-γ*, interferon gamma; *LL37*, Cathelicidin; *MAC-1*, macrophage 1 antigen; *MMP*, matrix metalloproteinase; *ox-LDL*, oxidized low density lipoprotein; *PSGL-1*, P-selectin glycoprotein ligand-1; *TLR*, toll-like receptor; *TNFα*, tumor necrosis factor alpha)

The beneficial effect of statins in atherosclerosis may relate in part to effects on neutrophils. Statins decrease the production of ROS by inhibiting neutrophil NADPH oxidase, as well as reducing neutrophil activation, adhesion molecule expression, and platelet interactions [6].

Proteases released by neutrophils also contribute to atherosclerosis (Fig. 5). Neutrophil-derived extracellular matrix-degrading proteases can promote endothelial dysfunction by degrading vascular basement membrane and type IV collagen. In turn, dysfunctional endothelium promotes additional neutrophil recruitment through increased expression of adhesion molecules (β2 integrins, P-selectin, E-selectin, ICAM-1) and IL-8. Neutrophils are an important source of MMPs which contribute to atherosclerosis. MMP-1, MMP-8, and MMP-13 have been shown to promote atherosclerosis in mice [48, 49] and to be overexpressed in human atherosclerotic plaque [50]. MMP-8 has also been associated with plaque instability [50], and genetic polymorphism in MMP-8 is related to progression of atherosclerosis

and plasma levels of the adhesion molecule VCAM-1 [49]. Neutrophils are also a primary source of MMP-9. MMP-9 promotes plaque instability and rupture through effects on extracellular matrix. Treatment with statins increases plaque stability in part by reducing neutrophil MMP-9 expression and neutrophil infiltration [51].

In humans, atherosclerotic plaque instability correlates with the presence of intra-plaque neutrophils [52]. Progression of aortic arch atheroma is associated with increased neutrophils in addition to increased risk of stroke and myocardial infarction [53]. In humans, an increase in neutrophil counts is associated with hypoechoic unstable carotid plaques, symptomatic carotid artery stenosis, and cerebral microembolization [54].

6 Neutrophil Response in Acute Ischemic Stroke: Humans

Neutrophils respond acutely to ischemic stroke, and remain activated for days to weeks (Fig. 1). CD11b expression increases on neutrophils by 15 min following experimental strokes and remains elevated for over 24 h [55]. Deep hypothermic cardiac arrest in humans immediately increases counts of activated neutrophils (CD11) [56]. After permanent middle cerebral artery occlusion (MCAO) in rodents, there is significant rolling and adherence of leukocytes in pial venules and arterioles by 2 h [20]. In rodent permanent MCAO models, neutrophil infiltration was moderate and typically within and adjacent to blood vessels bordering the infarcted cortex whereas temporary MCAO with reperfusion resulted in neutrophil infiltration throughout the infarcted cortex [57].

Labeled polymorphonuclear leukocytes (neutrophils) can be detected in the ischemic hemisphere by 1 h following cerebral ischemia in the dog [21]. Intravascular neutrophils in the rodent ischemic hemisphere peak around 6 h [22]. Neutrophil "invasion" into ischemic brain begins between 6 and 8 h after reperfusion in rodent stroke models [23, 24] (Fig. 1) and can peak in rodent brain as early as 24 h after MCAO [22, 25]. Cytokine-induced neutrophil chemoattractant (CINC) mRNA is increased by 6 h post MCAO, peaks at 12 h, and decreases but is still elevated at 24 h in a rat stroke model [58]. Animal studies that have tracked myeloperoxidase (MPO) find neutrophil numbers peak between 1 and 5 days following stroke and decrease thereafter whereas monocytes peak later between 3 and 7 days [24, 59–61]. One study disputes these time lines, finding that macrophages, lymphocytes, and dendritic cells enter cerebral infarcts prior to neutrophil entry (based upon flow cytometry), though equal numbers of neutrophils and activated microglia were observed at day 3 [62].

The inflammatory response post stroke can be quite prolonged, taking 12 months for fibrinogen and CRP levels to return to baseline levels [63]. IL-1ra, produced in part by neutrophils [64], takes 6 months to return to baseline following stroke whereas IL-6 levels, produced in part by neutrophils, return to baseline around 8 weeks after ischemic stroke [63]. Direct assessment of neutrophils in human stroke brain over time shows three groups: severe neutrophil accumulation that dramatically increased within 12 h after stroke onset and persisted beyond 30 days; those with moderate neutrophil accumulation that significantly decreased by 30 days; those with mild

neutrophil accumulation that decreased by 6–9 days [65]. In patients with severe neutrophil accumulation, neurological outcome was worse and infarction volume larger than in patients with less marked accumulation—suggesting the magnitude of the neutrophil response correlates with the magnitude of the brain injury [65]. Neutrophil numbers decrease in part after stroke because of apoptosis and engulfment by brain microglia [66] and engulfment by macrophages [60], and because of decreased release from bone marrow.

7 Neutrophil Biomarkers in Blood Following Acute Stroke: Humans

Additional evidence for neutrophil responses to acute stroke in humans comes from measurements of neutrophil biomarkers in serum and plasma. PSGL-1, P-selectin glycoprotein ligand 1 which binds to P-selectin on platelets and endothelial cells, showed increased expression on neutrophils and monocytes from day 1 to 90 after stroke as compared with control subjects ($p<0.05$) [67]. Neutrophil Mac-1 expression was significantly increased on days 1 and 7 after stroke compared to controls ($p<0.05$). Neutrophil PSGL-1 expression on day 1 was significantly higher in patients with early neurologic deterioration (END) ($p<0.01$) [67].

Neutrophil elastase from blood was elevated on the first day of a stroke (216.5+/−26.8 μg/L) and significantly increased by 7 days post stroke (384.7+/−31.4 μg/L) compared to control levels (67.4+/−5.2 μg/L) [68]. Plasma neutrophil gelatinase-associated lipocalin (NGAL) is increased in the first few days following acute ischemic stroke [69, 70] and can remain elevated for over a year following the stroke [70]. Neutrophil proteinase 4 (NP4) is also elevated in blood following ischemic stroke [69].

IL8, neutrophil chemotactic factor, is increased in serum within 24 h of ischemic stroke and is paralleled by an increase in blood neutrophils [71]. Though not specific for neutrophils, TNF-alpha, PAI-1, and tPA showed a significant predictive value of ischemic stroke, whereas IL-6, VICAM-1, ICAM-1, and neutrophil percentage showed only a slight or no association with stroke diagnosis [72]. Serum TNF-alpha, produced in part by neutrophils, rises early and remains elevated paralleling the prolonged leukocytosis following stroke [73].

8 Neutrophil Response to Acute Stroke: Animals

8.1 Neutrophils Detect Injury

The mechanisms by which neutrophils detect pathogens and organ injury and infiltrate tissue have been well studied [2, 74]. Though there have been an increasing number of studies on how neutrophils respond following stroke, many of the details are derived from peripheral blood vessels and the response to pathogens rather than

ischemic brain [2]. Thus, the following description is based upon a composite derived from various tissues.

Following a stroke, circulating neutrophils detect injury via changes in molecules on the endothelial surface and molecules released by damaged endothelial cells. Once attracted to an area of injury, neutrophil recruitment involves a series of steps: capture/tethering, slow rolling, arrest/adhesion, crawling, and transmigration which is mediated by a series of molecules (Fig. 3) [2, 74]. Attenuation of CXCR4 and activation of CXCR2 lead to neutrophil release from bone marrow in response to inflammation, accounting in part for increased circulating neutrophils [75]. Neutrophil recruitment to damaged endothelium is initiated by changes on the surface of endothelium that result from stimulation by inflammatory mediators including histamine, cysteinyl-leukotrienes (e.g., leukotrienes B4) and cytokines (e.g., IL-1β) and chemokines that are released by resident sentinel vascular leukocytes, brain endothelial cells, and brain microglia [2]. For example, chemokines activate neutrophil receptors including CCR1, CCR2, and CCR5 which mediate neutrophil recruitment to ischemic tissue [76]. CXCL1, -2, and -5 released from endothelial cells signal via neutrophil CXCR2 to activate neutrophils and promote adhesion to endothelium [2]. Endothelial cells can also be activated by DAMPS (HMGB1, Hsp72 from brain) that increase adhesion molecules.

Following infection or injury, P-selectin expression on endothelial cells increases within minutes after release from Weibel–Palade bodies, and E-selectin is synthesized in endothelial cells within 90 min of injury [2]. P-selectin and E-selectin on endothelial cell surfaces bind P-selectin glycoprotein ligand-1 on neutrophils to capture or tether them (Fig. 3) [74]. DAMPs released from injured endothelial and brain cells can also activate neutrophils (Fig. 2) which may promote adhesion.

Arrest, adhesion, and crawling are promoted by neutrophil integrins LFA-1 (also called α1β2 or β2 integrin C11a-CD18) and MAC1 (also called αM β2 or CD11b-CD18) (Fig. 3). Transmigration requires integrin interactions with CAMs (ICAM-1 or -2) for neutrophils to pass between endothelial cells (paracellular) or through endothelial cells (transcellular) and across the basement membrane [2, 74]. Once across the vessel wall, neutrophils infiltrate infected tissue via a second gradient of molecules [2]. However, following stroke it is not as clear whether neutrophils infiltrate tissue very much if at all. In fact most pathological studies show neutrophils on the luminal and abluminal sides of vessels in infarcted brain suggesting they are mostly perivascular [22]. A recent study challenges the concept that neutrophils actually enter ischemic brain tissue [77]. Using an in vivo transient MCAO model in mice, neutrophils were mainly restricted to luminal surfaces or perivascular spaces of cerebral vessels and virtually no PMNs entered the infarcted CNS parenchyma in a period from 1 h to 2 weeks after the stroke. Vascular PMN accumulation did not correlate with vessel permeability, expression of endothelial cell adhesion molecules, platelet aggregation, or release of NETs. The absence of PMN infiltration within infarcted brain per se was supported by studies of 25 human stroke specimens collected at early time points after infarction [77]. This study challenges the conceptual framework of many previous studies, and might suggest that neutrophil interactions with the BBB (endothelium, pericytes, basement membrane, perhaps astrocytes) might explain protective effects of modulating neutrophil functions.

9 Regulating Neutrophils Response in Acute Ischemic Stroke: Animal Models

Given the above biology, a large number of studies, some of which date back several decades, have suggested that modulating interactions between neutrophils and endothelium [78, 79] was associated with decreased neutrophil responses to ischemic brain [80, 81] and improved outcomes following ischemic stroke. Although many of the approaches were not specific for blocking neutrophils, they have provided proof of principle. A summary of selected studies shows there is still much to be learned and more specific approaches are needed in order to develop possible treatments for acute stroke targeted just at neutrophils.

9.1 Cellular Adhesion Molecules

In early experimental studies, ICAM-1 was shown to be upregulated following ischemic stroke. An antibody to ICAM-1 reduced ischemic cell damage after transient but not permanent MCAO in Wistar rats [82]. Knockout of ICAM-1 and immunodepletion of neutrophils decreased infarct volume and mortality and improved outcome following transient MCAO in mice [83]. Another study showed knockout of ICAM-1 decreased infarct volume in transient and permanent MCAO models without having much effect on neutrophil infiltration into ischemic brain, and depletion of neutrophils further decreased infarct volume following transient MCAO [84]. These and other studies led to a study with an antibody (Enlimomab) targeted against ICAM-1 which failed in human ischemic stroke [8] and is discussed below.

At baseline type 2 diabetic rats have higher neutrophil CD11 and endothelial sICAM, and then following stroke have higher levels of both molecules, have greater neutrophil adhesion and cell aggregates and worse outcomes following stroke [85]. The platelet inhibitor cilostazol appears to act on endothelial cells to inhibit expression of adhesion molecules including ICAM-1 and neutrophil adhesion induced by high glucose through increasing NO production [86]. Anti-VCAM-1 antibodies do not protect against focal cerebral ischemia in rats or mice [87]. A problem with targeting CAMs has generally been that this is nonspecific and would prevent adhesion of not only neutrophils but also other leukocytes.

9.2 CD11/CD18

Macrophage-1 antigen (Mac-1) (CD11b/CD18), also called complement receptor 3 (CR3), is a leukocyte beta2 integrin and facilitates leukocyte adhesion, transendothelial migration, phagocytosis, and respiratory burst, all of which may mediate reperfusion-induced injury to ischemic brain tissue. CD11b/CD18 is found not only on neutrophils, but also on monocytes/macrophages and Natural Killer cells and binds to endothelial cell ICAM-1. Pre-ischemic administration of an anti-CD18 monoclonal

antibody increased reflow in microvessels in a primate stroke model [88]. Postischemic treatment at 2–4 h [89, 90] and at 20 min after transient MCAO [91] with anti-CD11b/CD18b monoclonal antibodies decreased infarct volumes and neutrophil migration into ischemic brain. Mice with knockouts of CD18 have smaller infarcts, decreased mortality, and decrease of neutrophils in ischemic brain following transient MCAO [92, 93], though there were no differences following permanent MCAO [93].

9.3 Neutrophil Inhibitory Factor

Neutrophil inhibitory factor (NIF) blocks neutrophil adhesion by binding the CD11a and CD11b beta 2 integrins. Administration of recombinant neutrophil inhibitory factor (rNIF) beginning at 2–4 h after temporary MCAO in adult rats decreases infarct volumes at 7 days, but had no effect if treatment were continued for 6 h rather than 48 h, and had no effect in permanent MCAO [94, 95]. In another study, administration of NIF at 4 and 6 h post MCAO decreased infarct volume and brain swelling and improved functional outcomes [96]. A human trial of NIF failed to show improvement in acute ischemic stroke [9]. Though the reasons for the failure are discussed below, it is important to note that NIF would affect not only neutrophils but any beneficial roles of monocytes and Natural Killer cells.

9.4 CXCR1/2 and CCR2

CXCR1/2 receptors (IL8 receptors) are involved in recruitment of neutrophils into infected or injured tissue. IL8 is released by endothelial cells and monocytes (and neutrophils themselves) to act on CXCR1/2 receptors to recruit neutrophils. Blockade of CXCR1/2 chemokine receptors with the drug reparixin protected against brain damage in ischemic stroke in mice, decreased MPO staining in the stroke, decreased IL1beta, and improved motor outcomes in two studies [97, 98]. However, two other studies were negative. The CXC chemokine-binding protein Evasin-3 potently inhibits chemokine bioactivity and related neutrophilic inflammation in two mouse models of carotid atherosclerosis [46]. In carotid atherosclerosis, treatment with Evasin-3 was associated with reduction in intraplaque neutrophil and MMP-9 content and weak increase in collagen as compared with Vehicle. However, in acute stroke, Evasin-3 decreased neutrophil infiltration and oxidative stress but without improving stroke outcomes [46]. In a similar study, administration of a CXCR2 antagonist (SB225002) reduced expression of CXC chemokine subfamily of genes and neutrophil-related infiltration following SB225002 administration but did not improve outcome after cerebral ischemia-reperfusion [99]. Of note the CXCR2 ligand, CXCL5, is increased in CSF but not blood of patients within 24 h of ischemic stroke [100]. CXCL1, another neutrophil attractant, is increased in CSF but not serum of patients with ischemic stroke [101].

CCR2 is another neutrophil receptor that mediates recruitment. Though CCR2 knockout mice have decreased infarct volumes, decreased edema, and decreased

infiltration of both neutrophils and monocytes into ischemic brain [102], this has yet to be confirmed in independent studies. In addition, blocking CCR2 would not selectively block neutrophils.

9.5 Cannabinoid Receptors/CXCL2

Cannabinoid 2 receptor (CB(2)) agonists decrease ischemic brain injury in an MCAO model [103]. They do this by acting on neutrophil CB(2) receptors and activating p38 in neutrophils via a chemokine CXCL2 dependent mechanism which inhibits neutrophil entry into ischemic brain [103]. In addition cannabinoids may decrease cerebral infarct size in part by decreasing HGMB1 in plasma and decreasing TLR4 activation of neutrophils [104].

9.6 P- and E-Selectin

P- and E-selectin are constitutively expressed in endothelial cells and facilitate entry of leukocytes into brain. When P-selectin is stimulated by thrombin, it translocates from cytoplasmic granules to the plasma membrane. Pro-inflammatory mediators like TNF alpha and lipopolysaccharide (LPS) induce P-selectin mRNA. Administration of a humanized antihuman E- and P-selectin monoclonal antibody decreases stroke volume and improved functional outcomes in a primate stroke model without evidence of immunosuppression [105]. This antibody would bind endothelial E- and P-selectins and would tend to decrease infiltration of all leukocytes including neutrophils into ischemic brain [105]. P-selectin knockout mice have decreased damage to the BBB and marked decrease of neutrophil infiltration following MCAO compared to wild type mice [106] and P-selectin KO mice have smaller infarcts [107]. In one study post stroke anti-P-selectin antibody decreased infarct volumes [107], though in another pretreatment but not post treatment with a P-selectin antibody decreased infarct volume following transient MCAO [108]. An E-selectin antibody decreases infarct volume and neutrophil accumulation following transient MCAO in mice [109]. An L-selectin antibody did not protect against focal cerebral ischemia [110], even though it plays a role in adhesion of neutrophils and other leukocytes to endothelial cells [111].

9.7 CD47

CD47 is a cell surface glycoprotein that helps mediate neutrophil transmigration across blood vessels. Knockout of CD47 decreased infarct volumes at 24 and 72 h after a 90 min (temporary) MCAO [112]. CD47ko also decreased edema at 72 h, ameliorated decreases of claudin-5, markedly decreased MMP-9, and decreased neutrophil numbers in brain infarcts [112].

9.8 *Slit1/Robo1*

In the normal brain, the presence of Slit1 on PMNs, and Robo1 on cerebral endothelial cells, generated a repulsive force to prevent the infiltration of PMNs into the brain [113]. During stroke recovery, a transient reduction in Robo1 expression on the cerebral endothelial cells allowed the uncontrolled infiltration of Slit1-expressing PMNs into the brain causing inflammatory reactions [113]. Similarly, lack of the immune receptor CCR5 on brain cells was associated with larger infarcts and worse motor deficits compared to wild type mice, and this was associated with greater influx of neutrophils into the infarct [114].

9.9 *DAMPs/Toll-Like Receptors*

Ischemic brain injury results in the release of a number of DAMPs, including HMGB1, Hsp72, S100A9, peroxiredoxin, mitochondrial peptides, and extracellular nucleic acids (DNA, RNA) [115–117] (Figs. 1 and 2). DAMPs act on neutrophils through specific receptors (toll-like receptors (TLR), co-receptors, FPR1) [115], and result in a pro-inflammatory response involving the production of cytokines, proteases, and ROS [115]. Targeting DAMPS and TLRs has shown promise as a treatment in ischemic stroke. When HMGB1 levels in plasma are reduced with cannabinoids, there is a reduction in infarct size and activated neutrophils [104]. Neutralization of peroxiredoxin with antibodies reduces inflammatory response and infarct volume growth [117]. In patients with ischemic stroke, increased neutrophil expression of TLR4 on day 3 and 7 is associated with worse stroke outcome and infarct volume [118]. When TLR4 is knocked out in mice, ischemic brain injury is reduced [119]. Despite the absence of TLR4, MPO+ cells and Iba1+ microglial cells were increased in brain, suggesting an increase of brain MPO+ cells (which could be neutrophils or macrophage/microglia) does not necessarily equate to worsening of brain injury in stroke.

10 Neutrophil Mediated BBB Disruption in Ischemic Stroke

The BBB is disrupted following ischemic stroke. Neutrophils contribute to this disruption through the release of proteases (MMPs, elastase, cathepsin G, proteinase 3), ROS and during the process of migrating across cerebral endothelium [2, 77, 120] (Fig. 1). BBB disruption plays an important role in poststroke cerebral edema and hemorrhagic transformation (HT). The importance of neutrophils in BBB disruption and HT is highlighted by the fact that neutrophil proteins (MMP-9) and genes (LTF, NGAL, CEACAM8, CRISP3) are predictive of HT in patients with stroke [121]. Furthermore, inhibiting or depleting neutrophils reduces BBB disruption and the rate of HT [120, 122]. In contrast, promoting neutrophil activation with LPS enhances BBB disruption in rodent stroke [123]. Thus, targeting neutrophil mediated BBB disruption may have utility to reduce cerebral edema and the rate of HT post stroke.

10.1 Neutrophil MMP-9

Neutrophils are an important source of MMP-9 within the first 24 h of ischemic stroke. MMP-9 is increased in plasma within the first 2–6 h of stroke in patients, primates, and rodents [124, 125]. We found increased leukocyte MMP-9 mRNA within 3 h of stroke [126, 127]. Increased plasma MMP-9 levels correlate with BBB disruption and predict tPA related HT [128]. In humans with ischemic stroke, MMP-9-positive neutrophil infiltration is associated with BBB breakdown, basal lamina type IV collagen degradation, and hemorrhagic transformation (HT) [129].

Targeting neutrophil MMP-9 has been evaluated as a treatment in ischemic stroke. Mice lacking leukocyte MMP-9 have decreased BBB disruption and infarct size [130] which appears to be mediated by leukocyte MMP-9 and not brain MMP-9 [130, 131]. In neutrophils, MMP-9 is regulated by carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). Knocking down CEACAM1 promotes neutrophil MMP-9 expression and enhances BBB breakdown in ischemic stroke [132]. Pharmacological inhibition of MMP-9 post stroke also reduces BBB disruption and HT [133]. For example, the rate of HT in rats can be reduced with BB-94, a broad spectrum MMP inhibitor, and minocycline, an inhibitor of MMP-9 and microglia [134]. Ongoing studies are evaluating the role of minocycline to reduce HT in stroke patients. Though early inhibition of MMP-9 reduces HT in animals, delayed MMP-9 inhibition enhances brain injury and worsens stroke in part by impairing vascular remodeling [135].

Granulocyte colony-stimulating factor (G-CSF) has activity to induce neutrophil mobilization. In a rat MCAO stroke model, G-CSF increases peripheral neutrophil numbers at 24 h, and this is associated with an increase in MMP-9 and tPA related hemorrhagic transformation [136]. G-CSF was found to potentiate neutrophil release of MMP-9 in the presence of tPA. In humans, G-CSF administered following ischemic stroke has also been found to increase peripheral leukocyte count. This increase was not associated with any improvement in neurological outcome at 90 days, nor an increase risk of HT. Though the rationale as to why G-CSF did not show clinical benefit is unclear, it may have enhanced early mobilization of inflammatory neutrophils which offset any potential beneficial effect of G-CSF.

10.2 Neutrophil Elastase

Neutrophil elastase degrades basal lamina and extracellular matrix. In experimental stroke pharmacological inhibition of neutrophil elastase reduces BBB permeability, decreases cerebral edema, and improves neurological outcomes [137]. In mice lacking elastase, ischemia-induced BBB disruption is reduced, as is infarct volume, cerebral edema, and leukocyte-endothelial adhesion [138]. Furthermore, pharmacological inhibition of elastase in MMP-9-null mice further decreased infarct volume and BBB disruption, indicating effects independent of MMP-9 [138]. These results are important since they suggest that combined inhibition of MMP-9 and elastase may be more effective than either alone.

10.3 Neutrophil ROS

Neutrophils are an important source of ROS following stroke and reperfusion of ischemic brain [140, 148]. ROS disrupt the neurovascular unit through damage to endothelial cells, pericytes, smooth muscle cells, and astrocytes. This results in increased BBB permeability, cerebral edema, and hemorrhagic transformation.

Superoxide radicals are mediators of reperfusion-induced BBB disruption [139]. Neutrophils generate superoxide by the enzyme nicotinamide adenine dinucleotide phosphate oxidase (NOX). Inhibition of superoxide results in reduced BBB disruption in rodents. When NOX is inhibited with apocynin or by genetic ablation, the severity of BBB disruption is reduced, as are infarct volumes and the degree of neutrophil infiltration [140, 141].

Several studies have evaluated ROS as a target to reduce BBB disruption and HT in ischemic stroke. Though animal studies of the spin trap agent NXY-059 showed promise [142], it failed to improve stroke outcome or reduce hemorrhagic transformation (HT) in stroke patients [143]. Another spin trap agent, N-t-Butyl-Phenylnitrone (PBN), also reduces tPA related HT in rodent stroke [144] but worsens HT in rabbit stroke [145]. The free radical scavenger edaravone decreases BBB disruption and HT in rodent stroke [146] but increased HT in stroke patients [147]. Benidipine, a dihydropyridine calcium channel blocker, also inhibits neutrophil ROS production and improves stroke outcomes in rats [148]. Given these variable results, evaluation of candidate compounds in multiple animal models of HT may be beneficial [149].

11 Stroke, Infection, and Neutrophils

Infections, including pneumonia and urinary tract infections, worsen outcomes in patients with ischemic stroke [150–152]. The mechanisms for this worsening appear to be related in part to neutrophils. In one animal study, systemic inflammation was induced in mice by peripheral interleukin-1beta (IL-1beta) challenge and a transient MCAO performed [153]. This resulted in conversion of a transient to a sustained disruption of the tight junction protein, claudin-5, and also markedly exacerbated disruption to the cerebrovascular basal lamina protein, collagen-IV. These alterations were associated with a systemic inflammation-induced increase in neurovascular gelatinolytic activity that was mediated by a fivefold increase in neutrophil-derived MMP-9 in ischemic brain. MMP-9 inhibition attenuated the deleterious impact of systemic inflammation on brain damage, edema, neurological deficit, and incidence of hemorrhagic transformation [153]. Influenza infection has also been found to worsen rodent stroke in part by increasing release of cytokines and neutrophil-derived MMP-9 that are mediated by α7 nicotinic acetylcholine receptors [154].

Another study has implicated neutrophils in worsening outcome from systemic inflammation [123]. Systemic inflammation was produced by intraperitoneal LPS (a gram-negative bacteria coat protein) followed by a transient MCAO. Brain damage and neurological deficit were worsened by LPS. Administration of an IL-1 receptor antagonist abolished the effect of LPS on brain damage. Systemic IL-1 increased ischemic damage to a similar extent as LPS. Neutropenia abolished the damaging effects of systemic IL-1. The data show that LPS induction of neutrophil-induced IL-1, perhaps via neutrophil mobilization via CXC chemokine induction, worsens stroke outcomes following systemic inflammation. The results may help to explain the poorer outcome in stroke patients presenting with infection [123].

12 Protective Role of Neutrophils in Stroke

Though the cerebral ischemia field generally lumps all neutrophils into "bad guys," it would be important to know if neutrophils serve any neuroprotective functions. If they do have protective functions, it would be useful to avoid inhibiting these functions if anti-neutrophil treatments are to be designed to treat stroke. As described above, patients with stroke are at risk for infections, and infection clearly worsens in stroke in patients and in animal models of stroke [123, 154–156]. Therefore, significantly inhibiting the function of neutrophils in fighting infections might worsen outcomes—as discussed in the human trials below—and any neutrophil therapy for stroke should take this into consideration.

In addition, it has been suggested that neutrophils might adopt a pro-inflammatory N1 phenotype or an anti-inflammatory N2 phenotype in the CNS depending on environmental cues [157]. The N2, anti-inflammatory phenotype, may be adopted when neutrophil numbers exceed a critical threshold.

Clearly protective roles of neutrophils have been described including neutrophils performing wound debridement, and simply removing neutrophils from an inflamed tissue can result in more tissue pathology even in the setting of sterile injury [158]. MMP-9, derived either from brain or neutrophils, activates VEGF to promote revascularization at sites of injury [159]. Neutrophils facilitate recruitment of monocytes to inflamed tissue, and by promoting their own removal from tissue contribute to resolution of inflammation and promote repair [2]. During termination of inflammation, neutrophils release several anti-inflammatory molecules. These include annexin-1, lipoxin A4, resolvins, and protectins that decrease neutrophil migration and recruitment [2]. These molecules or their agonists might also be worth testing in acute ischemic stroke.

A recent dose escalation study of G-CSF in acute stroke did not detect any deleterious effects in spite of expected increases of both neutrophils and monocytes in a 12 h time window post stroke [160]. In animal studies, G-CSF knockout animals have larger infarcts than controls, and administration of G-CSF decreases infarct size whereas it increases peripheral but not brain neutrophil counts [161].

13 Human Stroke Trials Using Immunotherapy

Animal studies in the 1990s demonstrated excellent outcomes in animal stroke models following a variety of therapies that modulated the immune system, including antibodies against ICAM and modulating NIF/CD18 [42, 80, 82–84, 91, 93]. These studies led to two clinical trials both of which were negative and led to loss of enthusiasm for this approach. A third trial (LeukArrest, anti-CD11/CD18) was also performed and stopped during the trial because it was unlikely to show benefit [162], though the final results were never reported.

The results of the Enlimomab Acute Stroke Trial were published in 2001 [8]. Patients with ischemic stroke ($n=625$) were randomized to anti-ICAM antibody (Enlimomab) or placebo within 6 h of stroke onset and treatment continued for 5 days. At 90 days more patients died with Enlimomab and fewer were symptom free compared to placebo ($p=0.004$). There were more infections and fever with Enlimomab compared to placebo, and patients with fever had worse outcomes [8]. After the trial it was realized that the Enlimomab antibody may not have been sufficiently humanized and may have stimulated an immune response on its own [162]. A follow-up animal study to the human Enlimomab trial provided some insight into why this particular trial may have failed. It was found that administration to rats of a murine antibody preparation against ICAM-1, 1A29, elicits the production of host antibodies against the protein, activation of circulating neutrophils, complement activation, and sustained microvascular activation [163]. 1A29 did not improve stroke outcome in the rat or mouse, but pre-administration in the rats worsened stroke outcomes. Thus Enlimomab may have inadvertently stimulated an immune response that worsened stroke outcomes.

An additional reason the Enlimomab trial may not have shown benefit was the nonspecific leukocyte target ICAM-1. Blocking ICAM-1 would be expected to interfere with adhesion of almost all types of leukocytes and potentially decrease response to infection. Six enlimomab-treated stroke patients developed meningitis (none for placebo), suggesting that the anti-ICAM treatment impaired host responses to infection and this may have contributed to worsening of outcomes. Future immune therapy trials should carefully consider and test whether a potential therapy would significantly impair host responses to infections. Since rodents tend to be resistant to many infections, additional animal stroke models with vulnerability to infections similar to that of humans should be tested.

Another human trial of immunotherapy in stroke also failed to show benefit in ischemic stroke. In animals NIF had been shown to prevent neutrophil adhesion to endothelium by inhibition of CD11a and CD11b beta(2) integrins [164] and CD18 on PMNs [165]. In addition, UK-279,276 (NIF) reduced infarct volume in a rat MCAO reperfusion model [165]. Based on these and other animal data ASTIN (Acute Stroke Therapy by Inhibition of Neutrophils) was an adaptive phase 2 dose–response-finding, proof-of-concept study to establish whether UK-279,276 improved recovery in acute ischemic stroke [9]. 966 patients were entered into the trial and treated within 6 h of symptom onset. UK-279,276 did not improve recovery

in acute ischemic stroke patients [9], but it did not produce serious side effects and did not increase infections or fever. Reasons for the failure of the trial could have included: (1) blocking neutrophil adhesion does not improve stroke outcomes in humans but does so in rodents (2) the dose or timing of administration did not produce the desired biological effect (3) the effects of UK-279,276 on neutrophils are different in rodents compared to humans. This trial did suggest that failure was not related to an adverse effect on host immune response to pathogens.

14 Conclusions

The future of modulating neutrophils in ischemic stroke is thus uncertain. It seems clear that specific inhibition of this cell type might be desirable compared to a global suppression of the immune response. Moreover, approaches that maintain some response to pathogens would appear to be preferred. It is interesting that low dose G-CSF increased leukocyte counts post stroke in humans, including neutrophil counts, without worsening clinical status [166]. With a recent study suggesting neutrophils do not infiltrate ischemic brain but rather remain intravascular or perivascular [77], the role of targeting neutrophils to reduce ischemic brain injury seems unclear. Instead, targeting neutrophil effects on the BBB and neurovascular unit may be more fruitful. Though neutrophil MMPs and other proteases (elastase, cathepsin G) disrupt the BBB in stroke, additional studies are required to determine whether their inhibition can improve human ischemic stroke. Targeting neutrophils to prevent future stroke may also be beneficial. As our understanding advances regarding the roles of neutrophils thrombosis and atherosclerotic plaque rupture, modulating neutrophils may serve as a novel method to prevent stroke.

15 Author Contribution Statement

All authors contributed to the presented work and made critical revision of the manuscript for important intellectual content.

Acknowledgments We thank the National Institutes of Health/NINDS and the American Heart Association for support that made this review possible. Aspects of this manuscript have been published in the Journal of Cerebral Blood Flow and Metabolism (35(6):888–901).

References

1. Segel GB, Halterman MW, Lichtman MA. The paradox of the neutrophil's role in tissue injury. J Leukoc Biol. 2011;89(3):359–72.
2. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013;13(3):159–75.

3. Jin R, Yang G, Li G. Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. J Leukoc Biol. 2010;87(5):779–89.
4. Ruf W, Ruggeri ZM. Neutrophils release brakes of coagulation. Nat Med. 2010;16(8): 851–2.
5. Mocsai A. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. J Exp Med. 2013;210(7):1283–99.
6. Carbone F, Nencioni A, Mach F, Vuilleumier N, Montecucco F. Pathophysiological role of neutrophils in acute myocardial infarction. Thromb Haemost. 2013;110(3):501–14.
7. Muir KW, Tyrrell P, Sattar N, Warburton E. Inflammation and ischaemic stroke. Curr Opin Neurol. 2007;20(3):334–42.
8. Enlimomab Acute Stroke Trial Investigators. Use of anti-ICAM-1 therapy in ischemic stroke: results of the Enlimomab Acute Stroke Trial. Neurology. 2001;57(8):1428–34.
9. Krams M, Lees KR, Hacke W, Grieve AP, Orgogozo JM, Ford GA. Acute stroke therapy by inhibition of neutrophils (ASTIN): an adaptive dose–response study of UK-279,276 in acute ischemic stroke. Stroke. 2003;34(11):2543–8.
10. Ulm L, Harms H, Ohlraun S, Reimnitz P, Meisel A. Impact of infections on long-term outcome after severe middle cerebral artery infarction. J Neurol Sci. 2012;319(1–2):15–7.
11. Yousuf S, Atif F, Sayeed I, Wang J, Stein DG. Post-stroke infections exacerbate ischemic brain injury in middle-aged rats: immunomodulation and neuroprotection by progesterone. Neuroscience. 2013;239:92–102.
12. Emerich DF, Dean 3rd RL, Bartus RT. The role of leukocytes following cerebral ischemia: pathogenic variable or bystander reaction to emerging infarct? Exp Neurol. 2002;173(1):168–81.
13. Barreto GE, Sun X, Xu L, Giffard RG. Astrocyte proliferation following stroke in the mouse depends on distance from the infarct. PLoS One. 2011;6(11), e27881.
14. Ross AM, Hurn P, Perrin N, Wood L, Carlini W, Potempa K. Evidence of the peripheral inflammatory response in patients with transient ischemic attack. J Stroke Cerebrovasc Dis. 2007;16(5):203–7.
15. Kim J, Song TJ, Park JH, Lee HS, Nam CM, Nam HS, et al. Different prognostic value of white blood cell subtypes in patients with acute cerebral infarction. Atherosclerosis. 2012;222(2):464–7.
16. Buck BH, Liebeskind DS, Saver JL, Bang OY, Yun SW, Starkman S, et al. Early neutrophilia is associated with volume of ischemic tissue in acute stroke. Stroke. 2008;39(2):355–60.
17. Kumar AD, Boehme AK, Siegler JE, Gillette M, Albright KC, Martin-Schild S. Leukocytosis in patients with neurologic deterioration after acute ischemic stroke is associated with poor outcomes. J Stroke Cerebrovasc Dis. 2013;22(7):e111–7.
18. Gokhan S, Ozhasenekler A, Mansur Durgun H, Akil E, Ustundag M, Orak M. Neutrophil lymphocyte ratios in stroke subtypes and transient ischemic attack. Eur Rev Med Pharmacol Sci. 2013;17(5):653–7.
19. Tsai NW, Chang WN, Shaw CF, Jan CR, Lu CH. Leucocyte apoptosis in patients with acute ischaemic stroke. Clin Exp Pharmacol Physiol. 2010;37(9):884–8.
20. Kataoka H, Kim SW, Plesnila N. Leukocyte–endothelium interactions during permanent focal cerebral ischemia in mice. J Cereb Blood Flow Metab. 2004;24(6):668–76.
21. Hallenbeck JM, Dutka AJ, Tanishima T, Kochanek PM, Kumaroo KK, Thompson CB, et al. Polymorphonuclear leukocyte accumulation in brain regions with low blood flow during the early postischemic period. Stroke. 1986;17(2):246–53.
22. Garcia JH, Liu KF, Yoshida Y, Lian J, Chen S, del Zoppo GJ. Influx of leukocytes and platelets in an evolving brain infarct (Wistar rat). Am J Pathol. 1994;144(1):188–99.
23. Watcharotayangul J, Mao L, Xu H, Vetri F, Baughman VL, Paisansathan C, et al. Post-ischemic vascular adhesion protein-1 inhibition provides neuroprotection in a rat temporary middle cerebral artery occlusion model. J Neurochem. 2012;123 Suppl 2:116–24.
24. Barone FC, Hillegass LM, Tzimas MN, Schmidt DB, Foley JJ, White RF, et al. Time-related changes in myeloperoxidase activity and leukotriene B4 receptor binding reflect leukocyte influx in cerebral focal stroke. Mol Chem Neuropathol. 1995;24(1):13–30.

25. Tu XK, Yang WZ, Shi SS, Wang CH, Zhang GL, Ni TR, et al. Spatio-temporal distribution of inflammatory reaction and expression of TLR2/4 signaling pathway in rat brain following permanent focal cerebral ischemia. Neurochem Res. 2010;35(8):1147–55.
26. Darbousset R, Thomas GM, Mezouar S, Frere C, Bonier R, Mackman N, et al. Tissue factor-positive neutrophils bind to injured endothelial wall and initiate thrombus formation. Blood. 2012;120(10):2133–43.
27. von Bruhl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. J Exp Med. 2012;209(4):819–35.
28. Jin R, Yu S, Song Z, Zhu X, Wang C, Yan J, et al. Soluble CD40 ligand stimulates CD40-dependent activation of the beta2 Integrin Mac-1 and protein kinase C Zeda (PKCzeta) in neutrophils: implications for neutrophil–platelet interactions and neutrophil oxidative burst. PLoS One. 2013;8(6), e64631.
29. McCabe DJ, Harrison P, Mackie IJ, Sidhu PS, Purdy G, Lawrie AS, et al. Increased platelet count and leucocyte–platelet complex formation in acute symptomatic compared with asymptomatic severe carotid stenosis. J Neurol Neurosurg Psychiatry. 2005;76(9):1249–54.
30. Hallevi H, Hazan-Halevy I, Paran E. Modification of neutrophil adhesion to human endothelial cell line in acute ischemic stroke by dipyridamole and candesartan. Eur J Neurol. 2007;14(9):1002–7.
31. Vickers JD. Binding of polymerizing fibrin to integrin alpha(IIb)beta(3) on chymotrypsin-treated rabbit platelets decreases phosphatidylinositol 4,5-bisphosphate and increases cytoskeletal actin. Platelets. 1999;10(4):228–37.
32. Gavins FN, Russell J, Senchenkova EL, De Almeida PL, Damazo AS, Esmon CT, et al. Mechanisms of enhanced thrombus formation in cerebral microvessels of mice expressing hemoglobin-S. Blood. 2011;117(15):4125–33.
33. Massberg S, Grahl L, von Bruehl ML, Manukyan D, Pfeiler S, Goosmann C, et al. Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. Nat Med. 2010;16(8):887–96.
34. Faraday N, Schunke K, Saleem S, Fu J, Wang B, Zhang J, et al. Cathepsin g-dependent modulation of platelet thrombus formation in vivo by blood neutrophils. PLoS One. 2013;8(8), e71447.
35. Khan MM, Motto DG, Lentz SR, Chauhan AK. ADAMTS13 reduces VWF-mediated acute inflammation following focal cerebral ischemia in mice. J Thromb Haemost. 2012;10(8):1665–71.
36. Fuchs TA, Brill A, Wagner DD. Neutrophil extracellular trap (NET) impact on deep vein thrombosis. Arterioscler Thromb Vasc Biol. 2012;32(8):1777–83.
37. Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers Jr DD, et al. Extracellular DNA traps promote thrombosis. Proc Natl Acad Sci U S A. 2010;107(36):15880–5.
38. Brill A, Fuchs TA, Savchenko AS, Thomas GM, Martinod K, De Meyer SF, et al. Neutrophil extracellular traps promote deep vein thrombosis in mice. J Thromb Haemost. 2012;10(1):136–44.
39. Fischer S, Preissner KT. Extracellular nucleic acids as novel alarm signals in the vascular system. Mediators of defence and disease. Hamostaseologie. 2013;33(1):37–42.
40. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473(7347):317–25.
41. Johnson RC, Chapman SM, Dong ZM, Ordovas JM, Mayadas TN, Herz J, et al. Absence of P-selectin delays fatty streak formation in mice. J Clin Invest. 1997;99(5):1037–43.
42. Nageh MF, Sandberg ET, Marotti KR, Lin AH, Melchior EP, Bullard DC, et al. Deficiency of inflammatory cell adhesion molecules protects against atherosclerosis in mice. Arterioscler Thromb Vasc Biol. 1997;17(8):1517–20.
43. Braunersreuther V, Zernecke A, Arnaud C, Liehn EA, Steffens S, Shagdarsuren E, et al. Ccr5 but not Ccr1 deficiency reduces development of diet-induced atherosclerosis in mice. Arterioscler Thromb Vasc Biol. 2007;27(2):373–9.

44. Doring Y, Drechsler M, Wantha S, Kemmerich K, Lievens D, Vijayan S, et al. Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice. Circ Res. 2012;110(8):1052–6.
45. Wantha S, Alard JE, Megens RT, van der Does AM, Doring Y, Drechsler M, et al. Neutrophil-derived cathelicidin promotes adhesion of classical monocytes. Circ Res. 2013;112(5):792–801.
46. Copin JC, da Silva RF, Fraga-Silva RA, Capettini L, Quintao S, Lenglet S, et al. Treatment with Evasin-3 reduces atherosclerotic vulnerability for ischemic stroke, but not brain injury in mice. J Cereb Blood Flow Metab. 2013;33(4):490–8.
47. Mazor R, Shurtz-Swirski R, Farah R, Kristal B, Shapiro G, Dorlechter F, et al. Primed polymorphonuclear leukocytes constitute a possible link between inflammation and oxidative stress in hyperlipidemic patients. Atherosclerosis. 2008;197(2):937–43.
48. Dorweiler B, Torzewski M, Dahm M, Kirkpatrick CJ, Lackner KJ, Vahl CF. Subendothelial infiltration of neutrophil granulocytes and liberation of matrix-destabilizing enzymes in an experimental model of human neo-intima. Thromb Haemost. 2008;99(2):373–81.
49. Laxton RC, Hu Y, Duchene J, Zhang F, Zhang Z, Leung KY, et al. A role of matrix metalloproteinase-8 in atherosclerosis. Circ Res. 2009;105(9):921–9.
50. Lenglet S, Mach F, Montecucco F. Role of matrix metalloproteinase-8 in atherosclerosis. Mediators Inflamm. 2013;2013:659282.
51. Nakamura K, Sasaki T, Cheng XW, Iguchi A, Sato K, Kuzuya M. Statin prevents plaque disruption in apoE-knockout mouse model through pleiotropic effect on acute inflammation. Atherosclerosis. 2009;206(2):355–61.
52. Ionita MG, van den Borne P, Catanzariti LM, Moll FL, de Vries JP, Pasterkamp G, et al. High neutrophil numbers in human carotid atherosclerotic plaques are associated with characteristics of rupture-prone lesions. Arterioscler Thromb Vasc Biol. 2010;30(9):1842–8.
53. Sen S, Hinderliter A, Sen PK, Simmons J, Beck J, Offenbacher S, et al. Aortic arch atheroma progression and recurrent vascular events in patients with stroke or transient ischemic attack. Circulation. 2007;116(8):928–35.
54. Nasr N, Ruidavets JB, Arnal JF, Sie P, Larrue V. Association of neutrophil count with microembolization in patients with symptomatic carotid artery stenosis. Atherosclerosis. 2009;207(2):519–23.
55. Morrison H, McKee D, Ritter L. Systemic neutrophil activation in a mouse model of ischemic stroke and reperfusion. Biol Res Nurs. 2011;13(2):154–63.
56. Algra SO, Groeneveld KM, Schadenberg AW, Haas F, Evens FC, Meerding J, et al. Cerebral ischemia initiates an immediate innate immune response in neonates during cardiac surgery. J Neuroinflammation. 2013;10:24.
57. Barone FC, Schmidt DB, Hillegass LM, Price WJ, White RF, Feuerstein GZ, et al. Reperfusion increases neutrophils and leukotriene B4 receptor binding in rat focal ischemia. Stroke. 1992;23(9):1337–47. discussion 47–8.
58. Liu T, Young PR, McDonnell PC, White RF, Barone FC, Feuerstein GZ. Cytokine-induced neutrophil chemoattractant mRNA expressed in cerebral ischemia. Neurosci Lett. 1993;164(1–2):125–8.
59. Breckwoldt MO, Chen JW, Stangenberg L, Aikawa E, Rodriguez E, Qiu S, et al. Tracking the inflammatory response in stroke in vivo by sensing the enzyme myeloperoxidase. Proc Natl Acad Sci U S A. 2008;105(47):18584–9.
60. Weston RM, Jones NM, Jarrott B, Callaway JK. Inflammatory cell infiltration after endothelin-1-induced cerebral ischemia: histochemical and myeloperoxidase correlation with temporal changes in brain injury. J Cereb Blood Flow Metab. 2007;27(1):100–14.
61. Yamagami S, Tamura M, Hayashi M, Endo N, Tanabe H, Katsuura Y, et al. Differential production of MCP-1 and cytokine-induced neutrophil chemoattractant in the ischemic brain after transient focal ischemia in rats. J Leukoc Biol. 1999;65(6):744–9.
62. Gelderblom M, Leypoldt F, Steinbach K, Behrens D, Choe CU, Siler DA, et al. Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. Stroke. 2009;40(5):1849–57.
63. Beamer NB, Coull BM, Clark WM, Briley DP, Wynn M, Sexton G. Persistent inflammatory response in stroke survivors. Neurology. 1998;50(6):1722–8.

64. McColl SR, Paquin R, Menard C, Beaulieu AD. Human neutrophils produce high levels of the interleukin 1 receptor antagonist in response to granulocyte/macrophage colony-stimulating factor and tumor necrosis factor alpha. J Exp Med. 1992;176(2):593–8.
65. Akopov SE, Simonian NA, Grigorian GS. Dynamics of polymorphonuclear leukocyte accumulation in acute cerebral infarction and their correlation with brain tissue damage. Stroke. 1996;27(10):1739–43.
66. Neumann J, Sauerzweig S, Ronicke R, Gunzer F, Dinkel K, Ullrich O, et al. Microglia cells protect neurons by direct engulfment of invading neutrophil granulocytes: a new mechanism of CNS immune privilege. J Neurosci. 2008;28(23):5965–75.
67. Tsai NW, Chang WN, Shaw CF, Jan CR, Huang CR, Chen SD, et al. The value of leukocyte adhesion molecules in patients after ischemic stroke. J Neurol. 2009;256(8):1296–302.
68. Cojocaru IM, Cojocaru M, Burcin C. Evaluation of granulocyte elastase as a sensitive diagnostic parameter of inflammation in first ischemic stroke. Rom J Intern Med. 2006;44(3):317–21.
69. Elneihoum AM, Falke P, Axelsson L, Lundberg E, Lindgarde F, Ohlsson K. Leukocyte activation detected by increased plasma levels of inflammatory mediators in patients with ischemic cerebrovascular diseases. Stroke. 1996;27(10):1734–8.
70. Anwaar I, Gottsater A, Ohlsson K, Mattiasson I, Lindgarde F. Increasing levels of leukocyte-derived inflammatory mediators in plasma and cAMP in platelets during follow-up after acute cerebral ischemia. Cerebrovasc Dis. 1998;8(6):310–7.
71. Domac FM, Misirli H. The role of neutrophils and interleukin-8 in acute ischemic stroke. Neurosciences. 2008;13(2):136–41.
72. Tuttolomondo A, Pinto A, Corrao S, Di Raimondo D, Fernandez P, Di Sciacca R, et al. Immuno-inflammatory and thrombotic/fibrinolytic variables associated with acute ischemic stroke diagnosis. Atherosclerosis. 2009;203(2):503–8.
73. Intiso D, Zarrelli MM, Lagioia G, Di Rienzo F, Checchia De Ambrosio C, Simone P, et al. Tumor necrosis factor alpha serum levels and inflammatory response in acute ischemic stroke patients. Neurol Sci. 2004;24(6):390–6.
74. Dimasi D, Sun WY, Bonder CS. Neutrophil interactions with the vascular endothelium. Int Immunopharmacol. 2013;17(4):1167–75.
75. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. J Clin Invest. 2010;120(7): 2423–31.
76. Reichel CA, Khandoga A, Anders HJ, Schlondorff D, Luckow B, Krombach F. Chemokine receptors Ccr1, Ccr2, and Ccr5 mediate neutrophil migration to postischemic tissue. J Leukoc Biol. 2006;79(1):114–22.
77. Enzmann G, Mysiorek C, Gorina R, Cheng YJ, Ghavampour S, Hannocks MJ, et al. The neurovascular unit as a selective barrier to polymorphonuclear granulocyte (PMN) infiltration into the brain after ischemic injury. Acta Neuropathol. 2013;125(3):395–412.
78. Lindsberg PJ, Siren AL, Feuerstein GZ, Hallenbeck JM. Antagonism of neutrophil adherence in the deteriorating stroke model in rabbits. J Neurosurg. 1995;82(2):269–77.
79. Matsuo Y, Onodera H, Shiga Y, Nakamura M, Ninomiya M, Kihara T, et al. Correlation between myeloperoxidase-quantified neutrophil accumulation and ischemic brain injury in the rat. Effects of neutrophil depletion. Stroke. 1994;25(7):1469–75.
80. Clark WM, Lauten JD, Lessov N, Woodward W, Coull BM. The influence of antiadhesion therapies on leukocyte subset accumulation in central nervous system ischemia in rats. J Mol Neurosci. 1995;6(1):43–50.
81. Matsuo Y, Onodera H, Shiga Y, Shozuhara H, Ninomiya M, Kihara T, et al. Role of cell adhesion molecules in brain injury after transient middle cerebral artery occlusion in the rat. Brain Res. 1994;656(2):344–52.
82. Zhang RL, Chopp M, Jiang N, Tang WX, Prostak J, Manning AM, et al. Anti-intercellular adhesion molecule-1 antibody reduces ischemic cell damage after transient but not permanent middle cerebral artery occlusion in the Wistar rat. Stroke. 1995;26(8):1438–42. discussion 43.

83. Connolly Jr ES, Winfree CJ, Springer TA, Naka Y, Liao H, Yan SD, et al. Cerebral protection in homozygous null ICAM-1 mice after middle cerebral artery occlusion. Role of neutrophil adhesion in the pathogenesis of stroke. J Clin Invest. 1996;97(1):209–16.
84. Kitagawa K, Matsumoto M, Mabuchi T, Yagita Y, Ohtsuki T, Hori M, et al. Deficiency of intercellular adhesion molecule 1 attenuates microcirculatory disturbance and infarction size in focal cerebral ischemia. J Cereb Blood Flow Metab. 1998;18(12):1336–45.
85. Ritter L, Davidson L, Henry M, Davis-Gorman G, Morrison H, Frye JB, et al. Exaggerated neutrophil-mediated reperfusion injury after ischemic stroke in a rodent model of type 2 diabetes. Microcirculation. 2011;18(7):552–61.
86. Omi H, Okayama N, Shimizu M, Fukutomi T, Nakamura A, Imaeda K, et al. Cilostazol inhibits high glucose-mediated endothelial-neutrophil adhesion by decreasing adhesion molecule expression via NO production. Microvasc Res. 2004;68(2):119–25.
87. Justicia C, Martin A, Rojas S, Gironella M, Cervera A, Panes J, et al. Anti-VCAM-1 antibodies did not protect against ischemic damage either in rats or in mice. J Cereb Blood Flow Metab. 2006;26(3):421–32.
88. Mori E, del Zoppo GJ, Chambers JD, Copeland BR, Arfors KE. Inhibition of polymorphonuclear leukocyte adherence suppresses no-reflow after focal cerebral ischemia in baboons. Stroke. 1992;23(5):712–8.
89. Zhang ZG, Chopp M, Tang WX, Jiang N, Zhang RL. Postischemic treatment (2–4 h) with anti-CD11b and anti-CD18 monoclonal antibodies are neuroprotective after transient (2 h) focal cerebral ischemia in the rat. Brain Res. 1995;698(1–2):79–85.
90. Chopp M, Zhang RL, Chen H, Li Y, Jiang N, Rusche JR. Postischemic administration of an anti-Mac-1 antibody reduces ischemic cell damage after transient middle cerebral artery occlusion in rats. Stroke. 1994;25(4):869–75. discussion 75–6.
91. Yenari MA, Kunis D, Sun GH, Onley D, Watson L, Turner S, et al. Hu23F2G, an antibody recognizing the leukocyte CD11/CD18 integrin, reduces injury in a rabbit model of transient focal cerebral ischemia. Exp Neurol. 1998;153(2):223–33.
92. Soriano SG, Coxon A, Wang YF, Frosch MP, Lipton SA, Hickey PR, et al. Mice deficient in Mac-1 (CD11b/CD18) are less susceptible to cerebral ischemia/reperfusion injury. Stroke. 1999;30(1):134–9.
93. Prestigiacomo CJ, Kim SC, Connolly Jr ES, Liao H, Yan SF, Pinsky DJ. CD18-mediated neutrophil recruitment contributes to the pathogenesis of reperfused but not nonreperfused stroke. Stroke. 1999;30(5):1110–7.
94. Jiang N, Chopp M, Chahwala S. Neutrophil inhibitory factor treatment of focal cerebral ischemia in the rat. Brain Res. 1998;788(1–2):25–34.
95. Jiang N, Moyle M, Soule HR, Rote WE, Chopp M. Neutrophil inhibitory factor is neuroprotective after focal ischemia in rats. Ann Neurol. 1995;38(6):935–42.
96. Mackay KB, Bailey SJ, King PD, Patel S, Hamilton TC, Campbell CA. Neuroprotective effect of recombinant neutrophil inhibitory factor in transient focal cerebral ischaemia in the rat. Neurodegeneration. 1996;5(4):319–23.
97. Sousa LF, Coelho FM, Rodrigues DH, Campos AC, Barcelos Lda S, Teixeira MM, et al. Blockade of CXCR1/2 chemokine receptors protects against brain damage in ischemic stroke in mice. Clinics. 2013;68(3):391–4.
98. Villa P, Triulzi S, Cavalieri B, Di Bitondo R, Bertini R, Barbera S, et al. The interleukin-8 (IL-8/CXCL8) receptor inhibitor reparixin improves neurological deficits and reduces long-term inflammation in permanent and transient cerebral ischemia in rats. Mol Med. 2007;13(3–4):125–33.
99. Brait VH, Rivera J, Broughton BR, Lee S, Drummond GR, Sobey CG. Chemokine-related gene expression in the brain following ischemic stroke: no role for CXCR2 in outcome. Brain Res. 2011;1372:169–79.
100. Zaremba J, Skrobanski P, Losy J. The level of chemokine CXCL5 in the cerebrospinal fluid is increased during the first 24 hours of ischaemic stroke and correlates with the size of early brain damage. Folia Morphol. 2006;65(1):1–5.

101. Losy J, Zaremba J, Skrobanski P. CXCL1 (GRO-alpha) chemokine in acute ischaemic stroke patients. Folia Neuropathol. 2005;43(2):97–102.
102. Dimitrijevic OB, Stamatovic SM, Keep RF, Andjelkovic AV. Absence of the chemokine receptor CCR2 protects against cerebral ischemia/reperfusion injury in mice. Stroke. 2007;38(4):1345–53.
103. Murikinati S, Juttler E, Keinert T, Ridder DA, Muhammad S, Waibler Z, et al. Activation of cannabinoid 2 receptors protects against cerebral ischemia by inhibiting neutrophil recruitment. FASEB J. 2010;24(3):788–98.
104. Hayakawa K, Mishima K, Irie K, Hazekawa M, Mishima S, Fujioka M, et al. Cannabidiol prevents a post-ischemic injury progressively induced by cerebral ischemia via a high-mobility group box1-inhibiting mechanism. Neuropharmacology. 2008;55(8):1280–6.
105. Mocco J, Choudhri T, Huang J, Harfeldt E, Efros L, Klingbeil C, et al. HuEP5C7 as a humanized monoclonal anti-E/P-selectin neurovascular protective strategy in a blinded placebo-controlled trial of nonhuman primate stroke. Circ Res. 2002;91(10):907–14.
106. Jin AY, Tuor UI, Rushforth D, Kaur J, Muller RN, Petterson JL, et al. Reduced blood brain barrier breakdown in P-selectin deficient mice following transient ischemic stroke: a future therapeutic target for treatment of stroke. BMC Neurosci. 2010;11:12.
107. Connolly Jr ES, Winfree CJ, Prestigiacomo CJ, Kim SC, Choudhri TF, Hoh BL, et al. Exacerbation of cerebral injury in mice that express the P-selectin gene: identification of P-selectin blockade as a new target for the treatment of stroke. Circ Res. 1997;81(3):304–10.
108. Goussev AV, Zhang Z, Anderson DC, Chopp M. P-selectin antibody reduces hemorrhage and infarct volume resulting from MCA occlusion in the rat. J Neurol Sci. 1998;161(1):16–22.
109. Huang J, Choudhri TF, Winfree CJ, McTaggart RA, Kiss S, Mocco J, et al. Postischemic cerebrovascular E-selectin expression mediates tissue injury in murine stroke. Stroke. 2000;31(12):3047–53.
110. Bednar MM, Gross CE, Russell SR, Fuller SP, Ellenberger CL, Schindler E, et al. Humanized anti-L-selectin monoclonal antibody DREG200 therapy in acute thromboembolic stroke. Neurol Res. 1998;20(5):403–8.
111. Walcheck B, Kahn J, Fisher JM, Wang BB, Fisk RS, Payan DG, et al. Neutrophil rolling altered by inhibition of L-selectin shedding in vitro. Nature. 1996;380(6576):720–3.
112. Jin G, Tsuji K, Xing C, Yang YG, Wang X, Lo EH. CD47 gene knockout protects against transient focal cerebral ischemia in mice. Exp Neurol. 2009;217(1):165–70.
113. Gangaraju S, Sultan K, Whitehead SN, Nilchi L, Slinn J, Li X, et al. Cerebral endothelial expression of Robo1 affects brain infiltration of polymorphonuclear neutrophils during mouse stroke recovery. Neurobiol Dis. 2013;54:24–31.
114. Sorce S, Bonnefont J, Julien S, Marq-Lin N, Rodriguez I, Dubois-Dauphin M, et al. Increased brain damage after ischaemic stroke in mice lacking the chemokine receptor CCR5. Br J Pharmacol. 2010;160(2):311–21.
115. Prince LR, Whyte MK, Sabroe I, Parker LC. The role of TLRs in neutrophil activation. Curr Opin Pharmacol. 2011;11(4):397–403.
116. Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, et al. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature. 2010;464(7285):104–7.
117. Shichita T, Hasegawa E, Kimura A, Morita R, Sakaguchi R, Takada I, et al. Peroxiredoxin family proteins are key initiators of post-ischemic inflammation in the brain. Nat Med. 2012;18(6):911–7.
118. Brea D, Blanco M, Sobrino T, Ramos-Cabrer P, Castillo J. The levels of expression of toll-like receptors 2 and 4 in neutrophils are associated with the prognosis of ischaemic stroke patients. Rev Neurol. 2011;52(1):12–9.
119. Kilic U, Kilic E, Matter CM, Bassetti CL, Hermann DM. TLR-4 deficiency protects against focal cerebral ischemia and axotomy-induced neurodegeneration. Neurobiol Dis. 2008;31(1):33–40.
120. Petrault O, Ouk T, Gautier S, Laprais M, Gele P, Bastide M, et al. Pharmacological neutropenia prevents endothelial dysfunction but not smooth muscle functions impairment induced by middle cerebral artery occlusion. Br J Pharmacol. 2005;144(8):1051–8.

121. Fernandez-Cadenas I, Rio-Espinola AD, Domingues-Montanari S, Mendioroz M, Fernandez-Morales J, Penalba A, et al. Genes involved in hemorrhagic transformations that follow recombinant t-PA treatment in stroke patients. Pharmacogenomics. 2013;14(5):495–504.
122. Gautier S, Ouk T, Petrault O, Caron J, Bordet R. Neutrophils contribute to intracerebral haemorrhages after treatment with recombinant tissue plasminogen activator following cerebral ischaemia. Br J Pharmacol. 2009;156(4):673–9.
123. McColl BW, Rothwell NJ, Allan SM. Systemic inflammatory stimulus potentiates the acute phase and CXC chemokine responses to experimental stroke and exacerbates brain damage via interleukin-1- and neutrophil-dependent mechanisms. J Neurosci. 2007;27(16):4403–12.
124. Heo JH, Lucero J, Abumiya T, Koziol JA, Copeland BR, del Zoppo GJ. Matrix metalloproteinases increase very early during experimental focal cerebral ischemia. J Cereb Blood Flow Metab. 1999;19(6):624–33.
125. Kelly PJ, Morrow JD, Ning M, Koroshetz W, Lo EH, Terry E, et al. Oxidative stress and matrix metalloproteinase-9 in acute ischemic stroke: the biomarker evaluation for antioxidant therapies in stroke (BEAT-Stroke) study. Stroke. 2008;39(1):100–4.
126. Tang Y, Xu H, Du X, Lit L, Walker W, Lu A, et al. Gene expression in blood changes rapidly in neutrophils and monocytes after ischemic stroke in humans: a microarray study. J Cereb Blood Flow Metab. 2006;26(8):1089–102.
127. Stamova B, Xu H, Jickling G, Bushnell C, Tian Y, Ander BP, et al. Gene expression profiling of blood for the prediction of ischemic stroke. Stroke. 2010;41(10):2171–7.
128. Castellanos M, Sobrino T, Millan M, Garcia M, Arenillas J, Nombela F, et al. Serum cellular fibronectin and matrix metalloproteinase-9 as screening biomarkers for the prediction of parenchymal hematoma after thrombolytic therapy in acute ischemic stroke: a multicenter confirmatory study. Stroke. 2007;38(6):1855–9.
129. Rosell A, Cuadrado E, Ortega-Aznar A, Hernandez-Guillamon M, Lo EH, Montaner J. MMP-9-positive neutrophil infiltration is associated to blood–brain barrier breakdown and basal lamina type IV collagen degradation during hemorrhagic transformation after human ischemic stroke. Stroke. 2008;39(4):1121–6.
130. Gidday JM, Gasche YG, Copin JC, Shah AR, Perez RS, Shapiro SD, et al. Leukocyte-derived matrix metalloproteinase-9 mediates blood–brain barrier breakdown and is proinflammatory after transient focal cerebral ischemia. Am J Physiol Heart Circ Physiol. 2005;289(2): H558–68.
131. Wang G, Guo Q, Hossain M, Fazio V, Zeynalov E, Janigro D, et al. Bone marrow-derived cells are the major source of MMP-9 contributing to blood–brain barrier dysfunction and infarct formation after ischemic stroke in mice. Brain Res. 2009;1294:183–92.
132. Ludewig P, Sedlacik J, Gelderblom M, Bernreuther C, Korkusuz Y, Wagener C, et al. CEACAM1 inhibits MMP-9-mediated blood–brain-barrier breakdown in a mouse model for ischemic stroke. Circ Res. 2013;113(8):1013–22.
133. Lapchak PA. Hemorrhagic transformation following ischemic stroke: significance, causes, and relationship to therapy and treatment. Curr Neurol Neurosci Rep. 2002;2(1):38–43.
134. Fan X, Lo EH, Wang X. Effects of minocycline plus tissue plasminogen activator combination therapy after focal embolic stroke in type 1 diabetic rats. Stroke. 2013;44(3):745–52.
135. Zhao BQ, Wang S, Kim HY, Storrie H, Rosen BR, Mooney DJ, et al. Role of matrix metalloproteinases in delayed cortical responses after stroke. Nat Med. 2006;12(4):441–5.
136. Gautier S, Ouk T, Tagzirt M, Lefebvre C, Laprais M, Petrault O, et al. Impact of the neutrophil response to granulocyte colony-stimulating factor on the risk of hemorrhage when used in combination with tissue plasminogen activator during the acute phase of experimental stroke. J Neuroinflammation. 2014;11:96.
137. Ikegame Y, Yamashita K, Hayashi S, Yoshimura S, Nakashima S, Iwama T. Neutrophil elastase inhibitor prevents ischemic brain damage via reduction of vasogenic edema. Hypertens Res. 2010;33(7):703–7.
138. Stowe AM, Adair-Kirk TL, Gonzales ER, Perez RS, Shah AR, Park TS, et al. Neutrophil elastase and neurovascular injury following focal stroke and reperfusion. Neurobiol Dis. 2009;35(1):82–90.

139. Gasche Y, Copin JC, Sugawara T, Fujimura M, Chan PH. Matrix metalloproteinase inhibition prevents oxidative stress-associated blood–brain barrier disruption after transient focal cerebral ischemia. J Cereb Blood Flow Metab. 2001;21(11740200):1393–400.
140. Chen H, Song YS, Chan PH. Inhibition of NADPH oxidase is neuroprotective after ischemia-reperfusion. J Cereb Blood Flow Metab. 2009;29(7):1262–72.
141. Tang XN, Zheng Z, Giffard RG, Yenari MA. Significance of marrow-derived nicotinamide adenine dinucleotide phosphate oxidase in experimental ischemic stroke. Ann Neurol. 2011;70(4):606–15.
142. Lapchak PA, Araujo DM, Song D, Wei J, Purdy R, Zivin JA. Effects of the spin trap agent disodium- [tert-butylimino)methyl]benzene-1,3-disulfonate N-oxide (generic NXY-059) on intracerebral hemorrhage in a rabbit Large clot embolic stroke model: combination studies with tissue plasminogen activator. Stroke. 2002;33(6):1665–70.
143. Shuaib A, Lees KR, Lyden P, Grotta J, Davalos A, Davis SM, et al. NXY-059 for the treatment of acute ischemic stroke. N Engl J Med. 2007;357(6):562–71.
144. Asahi M, Asahi K, Wang X, Lo EH. Reduction of tissue plasminogen activator-induced hemorrhage and brain injury by free radical spin trapping after embolic focal cerebral ischemia in rats. J Cereb Blood Flow Metab. 2000;20(3):452–7.
145. Lapchak PA, Chapman DF, Zivin JA. Pharmacological effects of the spin trap agents N-t-butyl-phenylnitrone (PBN) and 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) in a rabbit thromboembolic stroke model: combination studies with the thrombolytic tissue plasminogen activator. Stroke. 2001;32(1):147–53.
146. Yamashita T, Kamiya T, Deguchi K, Inaba T, Zhang H, Shang J, et al. Dissociation and protection of the neurovascular unit after thrombolysis and reperfusion in ischemic rat brain. J Cereb Blood Flow Metab. 2009;29(4):715–25.
147. Mishina M, Komaba Y, Kobayashi S, Kominami S, Fukuchi T, Mizunari T, et al. Administration of free radical scavenger edaravone associated with higher frequency of hemorrhagic transformation in patients with cardiogenic embolism. Neurol Med Chir. 2008;48(7):292–7.
148. Matsubara M, Akizuki O, Ikeda J, Saeki K, Yao K, Sasaki K. Benidipine, an anti-hypertensive drug, inhibits reactive oxygen species production in polymorphonuclear leukocytes and oxidative stress in salt-loaded stroke-prone spontaneously hypertensive rats. Eur J Pharmacol. 2008;580(1–2):201–13.
149. Jickling GC, Liu D, Stamova B, Ander BP, Zhan X, Lu A, et al. Hemorrhagic transformation after ischemic stroke in animals and humans. J Cereb Blood Flow Metab. 2014;34(2):185–99.
150. Popovic N, Stefanovic-Budimkic M, Mitrovic N, Urosevic A, Milosevic B, Pelemis M, et al. The frequency of poststroke infections and their impact on early stroke outcome. J Stroke Cerebrovasc Dis. 2013;22(4):424–9.
151. Murray KN, Buggey HF, Denes A, Allan SM. Systemic immune activation shapes stroke outcome. Mol Cell Neurosci. 2013;53:14–25.
152. Hong KS, Kang DW, Koo JS, Yu KH, Han MK, Cho YJ, et al. Impact of neurological and medical complications on 3-month outcomes in acute ischaemic stroke. Eur J Neurol. 2008;15(12):1324–31.
153. McColl BW, Rothwell NJ, Allan SM. Systemic inflammation alters the kinetics of cerebrovascular tight junction disruption after experimental stroke in mice. J Neurosci. 2008;28(38):9451–62.
154. Muhammad S, Haasbach E, Kotchourko M, Strigli A, Krenz A, Ridder DA, et al. Influenza virus infection aggravates stroke outcome. Stroke. 2011;42(3):783–91.
155. Chamorro A, Amaro S, Vargas M, Obach V, Cervera A, Torres F, et al. Interleukin 10, monocytes and increased risk of early infection in ischaemic stroke. J Neurol Neurosurg Psychiatry. 2006;77(11):1279–81.
156. Dirnagl U. Pathobiology of injury after stroke: the neurovascular unit and beyond. Ann N Y Acad Sci. 2012;1268:21–5.

157. Easton AS. Neutrophils and stroke—can neutrophils mitigate disease in the central nervous system? Int Immunopharmacol. 2013;17(4):1218–25.
158. Fournier BM, Parkos CA. The role of neutrophils during intestinal inflammation. Mucosal Immunol. 2012;5(4):354–66.
159. Christoffersson G, Vagesjo E, Vandooren J, Liden M, Massena S, Reinert RB, et al. VEGF-A recruits a proangiogenic MMP-9-delivering neutrophil subset that induces angiogenesis in transplanted hypoxic tissue. Blood. 2012;120(23):4653–62.
160. Schabitz WR, Laage R, Vogt G, Koch W, Kollmar R, Schwab S, et al. AXIS: a trial of intravenous granulocyte colony-stimulating factor in acute ischemic stroke. Stroke. 2010;41(11): 2545–51.
161. Strecker JK, Sevimli S, Schilling M, Klocke R, Nikol S, Schneider A, et al. Effects of G-CSF treatment on neutrophil mobilization and neurological outcome after transient focal ischemia. Exp Neurol. 2010;222(1):108–13.
162. Becker KJ. Anti-leukocyte antibodies: LeukArrest (Hu23F2G) and Enlimomab (R6.5) in acute stroke. Curr Med Res Opin. 2002;18 Suppl 2:s18–22.
163. Furuya K, Takeda H, Azhar S, McCarron RM, Chen Y, Ruetzler CA, et al. Examination of several potential mechanisms for the negative outcome in a clinical stroke trial of enlimomab, a murine anti-human intercellular adhesion molecule-1 antibody: a bedside-to-bench study. Stroke. 2001;32(11):2665–74.
164. Lo SK, Rahman A, Xu N, Zhou MY, Nagpala P, Jaffe HA, et al. Neutrophil inhibitory factor abrogates neutrophil adhesion by blockade of CD11a and CD11b beta(2) integrins. Mol Pharmacol. 1999;56(5):926–32.
165. Zhang L, Zhang ZG, Zhang RL, Lu M, Krams M, Chopp M. Effects of a selective CD11b/CD18 antagonist and recombinant human tissue plasminogen activator treatment alone and in combination in a rat embolic model of stroke. Stroke. 2003;34(7):1790–5.
166. Moriya Y, Mizuma A, Uesugi T, Ohnuki Y, Nagata E, Takahashi W, et al. Phase I study of intravenous low-dose granulocyte colony-stimulating factor in acute and subacute ischemic stroke. J Stroke Cerebrovasc Dis. 2012;22(7):1088–97.

The Function of Cytokines in Ischemic Stroke

Christopher C. Leonardo and Keith R. Pennypacker

1 The Complex Sequelae Resulting from Ischemic Stroke

Ischemic stroke produces a complex injury profile that is characterized by multiple, successive waves of tissue injury. The acute phase primarily involves energy failure and excitotoxic injury, which are hallmarks of most neurodegenerative diseases and set the stage for delayed cell death. During the acute phase, neural tissue most directly irrigated by the occluded vessel experiences rapid energy failure resulting from oxygen and glucose deprivation. The resulting failure of cellular respiration necessitates the switch to anaerobic energy production, leading to acidosis. This metabolic switch, coupled with excessive release of the neurotransmitter glutamate from injured neurons and neighboring astrocytes, promotes ionic instability that compromises cell membranes and facilitates neuronal cell death. In addition to free radical injury, which is achieved in large part through the production of superoxide and peroxynitrite radicals, the release of sequestered intracellular calcium stores also promotes apoptotic signaling and cellular oedema [1].

These mechanisms of ischemic stroke pathology are well documented and have provided the basis for several decades of stroke research, whereby the primary goal was to limit excitotoxicity as means of limiting these injurious responses.

C.C. Leonardo, B.S., Ph.D. • K.R. Pennypacker, B.A., M.S., Ph.D. (✉)
Molecular Pharmacology and Physiology, University of South Florida,
12901 Bruce B Downs Blvd, Tampa, FL 33612, USA
e-mail: kpennypa@health.usf.edu; ccleonard@health.usf.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_15

Unfortunately, this approach was met with very limited success [2, 3], as relatively few stroke patients are eligible for the thrombolytic therapies (i.e., tissue plasminogen activator) that must be administered within 3–6 h of stroke onset. With few viable treatment options, the majority of patients are limited to supportive care during the period of infarct expansion. During this period, the viable tissue surrounding the core infarct becomes compromised through perpetuation of the above mechanisms coupled with detachment-induced injury from loss of synapses and neuroglial support. Without recanalization and restoration of blood flow, neural injury will progress based upon the severity of the initial insult. This will continue until endogenous remodeling mechanisms, such as glial scarring, are successful in protecting healthy surrounding tissue from the inflammatory microenvironment present within the infarction.

The rapid progression of neuronal injury, as well as the inability to diagnose ischemic stroke patients in time to receive thrombolytic therapy, has prompted investigations into other therapeutic targets. Among these was a broad category of neuroinflammatory responses that encompasses a wide range of deleterious phenomena. For example, depending upon the specific context, neuroinflammation may include blood–brain barrier (BBB) permeability, glial scar formation, extravasation of peripheral immune cells, and/or activation of resident immune cells [4]. Additionally, and somewhat paradoxically, immune cell chemotaxis is accompanied by the upregulation and release of cytokines, chemokines, prostaglandins, proteases, and other proinflammatory molecules that perpetuate cellular disruption and tissue injury [5]. To complicate matters, these signals collectively demonstrate dual and often overlapping roles that cloud interpretation of their individual actions within the grand scheme of stroke pathology. For these reasons, the inflammation that ensues following ischemic stroke produces a complicated injury profile with multiple mechanisms that need to be addressed in order for any therapeutic approach to be successful.

A critical component of any therapeutic is the ability to dampen proinflammatory signaling that contributes to delayed cell death. This delayed wave of neural injury is facilitated by BBB disruption in response to the initial ischemic event. Within hours to days of stroke onset, matrix metalloproteinases (MMPs) cleave tight junction proteins involved with sealing the barrier. The result is a biphasic opening of the BBB that is initiated by MMP-2 and enhanced through the actions of MMP-9 [6]. Immune cell signaling exacerbates the inflammatory milieu within the core infarct. Microglia respond to resident death signals, while peripheral leukocytes are activated by CNS antigens that have leaked into the systemic circulation. The result is a feed-forward proinflammatory microenvironment that is modulated by resident glia and peripheral leukocytes through the upregulation and release of cytokines. Thus, cytokine signaling has profound effects on the ultimate fate of the injured brain and is regulated by activated immune cells both within and outside of the brain parenchyma.

2 Cytokines as Therapeutic Targets for Ischemic Stroke

Cytokines are involved in a wide variety of biological processes, including embryonic development, stem cell differentiation, innate and adaptive immunity, cognition, progressive neural injury and neurodegenerative disease pathology. The complexity of cytokine actions is reflected in the distinct families of cytokines, which include interleukins, chemokines, mesenchymal growth factors, adipokines, and the tumor necrosis factor family [7]. Depending upon the cytokine, these signals can influence cell signaling by cell surface receptor binding, antibody binding, and direct DNA binding. Thus, this interesting class of molecules has attracted much attention as researchers have searched for new therapeutic targets.

Ischemic stroke produces a biphasic injury profile that can be characterized in terms of an "acute" excitotoxic response followed by a "delayed" neuroinflammatory response. Indeed, cytokines have emerged as important signaling molecules that play key roles in both injury phases and are intimately connected with immune cell activation. To date, there have been a multitude of studies aimed at uncovering the precise mechanisms by which cytokines influence cellular responses to cerebral ischemia. Results from clinical studies and animal models have identified several key cytokines that can be targeted for the treatment of ischemic stroke.

The body of work regarding cellular immune responses to ischemic stroke is extensive and is discussed in great detail throughout this book. Although it was not historically the case, neuroinflammation is now widely regarded as a principle cause of delayed infarct expansion that is driven, in large part, by cytokine signaling [8, 9]. Both the acute and progressive phases of stroke injury have been linked to the upregulation and release of proinflammatory cytokines [4, 5]. Animal models have offered the means to conduct controlled studies designed to elucidate the mechanisms by which cytokines influence injury and remodeling of the ischemic brain. This section reviews some of the key findings related to cytokine production and signaling after ischemic stroke. Additionally, promising therapeutic approaches predicated on modulating cytokine signaling are discussed within the context of neuroinflammation and delayed infarct expansion.

2.1 Tumor Necrosis Factor Alpha Signaling and Expression

Tumor necrosis factor alpha (TNFα) has long been identified as a key proinflammatory effector of ischemic tissue. Although it is principally produced by activated macrophages, virtually all lymphocytes have the capability of producing TNFα as part of the systemic inflammatory response. This cytokine binds two distinct receptors, TNF receptor type 1 (TNFR1) and TNF receptor type 2 (TNFR2), and TNFR signal transduction can have various consequences including induction of NF-κB and AP1 transcription factors, as well as initiation of apoptotic

signaling through caspase activation. To complicate matters, TNFR activation can also suppress inflammatory signaling and apoptosis.

The existence of distinct TNFRs and various intracellular signaling pathways coupled to TNFRs explains some of the inconsistencies from ischemic stroke studies. For example, activation of TNFRs can result in two antagonistic responses. Virtually all cells typically present within the brain after stroke express TNFR1. The intracellular death domain contained within TNFαR1 is linked to Fas-associated protein with a death domain (FADD) signaling, where activation can result in the induction of caspases and/or cytochrome c release. Alternatively, TNFα binding to TNFR2 can activate TNF receptor-associated factor 2 (TRAF2), which induces expression of cell survival-associated genes that are regulated by mitogen-activated protein kinase (MAPK), NF-κB and AP-1 transcription factors [10, 11]. Additionally, TNFR2 lacks an intracellular death domain and is expressed on the surfaces of cells present within the cerebral infarct, including resident glia (oligodendrocytes and astrocytes), cerebrovascular endothelial cells, T-cells, and monocytes. Thus, the dualistic nature of TNFα signaling can produce caveats concerning the measurement of cytokine levels in the brain. In fact, a recent study demonstrated that TNFα production increased in both microglia and monocytes between 24 and 72 h after transient middle cerebral artery occlusion (tMCAO). Additionally, this study showed a positive correlation between TNFα production and the phagocytic capacity of microglia [12], which might suggest a beneficial role in response to ischemia.

Since TNFR binding can result in a multitude of effects depending upon the receptor activated and the local environment (i.e., the cell types present and the extent of inflammatory signals), the timing of cytokine production and release has been a subject of great interest as researchers work to understand the functional consequences of TNFα signaling. Animal models of ischemia, particularly those investigating immunoinflammatory mechanisms, have been instrumental in characterizing protein and mRNA levels of various cytokines, including TNFα. Studies exploring ischemia/reperfusion injury in liver found that Kupffer cells and neutrophils increased expression of TNFα along with other inflammatory mediators including reactive oxygen species (ROS) and nitric oxide (NO) [13]. Importantly, elevated TNFα also correlates with damage to various other body tissues following ischemia [14].

Consistent with ischemic peripheral tissues, TNFα transcript and protein levels are consistently upregulated in the injured and ischemic brain. This cytokine is expressed at early time points following stroke and is a critically important facilitator of immune cell recruitment to the cerebral infarct. TNFα gene transcript was elevated as early as 6 h following tMCAO in mice and remained elevated at extended time points up to 96 h after stroke [8, 15, 16]. Furthermore, TNF-α was elevated in the systemic circulation 24 h after tMCAO in the same model system, but returned to normal levels by 96 h post-stroke [16].

A similar expression pattern was detected in the spleen and provides clues as to its role in progressive inflammation. The spleen is a reservoir of immune cells and plays a substantial role in systemic immune responses. There is now a body of work

implicating splenic signaling in progressive neural injury following various insults including ischemic stroke, hemorrhagic stroke, and traumatic brain injury (TBI). Because the BBB is breached as a result of these and many other insults, activated peripheral immune cells can exert effects both systemically and at the level of the brain. Mice subjected to tMCAO showed a splenic expression pattern where the expression of various cytokines, including TNFα, was increased between 1 and 2 days following stroke [15, 17, 18].

2.2 TNFα as a Therapeutic Target for Ischemic Stroke

Various experimental approaches have been used to determine whether TNFα is a viable target for the treatment of stroke. Some of these include exogenous administration and/or blockade of TNFα, the utilization of knockout mice lacking the TNFα gene ($TNF\alpha^{-/-}$) or key immune cell populations, and animals subjected to splenectomy prior to insult. These methodologies have provided valuable information as to the role of TNFα in proinflammatory signaling. Furthermore, data from these models provides insights into acute signals originating at the level of the brain as well as those that are regulated by the peripheral immune response.

Popular approaches to investigating cytokine signaling involve boosting levels of the protein, and conversely, deletion or blockade of the protein. Rodent stroke models, including tMCAO and permanent MCAO (pMCAO), have been useful in conducting such studies due to their cost-effectiveness and reproducibility. Studies show that the administration of TNFα increases infarct volume in these models [19], demonstrating a deleterious role for TNFα that is dose-dependent. Similarly, reducing TNFα by blocking the biosynthetic enzyme, TNFα converting enzyme (TACE), was effective in reducing infarct volume and functional deficits in rats subjected to pMCAO [20]. Consistent with these data, other data showed efficacy in reducing tMCAO injury following administration of TNFα neutralizing antibodies [21].

Indeed, this cytokine has traditionally been regarded as a proinflammatory mediator that exacerbates cellular injury due to its elevation in neurodegenerative disease and after brain injury. However, several lines of evidence indicate that TNFα may be at least moderately misunderstood since it also appears to play a beneficial role under certain conditions. Data showed that TNFα upregulates the antioxidant superoxide dismutase 2 (SOD2), which is important in converting superoxide into molecular oxygen or the less damaging radical hydrogen peroxide. Interestingly, SOD2 has been implicated in stroke preconditioning [11], which would suggest a protective role for TNFα. Consistent with a protective role for TNFα, mice lacking the TNFα gene actually demonstrated worse outcomes in terms of total infarct volume relative to control mice [22]. To further complicate the issue, deletion of the gene coding for TNFR1, but not TNFR2, protected mice against pMCAO [22]. These data are consistent with the pro-apoptotic and cell survival-associated roles of TNFR1 and TNFR2, respectively, and thus highlight the dual nature of TNFα.

From a strictly therapeutic perspective, there is evidence that selective targeting of TNFα itself or TNFRs may prove beneficial for the treatment of ischemic stroke. The promise of this approach comes from TNFR effects and altered TNF expression patterns in response to known neuroprotective treatments and strategies. For example, activation of TNFR2 triggered oligodendrocyte (OL) progenitor cell recruitment in a cuprizone-induced white matter injury model [23]. Since white matter injury is directly related to motor deficits in ischemic stroke pathology, this mechanism may prove beneficial to improve these outcomes. Other data showed that administration of cocaine- and amphetamine-regulated transcript (CART) decreased infarct volume at early and delayed time points following tMCAO. Furthermore, the observed neuroprotection was accompanied by reduced TNFα levels in the blood and a dampened proinflammatory T-cell response [16].

Interestingly, studies investigating immune cell function also point to a crucial role for TNFR signaling. Peripheral immune activation is now an emerging area of research due to a growing body of work demonstrating a clear link between exacerbation of brain injury and the activation of T-cells, monocytes, and peripheral macrophages. Removal of the spleen, a lymphoid organ that acts as a reservoir for peripheral immune cells, has been shown to elicit protection in models of stroke, TBI, and ischemia-reperfusion injury to peripheral body tissues [24–27]. Since splenectomized animals are protected against ischemia, both in peripheral tissues and at the level of the brain, researchers have also focused efforts on cytokine signaling in spleen-derived immune cells. One technique involves harvesting splenocytes from animals subjected to focal ischemia. These cells are then activated and assayed to determine the inflammatory profile, including cytokine production and release.

From splenectomy experiments and assays with cultured splenocytes from ischemic tissues, spleen-derived TNFα has emerged as a candidate target due to its response profile. For example, splenectomy caused reductions in hepatic injury and leukocyte infiltration that were accompanied by decreased TNFα release [25]. In addition, neuroprotection and improved spatial memory performance following splenectomy were associated with decreased TNFα in a model of TBI [27]. Splenocytes harvested from CART-protected animals showed a reduced capacity to produce proinflammatory cytokines, including TNFα [16]. In a model of tMCAO, splenocytes harvested from stroke mice showed upregulated TNFα expression after activation with CD3/CD28 antibodies [8]. These experiments were later replicated to show similar effects in splenocytes harvested up to 24 h following tMCAO, demonstrating a prolonged effect of TNFα production following ischemic stroke in which the cytokine was elevated as late as 96 h after the insult [16]. Alterations in splenic TNFα have also been observed in response to stem cell therapies. Treatment with hematopoetic stem cells (HSCs) blocked splenic TNFα gene transcript [17], while human umbilical cord blood (HUCB) cell treatment reduced the extent of concovalin A-induced TNF-α production and splenocyte proliferation [28].

To date, the various approaches to blocking TNFα signaling have been met with some success in animal models and as well as the clinical setting. Administration of MMP/TACE inhibitors has been effective in rodent models, with BB-94

demonstrating protection against transient global ischemia and KB-R7785 reducing neural injury following MCAO. In addition to inhibiting TACE, which is a key regulator of TNFα activation, another promising approach is blocking the soluble form of TNFα (sTNFα) and its receptors (sTNFRs). This method would act to blunt the effects of sTNFα binding, thus offering another level of regulation. Recently, the TNFα blocking compound etanercept has shown preclinical efficacy along with extended efficacy in reducing motoric and gait disturbances in a clinical trial [29, 30]. Although there is some promise in the clinic and various reports of preclinical efficacy, the utility of targeting TNFα remains debatable when considering its complex, dualistic role.

2.3 Interleukin-1 Beta (IL-1β) Signaling and Expression

Interleukin-1 beta (IL-1β) is another putative proinflammatory cytokine associated with exacerbated injury following ischemic stroke. A member of the IL-1 cytokine family, IL-1β is predominantly produced by macrophages as a proprotein that is proteolytically cleaved into its active form by caspase 1. In general, IL-1β functions in multiple capacities to increase cell proliferation and differentiation, facilitate immune cell chemotaxis through the activation of chemokines, and regulate apoptotic signaling pathways.

In human stroke patients, IL-1β is increased in plasma and cerebrospinal fluid during the acute phase [30, 31]. Together with TNFα and MMPs, IL-1β plays a key role in the BBB disruption that renders the brain susceptible to activated peripheral macrophages and monocytes. Perivascular astrocytes are a major source of neural IL-1β at this early time point [32]. In fact, a recent study demonstrated elevations in IL-1β as early as 1 h after tMCAO that appeared to be influenced by the activity of MMP-2 [33]. However, microglia begin to upregulate IL-1β as they transition from the early M2 phenotype, which is predominantly thought to be beneficial, to the putative M1 proinflammatory phenotype [34].

IL-1β has most frequently been examined by measuring mRNA content at selected time points following experimental stroke. Previous studies using the tMCAO model showed that IL-1β was elevated in ischemic brain at 6 h post-stroke and remained increased up to 96 h after insult [16].

2.4 IL-1β as a Therapeutic Target for Ischemic Stroke

Consistent with a proinflammatory role in disease pathology, animal studies showed that therapeutic doses of HSCs inhibited splenic chemokine and cytokine production, including IL-1β, when administered 24 h after tMCAO. Additionally, treatment reduced apoptotic neurons, activated macrophages/microglia, and T-cell infiltration [17]. Similarly, the deleterious effects of IL-1β signaling were demonstrated in a

model of neurodegenerative disease (ME7 prion disease) in which intrahippocampal injection of IL-1β induced astrocytic chemokine production and enhanced infiltration of T-cells, neutrophils, and monocytes into the brain parenchyma [35]. The recruitment of peripheral immune cells observed in this study is consistent with the IL-1β profile previously detected in the spleen following focal ischemia. For example, IL-1β gene transcript was elevated by 22 h post-reperfusion in a rodent model of tMCAO [15, 18] and remained elevated at 48 h after stroke [17]. Interestingly, the involvement of spleen-derived IL-1β in brain injury was also documented following TBI in rats. Removal of the spleen immediately following TBI reduced IL-1β production, and this effect occurred concomitantly with reductions in mortality and vasogenic oedema. Additionally, the same animals showed improved performance on the Morris Water Maze, demonstrating a spatial memory benefit that was directly correlated with reduced levels of Il-1β [27].

The preponderance of data demonstrating a role for IL-1β in perpetuating a proinflammatory microenvironment makes this cytokine an attractive target for stroke therapy. As such, many laboratories are investigating mechanisms of inhibiting IL-1β signaling. A recent study showed that mice deficient in lactadherin, an endogenous glycoprotein with IL-1β inhibitory actions, showed increased IL-1β production and greater infarct size following permanent distal MCAO relative to wildtype controls. Furthermore, the increase in cerebral infarction was negated in mice that were administered an IL-1R antagonist [36].

Despite these encouraging data, there are caveats associated with inhibiting IL-1β. This cytokine is a key modulator of immune cell function, and its complex regulation at the transcriptional level may pose problems in terms of selective targeting of the proinflammatory isoform. To the latter point, a recent study showed that a specific variant of the IL-1β gene produces a protein that was associated with higher recanalization rates in stroke patients following tPA administration [37]. In light of these data, it will be critical to understand the biological activity of all SNP variants to ensure that the appropriate IL-1β variant is being targeted.

2.5 Interleukin-6 Signaling and Expression

Interleukin-6 (IL-6) is another interesting cytokine in that experimental evidence suggests a dual nature, including proinflammatory and prosurvival effects. The cytokine has been implicated in the pathogenesis of many neurological diseases. Similar to the other cytokines discussed within this chapter, the purported involvement in ischemic injury (and therefore potential as a therapeutic target) has arisen largely from observed expression profiles in affected patients and experimental subjects.

One noteworthy distinction between IL-6 and many of the other cytokines investigated is that IL-6 serum levels have shown a positive correlation with negative outcomes and brain injury in human stroke patients [38]. In fact, of the clinical variables used to predict mortality, IL-6 remains among the most reliable [39].

Recent data from the Northern Manhattan Study (NOMAS) has expanded upon the predictive validity of IL-6 as a biomarker. The NOMAS study is an ongoing investigation of risk factors conducted using a multiethnic cohort living in the same community. In this study, researchers concluded that IL-6 was an accurate predictor of ischemic stroke when levels were higher than high-sensitivity C-reactive protein, another biomarker that has been useful as a stroke predictor [40].

Consistent with these clinical data, IL-6 gene transcript is also induced in mice after tMCAO [8], and the signaling pathways activated by IL-6 suggest that this cytokine may prove useful as a therapeutic target. IL-6 signaling is complex and signal transduction can result in very different effects on cells. The receptor complex that is activated by IL-6 is composed of IL-6R subunits alpha (IL-6Rα) and beta (gp130). Interestingly, the gp130 intracellular domain can initiate MAPK signaling or activate the Janus kinase/transducers and activators of transcription (Jak/STAT) pathway. Ultimately, these signal transduction pathways result in transcriptional regulation through binding to the promoters of genes driven by AP-1, NF-κB, and other regulatory elements.

The complexity of IL-6 signaling is mirrored by its diverse functions. This cytokine has been shown to play a role in normal physiological functions, including lipid and iron metabolism, osteoclast stimulation during bone remodeling, angiogenesis, and immune system regulation. In terms of the latter, IL-6 is involved in promoting neutrophil production and chemotaxis, enhancing proinflammatory Th17 cell production and inhibiting the putative proinflammatory cytokines TNFα and IL-1β [41]. As such, IL-6 has been implicated in various disease states including autoimmune disorders, diabetes, cancer, arthritis, Alzheimer's disease, and even some forms of psychopathology.

2.6 IL-6 as a Therapeutic Target for Ischemic Stroke

Data from experimental models has warranted a close look at IL-6 as researchers search for novel therapeutic targets. However, contradictory results have complicated the discussion as to whether the actions and effects of this cytokine can be modulated effectively. For example, one study using IL-6 knockout mice found that IL-6 gene deletion had no effect on cerebral infarction in the tMCAO model [42]. A separate study using IL-6 knockout mice in the same tMCAO model showed worsened long-term infarcts and functional outcomes relative to wildtype mice. This recent study attributed these effects to reduced capacity for angiogenesis and diminished STAT3 activation. Additionally, cultured neurons, endothelial cells, and mixed glia increased IL-6 gene transcript in response to IL-6 application, suggesting that resident cells of the brain are capable of upregulating IL-6 following exogenous administration [43].

Although these conflicting data highlight the difficulty in formulating conclusions from animal studies, other lines of evidence are consistent with the latter study in demonstrating benefits of IL-6 signaling. Intracerebroventricular (i.c.v.)

administration of recombinant IL-6 prior to pMCAO significantly decreased infarct volume in rats, suggesting that IL-6 is directly neuroprotective at the level of the brain [44]. These data are supported by another group that utilized a mouse model of tMCAO to demonstrate reduced infarct volume following i.c.v. administration of IL-6. Here, the afforded protection was linked to STAT3-mediated SOD2 production and this study included another group of IL-6R deficient mice that showed exacerbated neural injury relative to wildtype and IL-6-treated mice [45].

Successful cell therapies have also provided insights into mechanisms of IL-6 protection. For example, cultured NSCs that were preconditioned with IL-6 prior to transplantation effectively increased angiogenesis, reduced infarct volume, and improved functional outcomes in mice subjected to ischemic stroke. Once again, the protective effects in this study were associated with SOD2 induction [46]. In a separate study investigating the splenic contribution to ischemic stroke, NSCs blocked stroke-induced elevations in IL-6 at 24 h and were found to be in direct contact with $CD11b^+$ splenic macrophages at 3 days [18]. It is noteworthy that IL-6 is secreted by macrophages in response to pathogen-associated molecular patterns (PAMPs) and other inflammatory signals. Since IL-6 was upregulated prior to maximal infarct expansion and the blunted IL-6 production was linked to macrophages, it is conceivable that selective inhibition of macrophage-derived IL-6 could be a promising strategy. Interestingly, splenocytes harvested from mice 22 h after tMCAO, but not at other time points, increased IL-6 upon activation in vitro [8]. These data suggest that splenic immune cells might be preferentially responsive at a time point consistent with delayed BBB opening and infarct expansion. In this case, it may be possible to develop a more selective and/or time-sensitive approach to targeting IL-6 signaling.

2.7 Interleukin-10 Signaling and Expression

Interleukin-10 (IL-10) is widely considered anti-inflammatory and exerts its actions through multiple mechanisms including the suppression of proinflammatory cytokines and the direct activation of cell survival pathways [47]. This cytokine binds to IL-10R, which is composed of two IL-10R1 domains and two IL-10R2 domains. The IL-10 receptor is coupled to the Jak/STAT signal transduction pathway, which is activated when IL-10 binds to IL-10R1 and initiates the signal transduction pathway through the IL-10R2 subunits [48].

Interleukin-10 blocks proinflammatory signaling by macrophages/microglia, inhibits signaling pathways activated by death receptors, and is associated with the Th2 phenotype [48]. Importantly, Th2 cells have been identified and implicated as contributors to the protective effects of anti-inflammatory compounds targeting peripheral immune cell activation following ischemia. In agreement with its role as a protective cytokine, data from human patients showed that those with worsening scores (based upon the Canadian Stroke Scale) within 48 h of ischemic stroke also showed significantly lower levels of systemic IL-10 [49]. However, the clinical data

available is inconsistent. For example, a study examining cytokine levels before and after thrombolytic therapy found a reduction in IL-10 plasma levels in the acute phase of stroke but no significant change after tPA administration [31]. These data were similar to a previous study that documented reduced IL-10 serum levels after stroke, yet no significant relationship between IL-10 levels and clinical outcomes [50].

Although circumstantial, the clinical reduction in IL-10 suggests that this cytokine may be involved in stroke pathology. Furthermore, experimental data does mirror the clinical expression profile in some ways. Unfortunately, data from these models is inconsistent and therefore difficult to interpret. In the pMCAO model of ischemic stroke, IL-10 gene transcript was reduced in rats 12 h following occlusion [51]. Conversely, a separate study demonstrated elevations in IL-10 mRNA 6 h post-MCAO in rodents.

2.8 IL-10 as a Therapeutic Target for Ischemic Stroke

The protective role of IL-10 has been documented in several experimental ischemia models. For example, IL-10 knockout mice showed larger infarct volumes after permanent focal ischemia. In the same study, primary neuronal cultures from knockout animals showed heightened susceptibility to oxygen glucose deprivation (OGD), while application of recombinant IL-10 protected these neurons from OGD [52]. A later study replicated these deleterious outcomes in mice lacking the IL-10 gene [21] while a recent study showed that the beneficial effects of ischemic preconditioning were negated in mice treated with IL-10R blocking antibodies prior to myocardial infarction [53].

In contrast to the previously mentioned knockout study, a separate study showed only a modest effect on infarct volume in IL-10 knockout mice, with slight increases in infarct volume and neurological deficits. Interestingly, several proinflammatory cytokines were elevated 4 days after stroke but other putative anti-inflammatory proteins were increased between 4 and 7 days after ischemia [54]. Taken together, these data once again demonstrate the caveats associated with targeting cytokine signaling. In this case, it would appear that both compensatory responses and temporal effects are critical in cytokine regulation of the ischemic microenvironment.

Other recent studies have shown that regulatory immune cells, particularly T-regulatory (T-reg) and B-reg cells, may suppress proinflammatory pathways that exacerbate neural injury following stroke [15, 21, 55, 56]. B-cell-deficient mice showed increased neural injury and behavioral deficits relative to controls [57]. A follow-up study employed adoptive transfer of IL-10-enriched, splenic B-cells stimulated with LPS in vitro. These cells were administered intravenously to mice 1 day prior to tMCAO. Data showed reduced infarct volumes that were accompanied by reduced extravasation of T-cells and monocytes into the brain parenchyma [58].

These data are consistent with previous reports documenting the success of therapies boosting IL-10 signaling. For example, IL-10 overexpression in neuroglia and

brain endothelial cells reduced infarct volume in mice after pMCAO [59], and viral-mediated i.c.v. gene transfer of IL-10 reduced infarct volume after pMCAO and dampened hippocampal injury after global ischemia in rats [60]. Importantly, the selective targeting of IL-10 signaling may lie in the targeting of specific IL-10-expressing immune cell types, rather than the pan activation of IL-10 signaling pathways. Gene transcript for several cytokines discussed in this chapter, including IL-10, was downregulated within the ischemic brains of SCID mice that are deficient in functional lymphocytes [15]. A recent study using IL-10 knockout mice and Rag2-deficient mice, which lack mature lymphocytes, suggested that IL-10 is required for CD4+ T-cells to facilitate protection in a rat model of facial nerve axotomy [61]. The authors linked protective IL-10 signaling to Th2 cell activation based upon these data and a previous report demonstrating the neuroprotective effects of Th2-type CD4+ cells [62].

Although plausible, questions remain regarding the selective targeting of IL-10 and the immune cell phenotype(s) that should be targeted following cerebral ischemia. Since CD4+ T-cells include distinct T-reg phenotypes and also represent both Th1- and Th2-type lymphocytes, it is difficult to ascertain a precise cellular target from these studies alone. Instead, it may be more beneficial to target a specific time point following ischemia. The preponderance of evidence concerning IL-10, and in fact all cytokines that influence the fate of ischemic tissues, indicates that the timing after insult may hold the key to successful therapeutics.

2.9 Interferon-Gamma Signaling and Expression

Interferon-gamma (IFN-γ) is an important cytokine involved in innate immune responses and has also been identified as a potential therapeutic target for ischemic stroke treatment. Due to its potent proinflammatory effects, IFN-γ may be involved in several aspects of ischemic injury. The IFN-y receptor exhibits ubiquitous expression and is found on virtually all cell types. Similar to IL-10, the receptor complex consists of two IFNγR1 domains that confer IFN-γ binding and two IFNγR2 domains that mediate signal transduction through Jak/STAT phosphorylation. Lymphocytes are major producers of IFN-γ, which primarily targets macrophages to protect against pathogens through chemokine production and secretion [63].

In terms of regulation, IFN-γ production is increased through positive feedback by virtue of its own expression, as well as through the actions of IL-12. The net result of IFN-γ signaling can include a multipronged inflammatory milieu created through the induction of MCP chemokines, B-cell activation, and ROS production. These events, in turn, facilitate a switch in microglia/macrophages toward the M1 phenotype and priming of naïve T-cells toward the Th1 phenotype [60]. Since IFN-γ elicits such profound effects after insult, the expression levels of this cytokine have been investigated both clinically and in preclinical stroke paradigms.

Data from multiple studies has shown increased IFN-γ production at various time points following ischemic stroke. For example, IFN-γ mRNA was increased

2 days following tMCAO and remained elevated 6 days after stroke [51]. Another study found elevated plasma levels of IFN-γ 24 h after tMCAO that returned to control levels by the 4-day time point [16]. Consistent with a role in cerebral infarct expansion, IFN-y increased 72 h after stroke in the brains of rats subjected to pMCAO [64] and mice subjected to tMCAO [65]. Thus, a role for IFN-γ in post-stroke signaling is evident according to data from studies employing multiple models and species.

A hallmark of stroke, both clinically and in animal models, includes infiltration of peripheral leukocytes into ischemic brain tissue. The extravasation of peripheral immune cells results from several processes, including proteolytic cleavage of BBB proteins by MMPs, induction of endothelial adhesion molecules, recruitment of polymorphonuclear cells, and extravasation of other immune cell types through a compromised BBB [33]. With regard to immune cell populations, the spleen is a known mediator of systemic inflammation and has been shown to contribute to ischemic injury in multiple tissues, including brain. Indeed, splenic IFN-γ gene transcript was increased 22 h after focal ischemia in rodents [15].

One major source of IFN-γ is natural killer (NK) cells, which were aptly named due to their ability to destroy cells infected by pathogens. These immune cells were detected in the brains of ischemic stroke patients and peaked within 2–5 days after onset. In the same study, a mouse model of pMCAO was utilized to further examine NK cell infiltration and cytokine expression. These data showed that IFN-γ-expressing NK cells peaked in the ischemic mouse brain 12 h post-stroke, while the interferon-inducible chemokine IP-10 was detected at both acute and delayed time points after stroke [66]. IP-10 expression is induced by IFN-γ and facilitates the Th1 inflammatory response through dual actions, including the direct activation of the CXCR3 receptor on Th1 cells and antagonism of the CCR3 receptor present on Th2 cells. Thus, IP-10 induction through IFN-γ signaling can amplify the Th1 response and dampen the Th2 response simultaneously, resulting in perpetuated tissue inflammation [67]. Importantly, T-cells and NK cells upregulate IFN-γ in response to signals from activated macrophages. In ischemic tissues, these macrophages are activated through toll-like receptors (TLRs) seated on the plasma membrane. In addition to PAMPs, which are the typical TLR ligands in cases of infection, these receptors also bind proteolytic fragments and epitopes of intracellular proteins that are released following cellular necrosis. These signals trigger macrophage secretion of cytokines that ultimately drive the production of IFN-γ in NK cells and T-cells. Thus, there are multiple ways in which IFN-γ production is induced and perpetuated following brain ischemia.

2.10 IFN-γ as a Therapeutic Target for Ischemic Stroke

By virtue of the mechanisms through which IFN-γ is activated, the microenvironment resulting from cerebral ischemia is ripe for IFN-γ production and signaling. Likewise, the fact that IFN-γ is a major orchestrator of the proinflammatory Th1

response suggests that tissue injury resulting from peripheral immune cell actions may be due, in large part, to IFN-γ signaling. Interestingly, there is some clinical data that indirectly supports this mechanism. Patients that developed post-stroke infections exhibited worse outcomes than their non-infected counterparts regardless of stroke severity. These outcomes were also linked to Th1 responsiveness to the brain antigens myelin basic protein (MBP) and glial fibrillary acidic protein (GFAP), which come into contact with T-cells as a consequence of BBB disruption, and patients with elevated Th1 responses 3 months post-stroke showed worsened outcomes [68]. Although these data are far from a smoking gun, they do support the notion that IFN-γ exerts actions involving immune cell modulation of ischemic tissue injury.

In terms of therapeutic potential, multiple lines of evidence support the selective targeting of IFN-γ as means of mitigating ischemic stroke injury. In one study, administration of simvastatin reduced infarct volume and IFN-γ levels in mice subjected to tMCAO. Interestingly, protection was also afforded by splenectomy, including reduced levels of IFN-γ in brain, and these effects were negated in splenectomized mice that received adoptive transfer of splenocytes [65]. These data linked neuroprotection with peripheral splenic immune cells and IFN-γ signaling, as did reports from other laboratories. For example, i.c.v. administration of IFN-γ blocking antibodies reduced infarct volume following tMCAO in mice. In this study, removal of protective T-reg cell populations augmented the activation of resident microglia and infiltration of T-cells, which produced IFN-γ [21].

Consistent with a peripheral source of IFN-γ, levels of this cytokine were elevated in the spleen 24 h following pMCAO while removal of the spleen reduced neural IFN-γ expression by 72 h [64]. Additionally, splenocytes harvested from mice subjected to focal ischemia demonstrated an increased capacity for IFN-γ production. In similar experiments involving harvested splenocytes but assessing the efficacy of HUCB cell therapy, data showed that protective doses of HUCB cells also reduced splenocyte proliferation and production of IFN-γ in vitro upon stimulation with concovalin A [28]. The ability of protective therapies to blunt IFN-γ signaling was also documented following administration of CART in mice subjected to tMCAO. Data showed that CART administration at the time of reperfusion reduced infarct volume and IFN-γ plasma levels 24 h after reperfusion. In addition, harvested splenocytes failed to produce IFN-γ following artificial stimulation in vitro [16].

Neuroprotection studies and in vitro assays have expanded our understanding of cytokine signaling and the potential for selective targeting in brain disease, including ischemic stroke. Knockout studies are another valuable tool for identifying mechanisms of action and have been utilized to explore the role of IFN-γ in cerebral ischemia. Mice lacking IFN-y have decreased infarcts compared to WT mice, consistent with a role in exacerbating neural injury. Interestingly, these knockout mice exhibit infarcts that are similar in magnitude to Rag1 knockout mice lacking T-cells and B-cells [69], suggesting a prominent role for peripheral immune cell-derived IFN-γ. In separate studies utilizing a variety of knockouts for IFN-γ and various immune cell populations, the protection afforded by deletion of RAG1 was reversed

by adoptive transfer of splenocytes from wildtype mice, but only partially restored upon transfer from IFN-$\gamma^{-/-}$ mice. Although none of the mice showed improvements in neurological scores [69], these experiments linked splenic IFN-γ to brain infarction following ischemic stroke. Another study utilizing SCID mice, a transgenic line similar to Rag1$^{-/-}$ in T- and B-cell deficiency, found reduced cortical infarction in SCID mice 22 h after tMCAO that was accompanied by elevations in splenic IFN-γ mRNA [15].

Taken together, these data provide strong support for selective targeting of IFN-γ and/or immune cell targets that are primarily responsible for IFN-γ-mediated infarct expansion. Although experimental models have improved our understanding of the key players regulating the proinflammatory microenvironment, the complexity of this environment mandates a cautious, deliberate approach. Future studies will determine whether IFN-γ can be modulated effectively to expand the therapeutic window for ischemic stroke treatment.

2.11 Leukemia Inhibitory Factor Signaling and Expression

Leukemia inhibitory factor (LIF), although far less studied compared to the other cytokines discussed in this chapter, may hold important therapeutic potential for the treatment of ischemic stroke and other neurological disorders. Under normal conditions, LIF is instrumental in facilitating cellular functions related to proliferation, differentiation, and cell survival. Under pathological conditions, however, LIF appears to be beneficial through its ability to induce antioxidant- and prosurvival-related gene expression [70]. This anti-inflammatory cytokine is a member of the IL-6 cytokine family [71] and binds to a heteromeric receptor dimer consisting of a LIF-binding chain (LIFR) and a converter subunit (gp130). Receptor activation by LIF can transduce signaling of multiple pathways, particularly those regulated by MAPK [72], phosphatidylinositol-3-kinase (PI3K)/Akt [73], and JAK/STAT [74, 75].

To date, few studies have conducted detailed investigations of LIF expression following brain ischemia. Nevertheless, LIF expression has been documented both clinically and experimentally to some degree. Postmortem tissues from human stroke patients revealed increased LIF expression in peri-infarct zones that localized to neurons and endothelial cells, while LIF plasma levels were found to be reduced within the first 6 h following ischemia. Alterations in LIF expression were also documented in rats after MCAO, with greatest levels detected 90 min post-stroke [76]. In response to experimental cortical lesion, rats showed a 30-fold increase in astrocytic LIF mRNA relative to controls. Specifically, LIF gene transcript was elevated as early as 6 h post-insult and reached its peak at the 24h time point. Closer examination revealed an expression pattern localized predominantly to astrocytes, along with a limited population of microglial cells [77].

The known protective role of astroglia following neural injury, coupled with the induction of LIF gene transcript, suggests that this cytokine may be instrumental in

the endogenous response to injury. The ability to upregulate antioxidant gene expression would be highly beneficial following brain ischemia, as free oxygen and nitrogen radicals are major contributors to the acute phase of injury. Similarly, the ability to bolster expression of prosurvival genes would be beneficial at both early and delayed time points following stroke. With this in mind, the following section summarizes key findings related to the therapeutic potential of targeting LIF for ischemic stroke treatment.

2.12 LIF as a Therapeutic Target for Ischemic Stroke

The prosurvival pathways elicited by LIF, particularly the PI3K/Akt pathway, are now regarded as promising targets for the treatment of any injury involving oxidative injury, tissue necrosis, and apoptotic signaling. The promise of LIF has been demonstrated in several experimental injury models, including those that produce white matter injury. Data showed that exogenous LIF administration reduced demyelinating injury in experimental autoimmune encephalomyelitis (EAE), a model that recapitulates white matter damage reminiscent of multiple sclerosis, by preserving oligodendrocytes (OLs) [78]. Additionally, LIF effectively improved outcomes following spinal cord transection in mice [79]. The protection afforded in the latter case was attributed to activation of JAK/STAT and Akt signaling and was also associated with the induction of anti-apoptotic proteins. Similarly, LIF reduced motor nerve degeneration in the SOD1 G93A murine model of familiar amyotrophic lateral sclerosis (ALS) [80].

Importantly, the benefits of LIF treatment observed in these earlier studies have now been extended to models of cerebral ischemia. In these recent studies, similar pathways appear to mediate the protective effects of LIF. Data showed that LIF effectively decreased infarct volume and white matter injury when administered to rats following pMCAO. In addition to mitigating neural injury, LIF treatment also improved functional outcomes. Further investigation revealed a mechanism operating through Akt-mediated induction of the antioxidant protein peroxiredoxin 4 (Prdx4), as LIF efficacy in protecting OL primary cultures from OGD was negated by co-incubation with Akt inhibitors and Prdx4 neutralizing antibodies [81]. Although neuronal cultures were not used in these studies, neurons and OLs are both susceptible to oxidative injury and therefore benefit from the induction of antioxidant proteins in the wake of ischemia. Likewise, stroke injury includes OL cell death and neuronal injury results, in part, from anoikis (detachment-induced cell death) following the disruption of astroglial junctions.

The therapeutic potential of LIF, although promising, lies in the ability to target LIF signaling at time points prior to maximal infarction. Since the infarct expands from hours to days following stroke onset, it is likely that the therapeutic window can be extended providing that the LIF safety profile is acceptable. To date, LIF has been used clinically for separate indications and has demonstrated a good safety profile. Nevertheless, a better option would be to stimulate the endogenous pathways

activated by LIF (i.e., Akt signaling) as means of circumventing potential issues with safety and dosing. Since LIF can be released by several cell types following injury [77, 82, 83], it will be crucial to characterize the sources of endogenous LIF signaling. Once the temporal and cell-specific responses are uncovered with regard to LIF signaling, there will be more therapeutic options to mimic the endogenous protection afforded by this cytokine.

References

1. Lipton P. Ischemic cell death in brain neurons. Physiol Rev. 1999;79:1431–568.
2. Citron M, Westaway D, Xia W, Carlson G, Diehl T, Levesque G, et al. Mutant presenilins of Alzheimer's disease increase production of 42 residue amyloid B-protein in both transfected cells and transgenic mice. Nat Med. 1997;3:67–72.
3. Davis SM, Lees KR, Albers GW, Diener HC, Markabi S, Karlsson G, et al. Selfotel in acute ischemic stroke: possible neurotoxic effects of an NMDA antagonist. Stroke. 2000;31(2): 347–54.
4. Danton GH, Dietrich WD. Inflammatory mechanisms after ischemia and stroke. J Neuropathol Exp Neurol. 2003;62(2):127–36.
5. Allan SM, Rothwell NJ. Cytokines and acute neurodegeneration. Nat Rev Neurosci. 2001;2(10):734–44.
6. Candelario-Jalil E, Yang Y, Rosenberg GA. Diverse roles of matrix metalloproteinases and tissue inhibitors of metalloproteinases in neuroinflammation and cerebral ischemia. Neuroscience. 2009;158(3):983–94. doi:10.1016/j.neuroscience.2008.06.025.
7. Dinarello CA. Historical insights into cytokines. Eur J Immunol. 2007;37 Suppl 1:S34–45. doi:10.1002/eji.200737772.
8. Offner H, Subramanian S, Parker SM, Afentoulis ME, Vandenbark AA, Hurn PD. Experimental stroke induces massive, rapid activation of the peripheral immune system. J Cereb Blood Flow Metab. 2006;26(5):654–65.
9. Streit WJ, Mrak RE, Griffin WS. Microglia and neuroinflammation: a pathological perspective. J Neuroinflammation. 2004;1(1):14.
10. Speeckaert MM, Speeckaert R, Laute M, Vanholder R, Delanghe JR. Tumor necrosis factor receptors: biology and therapeutic potential in kidney diseases. Am J Nephrol. 2012;36(3):261–70. doi:10.1159/000342333.
11. Hallenbeck JM. The many faces of tumor necrosis factor in stroke. Nat Med. 2002;8(12):1363–8. doi:10.1038/nm1202-1363.
12. Ritzel RM, Patel AR, Grenier JM, Crapser J, Verma R, Jellison ER, et al. Functional differences between microglia and monocytes after ischemic stroke. J Neuroinflammation. 2015;12:106. doi:10.1186/s12974-015-0329-1.
13. Jaeschke H. Reactive oxygen and mechanisms of inflammatory liver injury. J Gastroenterol Hepatol. 2000;15(7):718–24.
14. Fan C, Zwacka RM, Engelhardt JF. Therapeutic approaches for ischemia/reperfusion injury in the liver. J Mol Med. 1999;77(8):577–92.
15. Hurn PD, Subramanian S, Parker SM, Afentoulis ME, Kaler LJ, Vandenbark AA, et al. T- and B-cell-deficient mice with experimental stroke have reduced lesion size and inflammation. J Cereb Blood Flow Metab. 2007;27(11):1798–805.
16. Chang L, Chen Y, Li J, Liu Z, Wang Z, Chen J, et al. Cocaine-and amphetamine-regulated transcript modulates peripheral immunity and protects against brain injury in experimental stroke. Brain Behav Immun. 2011;25(2):260–9. doi:10.1016/j.bbi.2010.09.017.

17. Schwarting S, Litwak S, Hao W, Bahr M, Weise J, Neumann H. Hematopoietic stem cells reduce postischemic inflammation and ameliorate ischemic brain injury. Stroke. 2008;39(10): 2867–75.
18. Lee ST, Chu K, Jung KH, Kim SJ, Kim DH, Kang KM, et al. Anti-inflammatory mechanism of intravascular neural stem cell transplantation in haemorrhagic stroke. Brain. 2008;131(Pt 3):616–29.
19. Barone FC, Arvin B, White RF, Miller A, Webb CL, Willette RN, et al. Tumor necrosis factor-alpha. A mediator of focal ischemic brain injury. Stroke. 1997;28(6):1233–44.
20. Wang X, Feuerstein GZ, Xu L, Wang H, Schumacher WA, Ogletree ML, et al. Inhibition of tumor necrosis factor-alpha-converting enzyme by a selective antagonist protects brain from focal ischemic injury in rats. Mol Pharmacol. 2004;65(4):890–6. doi:10.1124/mol.65.4.890.
21. Liesz A, Suri-Payer E, Veltkamp C, Doerr H, Sommer C, Rivest S, et al. Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. Nat Med. 2009;15(2): 192–9. doi:10.1038/nm.1927.
22. Lambertsen KL, Clausen BH, Babcock AA, Gregersen R, Fenger C, Nielsen HH, et al. Microglia protect neurons against ischemia by synthesis of tumor necrosis factor. J Neurosci. 2009;29(5):1319–30. doi:10.1523/JNEUROSCI.5505-08.2009.
23. Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK, Ting JP. TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. Nat Neurosci. 2001;4(11): 1116–22. doi:10.1038/nn738.
24. Okuaki Y, Miyazaki H, Zeniya M, Ishikawa T, Ohkawa Y, Tsuno S, et al. Splenectomy-reduced hepatic injury induced by ischemia/reperfusion in the rat. Liver. 1996;16(3):188–94.
25. Jiang H, Meng F, Li W, Tong L, Qiao H, Sun X. Splenectomy ameliorates acute multiple organ damage induced by liver warm ischemia reperfusion in rats. Surgery. 2007;141(1):32–40.
26. Savas MC, Ozguner M, Ozguner IF, Delibas N. Splenectomy attenuates intestinal ischemia-reperfusion-induced acute lung injury. J Pediatr Surg. 2003;38(10):1465–70.
27. Li M, Li F, Luo C, Shan Y, Zhang L, Qian Z, et al. Immediate splenectomy decreases mortality and improves cognitive function of rats after severe traumatic brain injury. J Trauma. 2011; 71(1):141–7.
28. Vendrame M, Gemma C, Pennypacker KR, Bickford PC, Davis Sanberg C, Sanberg PR, et al. Cord blood rescues stroke-induced changes in splenocyte phenotype and function. Exp Neurol. 2006;199(1):191–200.
29. Tuttolomondo A, Pecoraro R, Pinto A. Studies of selective TNF inhibitors in the treatment of brain injury from stroke and trauma: a review of the evidence to date. Drug Des Devel Ther. 2014;8:2221–38. doi:10.2147/DDDT.S67655.
30. Schonbeck U, Mach F, Libby P. Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. J Immunol. 1998;161(7):3340–6.
31. Mazzotta G, Sarchielli P, Caso V, Paciaroni M, Floridi A, Floridi A, et al. Different cytokine levels in thrombolysis patients as predictors for clinical outcome. Eur J Neurol. 2004;11(6):377–81. doi:10.1111/j.1468-1331.2004.00798.x.
32. Rosenberg GA, Estrada EY, Dencoff JE. Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain. Stroke. 1998;29(10):2189–95.
33. Amantea D, Micieli G, Tassorelli C, Cuartero MI, Ballesteros I, Certo M, et al. Rational modulation of the innate immune system for neuroprotection in ischemic stroke. Front Neurosci. 2015;9:147. doi:10.3389/fnins.2015.00147.
34. Hu X, Li P, Guo Y, Wang H, Leak RK, Chen S, et al. Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. Stroke. 2012;43(11):3063–70. doi:10.1161/STROKEAHA.112.659656.
35. Hennessy E, Griffin EW, Cunningham C. Astrocytes are primed by chronic neurodegeneration to produce exaggerated chemokine and cell infiltration responses to acute stimulation with the cytokines IL-1beta and TNF-alpha. J Neurosci. 2015;35(22):8411–22. doi:10.1523/JNEUROSCI.2745-14.2015.

36. Deroide N, Li X, Lerouet D, Van Vre E, Baker L, Harrison J, et al. MFGE8 inhibits inflammasome-induced IL-1beta production and limits postischemic cerebral injury. J Clin Invest. 2013;123(3):1176–81. doi:10.1172/JCI65167.
37. Fernandez-Cadenas I, Del Rio-Espinola A, Giralt D, Domingues-Montanari S, Quiroga A, Mendioroz M, et al. IL1B and VWF variants are associated with fibrinolytic early recanalization in patients with ischemic stroke. Stroke. 2012;43(10):2659–65. doi:10.1161/STROKEAHA.112.657007.
38. Beamer NB, Coull BM, Clark WM, Hazel JS, Silberger JR. Interleukin-6 and interleukin-1 receptor antagonist in acute stroke. `. 1995;37(6):800–5. doi:10.1002/ana.410370614.
39. Rallidis LS, Vikelis M, Panagiotakos DB, Rizos I, Zolindaki MG, Kaliva K, et al. Inflammatory markers and in-hospital mortality in acute ischaemic stroke. Atherosclerosis. 2006;189(1):193–7. doi:10.1016/j.atherosclerosis.2005.11.032.
40. Luna JM, Moon YP, Liu KM, Spitalnik S, Paik MC, Cheung K, et al. High-sensitivity C-reactive protein and interleukin-6-dominant inflammation and ischemic stroke risk: the northernManhattanstudy.Stroke.2014;45(4):979–87.doi:10.1161/STROKEAHA.113.002289.
41. Mihara M, Hashizume M, Yoshida H, Suzuki M, Shiina M. IL-6/IL-6 receptor system and its role in physiological and pathological conditions. Clin Sci (Lond). 2012;122(4):143–59. doi:10.1042/CS20110340.
42. Clark WM, Rinker LG, Lessov NS, Hazel K, Hill JK, Stenzel-Poore M, et al. Lack of interleukin-6 expression is not protective against focal central nervous system ischemia. Stroke. 2000;31(7):1715–20.
43. Gertz K, Kronenberg G, Kalin RE, Baldinger T, Werner C, Balkaya M, et al. Essential role of interleukin-6 in post-stroke angiogenesis. Brain. 2012;135(Pt 6):1964–80. doi:10.1093/brain/aws075.
44. Loddick SA, Turnbull AV, Rothwell NJ. Cerebral interleukin-6 is neuroprotective during permanent focal cerebral ischemia in the rat. J Cereb Blood Flow Metab. 1998;18(2):176–9. doi:10.1097/00004647-199802000-00008.
45. Jung JE, Kim GS, Chan PH. Neuroprotection by interleukin-6 is mediated by signal transducer and activator of transcription 3 and antioxidative signaling in ischemic stroke. Stroke. 2011;42(12):3574–9. doi:10.1161/STROKEAHA.111.626648.
46. Sakata H, Narasimhan P, Niizuma K, Maier CM, Wakai T, Chan PH. Interleukin 6-preconditioned neural stem cells reduce ischaemic injury in stroke mice. Brain. 2012;135(Pt 11):3298–310. doi:10.1093/brain/aws259.
47. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol. 2001;19:683–765. doi:10.1146/annurev.immunol.19.1.683.
48. Doll DN, Barr TL, Simpkins JW. Cytokines: their role in stroke and potential use as biomarkers and therapeutic targets. Aging Dis. 2014;5(5):294–306. doi:10.14336/AD.2014.0500294.
49. Vila N, Castillo J, Davalos A, Esteve A, Planas AM, Chamorro A. Levels of anti-inflammatory cytokines and neurological worsening in acute ischemic stroke. Stroke. 2003;34(3):671–5. doi:10.1161/01.STR.0000057976.53301.69.
50. Perini F, Morra M, Alecci M, Galloni E, Marchi M, Toso V. Temporal profile of serum anti-inflammatory and pro-inflammatory interleukins in acute ischemic stroke patients. Neurol Sci. 2001;22(4):289–96.
51. Li HL, Kostulas N, Huang YM, Xiao BG, van der Meide P, Kostulas V, et al. IL-17 and IFN-gamma mRNA expression is increased in the brain and systemically after permanent middle cerebral artery occlusion in the rat. J Neuroimmunol. 2001;116(1):5–14.
52. Grilli M, Barbieri I, Basudev H, Brusa R, Casati C, Lozza G, et al. Interleukin-10 modulates neuronal threshold of vulnerability to ischaemic damage. Eur J Neurosci. 2000;12(7):2265–72.
53. Cai G, Nie X, Zhang W, Wu B, Lin J, Wang H, et al. A regulatory role for IL-10 receptor signaling in development and B cell help of T follicular helper cells in mice. J Immunol. 2012;189(3):1294–302. doi:10.4049/jimmunol.1102948.

54. Puig J, Blasco G, Daunis IEJ, Thomalla G, Castellanos M, Figueras J, et al. Decreased corticospinal tract fractional anisotropy predicts long-term motor outcome after stroke. Stroke. 2013;44(7):2016–8. doi:10.1161/STROKEAHA.111.000382.
55. Frenkel D, Huang Z, Maron R, Koldzic DN, Moskowitz MA, Weiner HL. Neuroprotection by IL-10-producing MOG CD4+ T cells following ischemic stroke. J Neurol Sci. 2005;233(1–2):125–32.
56. Carter NA, Vasconcellos R, Rosser EC, Tulone C, Munoz-Suano A, Kamanaka M, et al. Mice lacking endogenous IL-10-producing regulatory B cells develop exacerbated disease and present with an increased frequency of Th1/Th17 but a decrease in regulatory T cells. J Immunol. 2011;186(10):5569–79. doi:10.4049/jimmunol.1100284.
57. Ren X, Akiyoshi K, Vandenbark AA, Hurn PD, Offner H. CD4+FoxP3+ regulatory T-cells in cerebral ischemic stroke. Metab Brain Dis. 2010;26(1):87–90.
58. Bodhankar S, Chen Y, Vandenbark AA, Murphy SJ, Offner H. Treatment of experimental stroke with IL-10-producing B-cells reduces infarct size and peripheral and CNS inflammation in wild-type B-cell-sufficient mice. Metab Brain Dis. 2014;29(1):59–73. doi:10.1007/s11011-013-9474-3.
59. de Bilbao F, Arsenijevic D, Moll T, Garcia-Gabay I, Vallet P, Langhans W, et al. In vivo overexpression of interleukin-10 increases resistance to focal brain ischemia in mice. J Neurochem. 2009;110(1):12–22.
60. Ooboshi H, Ibayashi S, Shichita T, Kumai Y, Takada J, Ago T, et al. Postischemic gene transfer of interleukin-10 protects against both focal and global brain ischemia. Circulation. 2005;111(7):913–9.
61. Xin J, Wainwright DA, Mesnard NA, Serpe CJ, Sanders VM, Jones KJ. IL-10 within the CNS is necessary for CD4+ T cells to mediate neuroprotection. Brain Behav Immun. 2011;25(5):820–9. doi:10.1016/j.bbi.2010.08.004.
62. DeBoy CA, Byram SC, Serpe CJ, Wisuri D, Sanders VM, Jones KJ. CD4+CD25+ regulatory T cells and CD1-restricted NKT cells do not mediate facial motoneuron survival after axotomy. J Neuroimmunol. 2006;176(1–2):34–8. doi:10.1016/j.jneuroim.2006.04.006.
63. Groom JR, Luster AD. CXCR3 ligands: redundant, collaborative and antagonistic functions. Immunol Cell Biol. 2011;89(2):207–15. doi:10.1038/icb.2010.158.
64. Seifert HA, Leonardo CC, Hall AA, Rowe DD, Collier LA, Benkovic SA, et al. The spleen contributes to stroke induced neurodegeneration through interferon gamma signaling. Metab Brain Dis. 2012;27(2):131–41. doi:10.1007/s11011-012-9283-0.
65. Jin R, Zhu X, Liu L, Nanda A, Granger DN, Li G. Simvastatin attenuates stroke-induced splenic atrophy and lung susceptibility to spontaneous bacterial infection in mice. Stroke. 2013;44(4):1135–43. doi:10.1161/STROKEAHA.111.000633.
66. Zhang Y, Gao Z, Wang D, Zhang T, Sun B, Mu L, et al. Accumulation of natural killer cells in ischemic brain tissues and the chemotactic effect of IP-10. J Neuroinflammation. 2014;11:79. doi:10.1186/1742-2094-11-79.
67. Loetscher P, Pellegrino A, Gong JH, Mattioli I, Loetscher M, Bardi G, et al. The ligands of CXC chemokine receptor 3, I-TAC, Mig, and IP10, are natural antagonists for CCR3. J Biol Chem. 2001;276(5):2986–91.
68. Becker KJ, Kalil AJ, Tanzi P, Zierath DK, Savos AV, Gee JM, et al. Autoimmune responses to the brain after stroke are associated with worse outcome. Stroke. 2011;42(10):2763–9. doi:10.1161/STROKEAHA.111.619593.
69. Yilmaz G, Arumugam TV, Stokes KY, Granger DN. Role of T lymphocytes and interferon-gamma in ischemic stroke. Circulation. 2006;113(17):2105–12.
70. Alonzi T, Middleton G, Wyatt S, Buchman V, Betz UA, Müller W, et al. Role of STAT3 and PI 3-kinase/Akt in mediating the survival actions of cytokines on sensory neurons. Mol Cell Neurosci. 2001;18(3):270–82.
71. Metcalfe SM, Watson TJ, Shurey S, Adams E, Green CJ. Leukemia inhibitory factor is linked to regulatory transplantation tolerance. Transplantation. 2005;79(6):726–30.
72. Boulton TG, Stahl N, Yancopoulos GD. Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common

set of proteins overlapping those induced by other cytokines and growth factors. J Biol Chem. 1994;269(15):11648–55.
73. Oh H, Fujio Y, Kunisada K, Hirota H, Matsui H, Kishimoto T, et al. Activation of phosphatidylinositol 3-kinase through glycoprotein 130 induces protein kinase B and p70 S6 kinase phosphorylation in cardiac myocytes. J Biol Chem. 1998;273(16):9703–10. doi:10.1074/jbc.273.16.9703.
74. Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, et al. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. Science. 1994;263(5143):92–5.
75. Stahl N, Farruggella TJ, Boulton TG, Zhong Z, Darnell J, Yancopoulos GD. Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. Science. 1995;267(5202):1349–53.
76. Slevin M, Krupinski J, Mitsios N, Perikleous C, Cuadrado E, Montaner J, et al. Leukaemia inhibitory factor is over-expressed by ischaemic brain tissue concomitant with reduced plasma expression following acute stroke. Eur J Neurol. 2008;15(1):29–37. doi:10.1111/j.1468-1331.2007.01995.x.
77. Banner LR, Moayeri NN, Patterson PH. Leukemia inhibitory factor is expressed in astrocytes following cortical brain injury. Exp Neurol. 1997;147(1):1–9.
78. Butzkueven H, Zhang J-G, Soilu-Hanninen M, Hochrein H, Chionh F, Shipham KA, et al. LIF receptor signaling limits immune-mediated demyelination by enhancing oligodendrocyte survival. Nat Med. 2002;8(6):613–9.
79. Azari MF, Profyris C, Karnezis T, Bernard CC, Small DH, Cheema SS, et al. Leukemia inhibitory factor arrests oligodendrocyte death and demyelination in spinal cord injury. J Neuropathol Exp Neurol. 2006;65(9):914–29.
80. Azari MF, Galle A, Lopes EC, Kurek J, Cheema SS. Leukemia inhibitory factor by systemic administration rescues spinal motor neurons in the SOD1 G93A murine model of familial amyotrophic lateral sclerosis. Brain Res. 2001;922(1):144–7.
81. Rowe DD, Collier LA, Seifert HA, Chapman CB, Leonardo CC, Willing AE, et al. Leukemia inhibitor factor promotes functional recovery and oligodendrocyte survival in rat models of focal ischemia. Eur J Neurosci. 2014;40(7):3111–9.
82. Chen C-W, Okada M, Proto JD, Gao X, Sekiya N, Beckman SA, et al. Human pericytes for ischemic heart repair. Stem Cells. 2013;31(2):305–16.
83. Laterza C, Merlini A, De Feo D, Ruffini F, Menon R, Onorati M, et al. iPSC-derived neural precursors exert a neuroprotective role in immune-mediated demyelination via the secretion of LIF. Nat Commun. 2013;4:2597. doi:10.1038/ncomms3597.

Part IV
White Matter Injury and Repair in Stroke

Ischemic Injury to White Matter: An Age-Dependent Process

Sylvain Brunet, Chinthasagar Bastian, and Selva Baltan

Abbreviations

AMPA/KA	AMPA/kainate
CKA	7-Chlorokynurenic acid
CNS	Central nervous system
GS	Glutamate synthetase
KB-R	2-[2-[4(4-Nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate
MON	Mouse optic nerve
NCX	Na^+–Ca^{2+} exchanger
NMDAR	NMDA-type receptors
OGD	Oxygen glucose deprivation
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
WM	White matter

S. Brunet, B.Sc., Ph.D. • S. Baltan, M.D., Ph.D. (✉)
Department of Molecular Medicine, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, 9500 Euclid Avenue (NB21), Cleveland, OH 44195, USA

Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue (NB30), Cleveland, OH 44195, USA
e-mail: brunets@ccf.org; baltans@ccf.org

C. Bastian, M.B.B.S., Ph.D.
Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, 9500 Euclid Avenue (NB30), Cleveland, OH 44195, USA
e-mail: bastiac@ccf.org

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_16

1 Introduction

In the United States, someone experiences a stroke every 40 s [1]. Axonal injury and dysfunction are responsible for most of the disability observed after a stroke [2] and aging is one of the most significant risk factors for stroke. The human brain comprises equal proportions of gray matter and white matter (WM) and WM is injured in most strokes [2]. However, most research efforts have traditionally been dedicated to protecting the gray matter. While effective in rodents, this approach has failed to translate to humans. Many reasons may underlie this failure, but major differences between humans and rodents include the greater proportion of WM in the human brain and the lack of significant WM involvement following middle cerebral artery occlusion in rodents, which is one of the most widely used animal models of stroke. Thus, over the past several years we and others have focused on examining how WM responds to ischemic injury, with an emphasis on the impact of aging [3–5].

2 WM Is Sensitive to Ischemic Injury

WM axons are dependent on a constant supply of oxygen and glucose to transmit signals. Central nervous system (CNS) WM electrical function is remarkably tolerant to anoxia [6], while there is regional heterogeneity in the ability to function and survive anoxia [7]. On the other hand, young adult WM is susceptible to ischemia induced by combined oxygen and glucose deprivation (OGD, Fig. 1). Mechanisms underlying ischemic WM injury proved to be unpredictably complex (Fig. 2) [8–16]. WM is composed of axons, oligodendrocytes, microglia, and astrocytes [2, 17]. Axons are myelinated by oligodendrocytes and exhibit patterns/gaps called nodes of Ranvier. Astrocytes support axons metabolically and restore the extracellular ionic environment following axonal activity. Microglia is partly responsible for immune surveillance. Thus, WM is composed of a complex cellular environment in which glial cell–cell interactions intricately maintain axon function. During ischemia, WM cellular elements are individually under attack but remain interactive with each other in intricate mechanisms that are currently under investigation.

3 Mechanisms of WM Ischemic Injury

It is now well-established that ischemia in WM sequentially activates three different injury pathways: the ionic, the excitotoxic, and the oxidative stress injury pathways. The ionic pathway attacks axons by collapsing their ionic homeostasis, which is initiated by the failure of the Na^+–K^+ pump, cell membrane depolarization, Na^+ channel activation, reversal of the Na^+/Ca^{2+} exchanger, and Ca^{2+} channel activation, resulting in the accumulation of intracellular Na^+ and Ca^{2+} (Fig. 2, yellow) [18–23]. This increased intracellular Na^+ leads to the reversal of the Na^+-glutamate transporter and the release of glutamate from astrocytes [16].

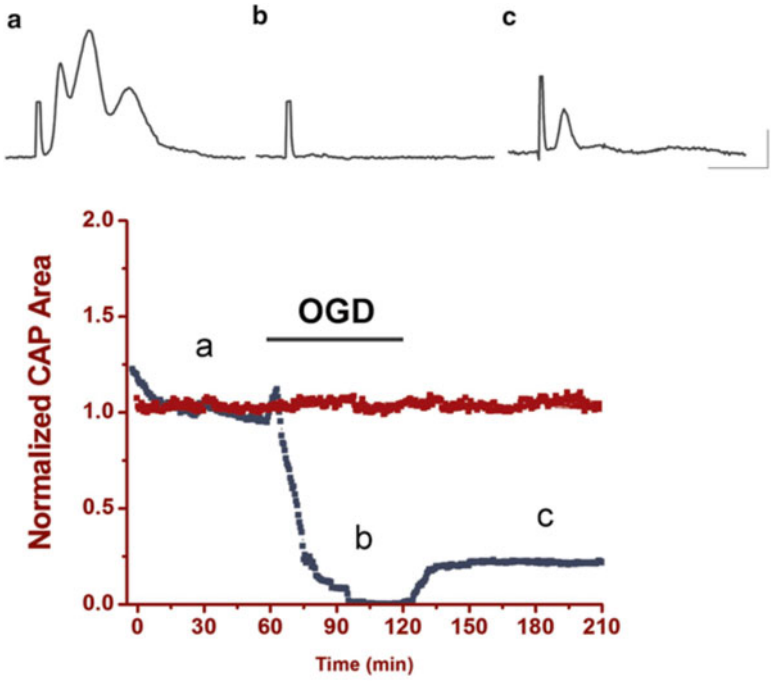

Fig. 1 WM is susceptible to ischemic injury. Axon function is quantified as the area under the compound action potential (CAP), normalized to control area, and plotted against time. Under normal conditions, CAP area is stable for long periods of time (*brown*). A 60 min period of OGD gradually depresses CAP area until conduction along axons is completely lost (*gray*). Restoring oxygen and glucose leads to ~25 % axon function recovery. Sample traces from control (*a*), OGD (*b*), and recovery (*c*) periods are shown above the graph (Reproduced in part from Baltan (2014) [18])

Subsequently, the excitotoxic pathway is initiated by an increase in extracellular glutamate (Fig. 2, green). The excitotoxic pathway mainly targets oligodendrocytes by overactivating AMPA/KA receptors [11, 14, 16, 24–27] (redox) [28], which leads to increases in intracellular Na^+ and Ca^{2+} and the activation of downstream toxic intracellular pathways to mediate WM injury.

In parallel with the excitotoxic pathway, increased extracellular glutamate also leads to the activation of the oxidative stress pathway (Fig. 2, blue). The oxidative pathway damages WM components due to the formation of reactive oxygen species (ROS), which arises because the increased extracellular glutamate competes with cysteine at the glutamate-cysteine pump [29], depleting intracellular cysteine to reduce glutathione levels [30] and to cause mitochondrial dysfunction. In addition, the increase in intracellular Ca^{2+} activates NOS to produce nitric oxide [31, 32], which readily reacts with ROS to produce reactive nitrogen species (RNS). ROS and RNS can then attack multiple cellular elements (phospholipids, proteins, DNA, RNA) to mediate injury.

The ionic pathway triggers the injury process, which subsequently reverses the glutamate transporter; however, it is the accumulation of glutamate that dictates the threshold for irreversible injury. Therefore, if the injury is short and only involves

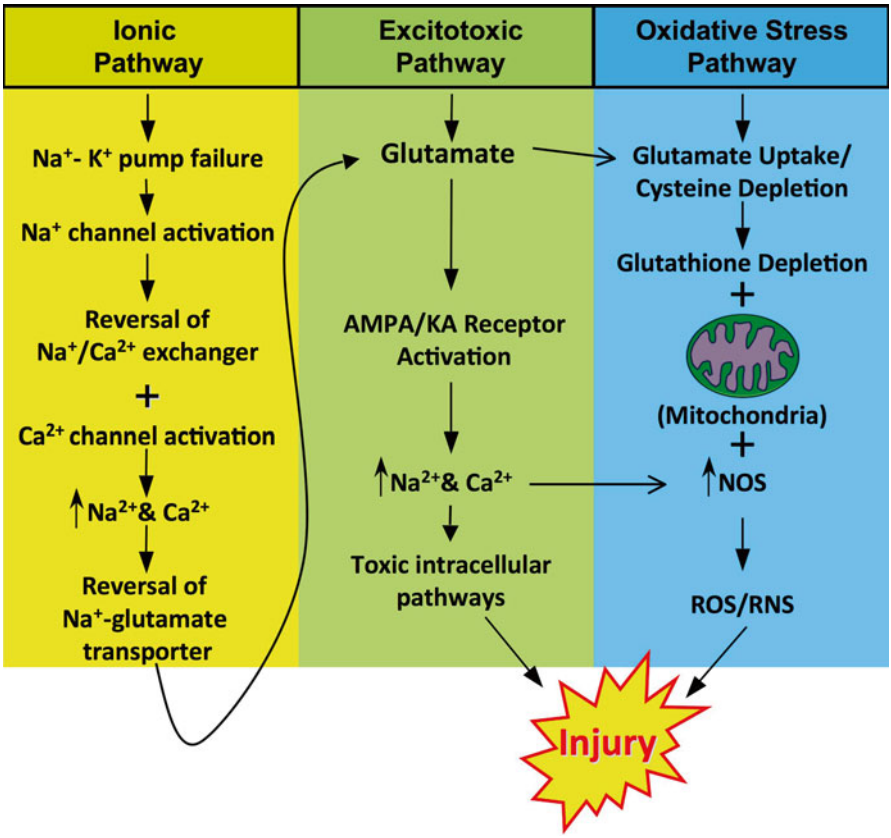

Fig. 2 Ischemia activates three pathways to mediate WM injury. Ischemia leads to the activation of the ionic pathway, which then leads to the sequential activation of the excitotoxic and oxidative stress pathways, which converge to cause irreversible injury to WM during ischemia. Note that glutamate release due to reversed Na^+-dependent transport dictates the irreversible nature of the injury. *ROS* reactive oxygen species (Reproduced in part from Baltan (2009) [2])

the ionic pathway, then the ischemic injury is completely reversible. Unlike gray matter, this sequential order of events is necessary for the injury to develop, such that bypassing the ionic pathway and applying exogenous glutamate (or glutamate analogues) fails to cause WM injury [16].

4 Mouse Optic Nerve: An Ideal Model to Investigate WM

The mouse optic nerve (MON) is ideal for ischemic studies of WM. The optic nerve, the second cranial nerve, is a purely myelinated central nervous system WM tract and is sensitive to ischemia and to the aging process [33, 34]. In addition, tissue

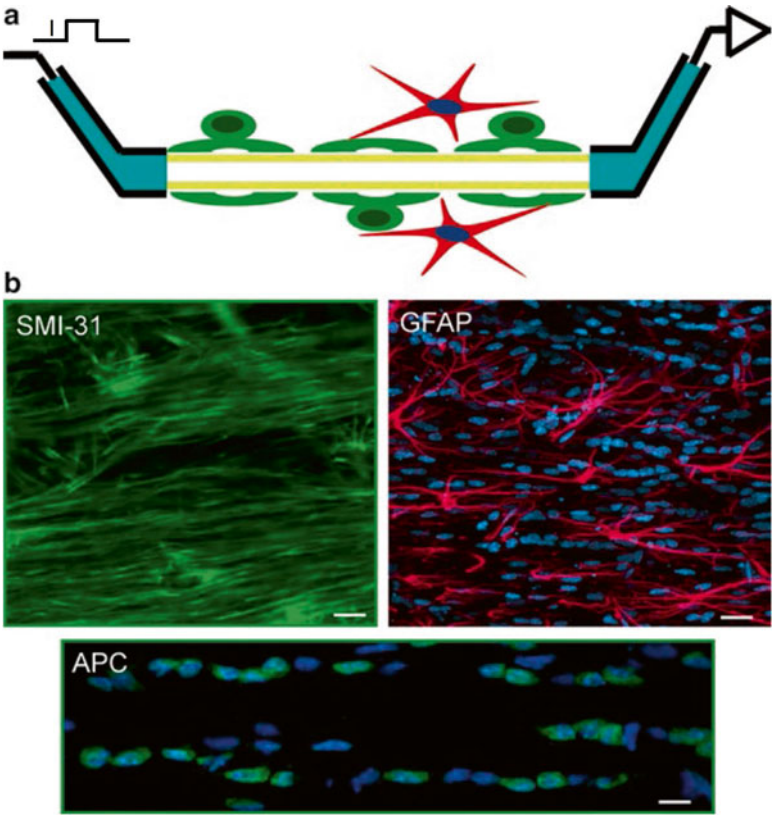

Fig. 3 The MON model is ideal for monitoring WM electrical function and cellular architecture. (**a**) Use of suction electrodes allows all axons to be stimulated and a CAP to be recorded. Cartoon of mouse optic nerve between two suction electrodes, where the left suction electrode stimulates and the right suction electrode records the CAP. Axons are represented in *yellow*, oligodendrocytes are in *green*, and astrocytes are in *red*. *I* current. (**b**) Using cell-specific antibodies, WM axons are labeled with SMI-31 for neurofilament (*green*), GFAP for astrocytes (*magenta*), and APC for mature oligodendrocyte cell bodies (*green*). Sytox (+) glial nuclei are in *blue*. Scale bar = 50 μm for SMI-31 and GFAP, 10 μm for APC (Reproduced in part from Baltan (2014) [18])

isolation does not require extensive surgical interventions; therefore there is negligible surgical injury, its small diameter allows sufficient glucose diffusion [6, 35], there are no neurons or synapses to contribute indirectly to the ischemic injury, and electrical function can be monitored by recording evoked compound action potentials (CAPs). MONs are stable both structurally and electrically for long durations (18 h) and glial cells and axons retain their native relationships to one another within a three-dimensional spatial organization (Fig. 3a). Furthermore, the cellular components can reliably be identified using immunohistochemistry (Fig. 3b), glutamate release can be measured by HPLC [36], proteins of interest can be quantified by Western blot analysis [3], and intravitreal injections provide a path for axonal

delivery [37]. Finally, the current and future availability of genetically engineered mice to experimentally test the role(s) of specific injury pathway components make the MON a model ideally suited to study WM injury mechanisms.

5 Why Is Aging WM More Susceptible to Ischemic Injury?

5.1 A Ca^{2+}-Independent Excitotoxicity Pathway in Aging WM

In aging WM, there is an enhanced contribution of a Ca^{2+}-independent excitotoxicity pathway to ischemic injury. In young WM, intracellular Ca^{2+} accumulation is important in the development of WM ischemic injury, and removal of extracellular Ca^{2+}, or blockade of Ca^{2+} entry via reversal of the Na^+–Ca^{2+} exchanger (NCX) with 2-[2-[4(4-nitrobenzyloxy)phenyl]ethyl]isothiourea mesylate (KB-R), improves WM electrical function (Fig. 4A, left). In contrast, in older WM these interventions fail to improve injury. In fact, OGD applied in Ca^{2+}-free conditions harms recovery, suggesting that extracellular Ca^{2+} entry does not significantly contribute to ischemic injury in aging WM (Fig. 4A, right). In addition, the lack of protection afforded by blockade of Ca^{2+} entry secondary from the reversal of the NCX confirms the Ca^{2+}-independent nature of ischemic injury in aging WM. On the other hand, in aging MONs, it is possible that Ca^{2+} release from intracellular Ca^{2+} stores becomes accentuated during ischemia. However, the exact relationship between transmembrane Ca^{2+} influx and intracellular Ca^{2+} concentration, and the role of intracellular Ca^{2+} stores in aging WM, all remain to be established. Though these results suggest an attenuated role for the ionic pathway with aging, whether WM injury can be triggered directly at the excitotoxic pathway needs to be tested.

Overactivation of AMPA/kainate (AMPA/KA) receptors mediates excitotoxic injury in both young and aging WM. Thus, blockade of these receptors protects axon function against OGD in both young and aging WM. Interestingly, unless combined with a brief period of OGD [16], activating AMPA/KA receptors with glutamate (or glutamate agonists) does not cause WM injury. This is presumably because of efficient uptake of glutamate from the extracellular compartment by Na^+-dependent transporters, such as GLT-1, as long as energy supply is maintained (Figs. 2 and 5). These findings suggest that OGD needs to activate ionic dysfunction to prime WM to the toxic effects of glutamate [16]. In aging WM, the Ca^{2+}-independent nature of excitotoxicity raises the question as to how activation of AMPA/KA receptors, which plays a prevalent role during ischemia in older WM, mediates injury. Either OGD-induced injury in older WM is specific to Ca^{2+} entry through AMPA/KA receptors or Na^+ entry through AMPA/KA receptors. The Ca^{2+}-mediated neurotoxicity requires distinct signaling pathways, such that some pathways are more efficiently triggered when Ca^{2+} ions enter at specific entry points such as the Ca^{2+}-permeable glutamate receptors [38]. In addition, overload of neurons by intracellular Na^+ exhibits irreversible neurotoxic swelling in the absence of extracellular Ca^{2+} [39]. Moreover, an increase in intracellular Na^+ leads to the reversal of the Na^+-dependent glutamate transporter, leading to an increase in extracellular

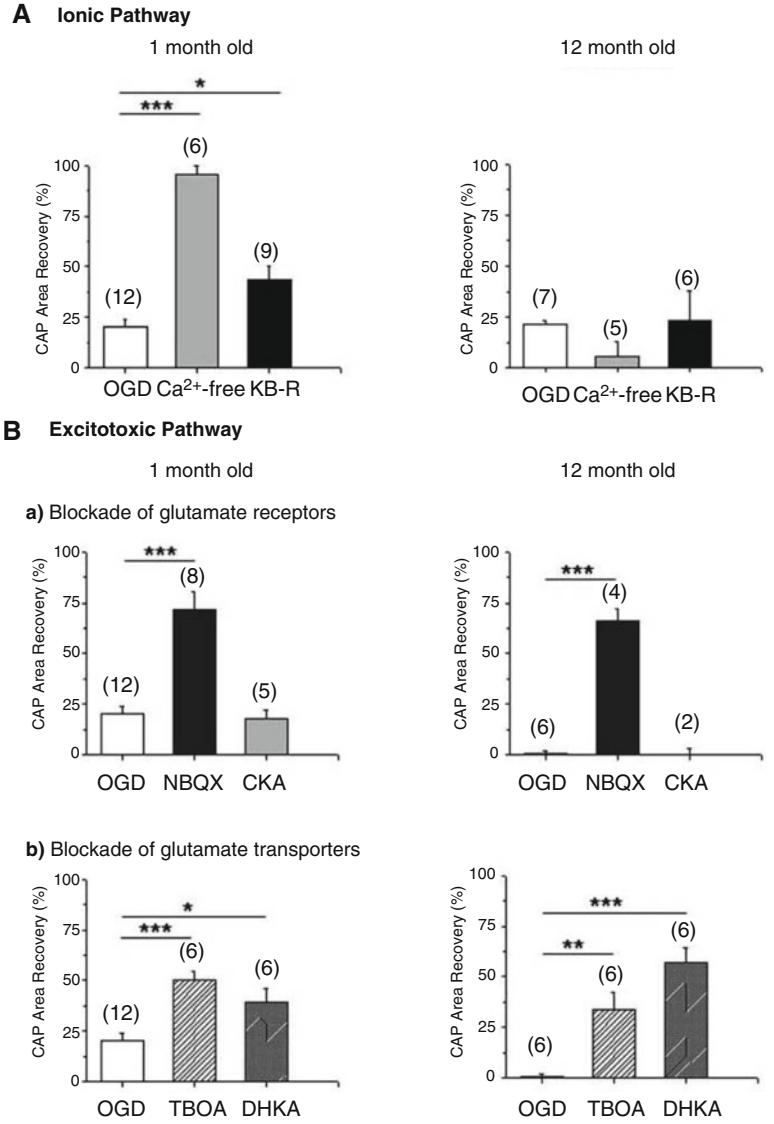

Fig. 4 Ca^{2+}-independent excitotoxicity mediates ischemic injury in older WM. (**A**) The role of the ionic pathway in ischemic injury diminishes in older WM. Removal of extracellular Ca^{2+} or blockade of Ca^{2+} entry via reversing the Na^+–Ca^{2+} exchanger (NCX) with KB-R (2-[2-[4(4-nitrobenzyloxy) phenyl]ethyl]isothiourea mesylate) is protective of axon function in 1-month-old WM after 60 min of OGD (*left*). KB-R failed to improve axon function in 12-month-old WM, even after 45 min of OGD (*right*). Note the reduced recovery in Ca^{2+}-free conditions of 12-month-old WM (right). (**B**, **a**) Blockade of AMPA/KA receptors with NBQX (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*] quinoxaline-7-sulfonamide) provides identical protection to axon function in 1- and 12-month-old WM after 60 min of OGD. Blockade of NMDA-type receptors (NMDARs) with chlorokynurenic acid (CKA) does not improve axon function recovery in 1- or 12-month-old WM. (**b**) Blockade of Na^+-dependent glutamate transporters improves axon function recovery in 1- and 12-month-old WM. Note that dihydrokainic acid (DHKA) afforded greater protection in aging axons. CAP compound action potential; TBOA (DL-threo-β-benzyloxyaspartate). $*p<0.05$, $**p<0.01$, $***p<0.001$, two-way ANOVA. Data replotted from Tekkok et al. (2007) [16] and Baltan et al. (2008) [3]) (Reproduced from Baltan (2009) [2])

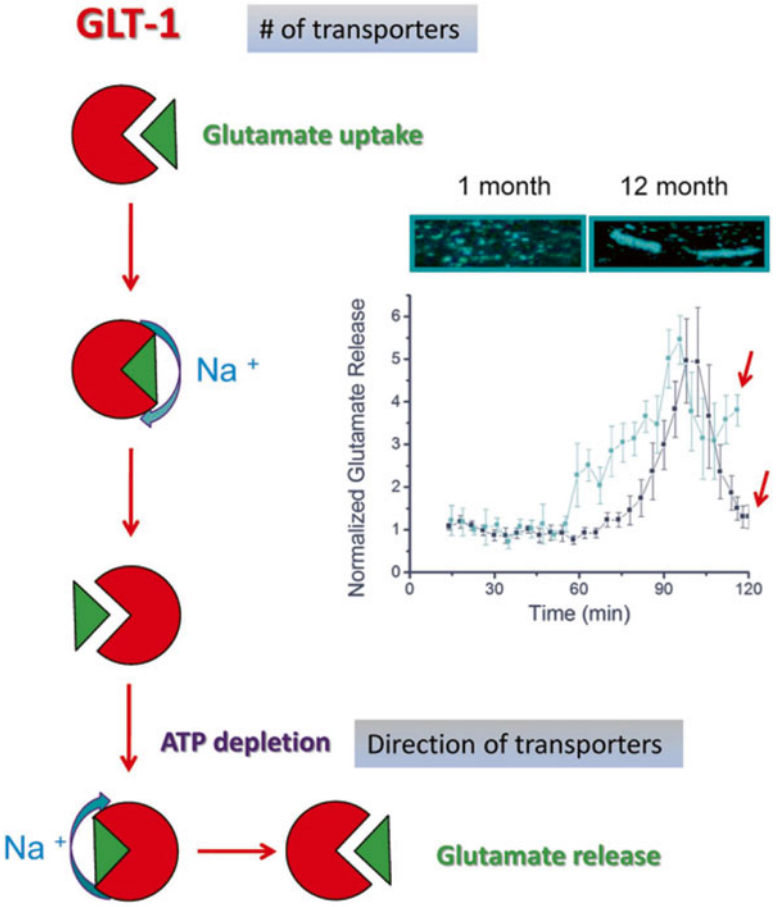

Fig. 5 In aging WM, enhanced excitotoxicity due to impaired mitochondrial function leads to early and robust glutamate release. The principal Na^+-dependent glutamate transporter, GLT-1, usually takes up glutamate with co-transport of Na^+. During ATP depletion, due to increased intracellular Na^+ levels, the transporter reverses and releases glutamate. Therefore, the number of transporters determines the capacity of the system, but it is the ATP levels that determine the direction of the transport (to either remove or release glutamate). Mitochondria in aging axons become longer and thicker compared to young axons, which may hinder ATP production and drive GLT-1 in reverse mode. Consistent with this, there is an early and robust glutamate release in aging WM (*blue*) compared to young MONs (*gray*). Note that the glutamate levels return to baseline in young WM but remain elevated in aging WM (*red arrows*) (Reproduced from in part from Baltan (2008) [3] and Baltan (2014) [18])

glutamate [40]. Together with the upregulation in GLT-1 expression in aged WM [3], increased intracellular Na^+ may cause increased and early release of glutamate, overactivating AMPA/KA receptors and creating a vicious cycle that underlies the vulnerability of aging WM to ischemia.

The NMDA-type receptors (NMDAR) are activated during ischemia by glutamate [41], and the resultant increases in intracellular Ca^{2+} lead to myelin injury [42] or detachment of oligodendrocyte processes [43]. Consequently, axon function recovery would be predicted to improve following OGD if NMDARs are blocked. However, blockade of NMDAR in 1-month-old MONs (Fig. 4B, left) [16] or corpus callosum [14] does not improve axon function recovery following OGD. Likewise, blockade of NMDARs with 7-chlorokynurenic acid (CKA) does not protect axon function in older animals (Fig. 4B, right). Moreover, even after shorter durations of OGD (30–45 min), blockade of NMDARs worsens axon function recovery in older WM (data not shown). These results suggest that NMDAR activation during OGD does not contribute to failure of axon function and raises caution for the therapeutic use of NMDAR antagonists during ischemia, particularly in aging WM. These results do not negate activation of NMDARs under ischemic conditions, but suggest that their activation does not specifically contribute to axonal injury.

6 Reorganization of Glutamate Homeostasis in Aging WM

WM glutamate homeostasis and related regulatory proteins also go through age-related remodeling (Fig. 6). Expression of GLT-1, the dominant glutamate transporter, is upregulated in aging WM [3]. GLT-1 plays a key role in the removal of glutamate from the extracellular space to maintain glutamate below neurotoxic levels [44, 45]. Even though GLT-1 is predominantly expressed on astrocytes in young WM, it extends to additional structures with aging, implying that additional WM elements may contribute to the toxic glutamate accumulation in aging WM [3].

In addition to GLT-1, other essential elements for maintaining glutamate homeostasis include GLAST, glutamate, and glutamate synthetase (GS). WM glutamate content increases significantly with aging, which correlates with increased GS levels (Fig. 6). Together with a two-fold increase in GLT-1 levels in older WM [3], these adjustments may support an age-dependent adaptive mechanism in WM to remove glutamate from the extracellular space and to convert excessive glutamate to glutamine to maintain glutamate homeostasis. As a result, glutamate levels [3] and axon conduction across aging axons are stable under normal conditions.

The number of GLT-1 transporters determines the capacity of WM to move glutamate between the intracellular and extracellular space (Fig. 5). However, the direction of the GLT-1 transporter acts to either protect or injure WM. During ischemia, GLT-1 transporter reversal acts to injure the WM due to an accelerated Na^+ overload as a result of decreased tolerance to energy deprivation in aging WM (Fig. 7). Therefore, in old WM, more GLT-1 transporters are reversed, leading to earlier and more robust release of glutamate and enhanced excitotoxicity (Fig. 7). Moreover, in young WM, glutamate levels return to baseline after the end of OGD, which suggests efficient uptake of glutamate by astrocytes. However, in aging WM,

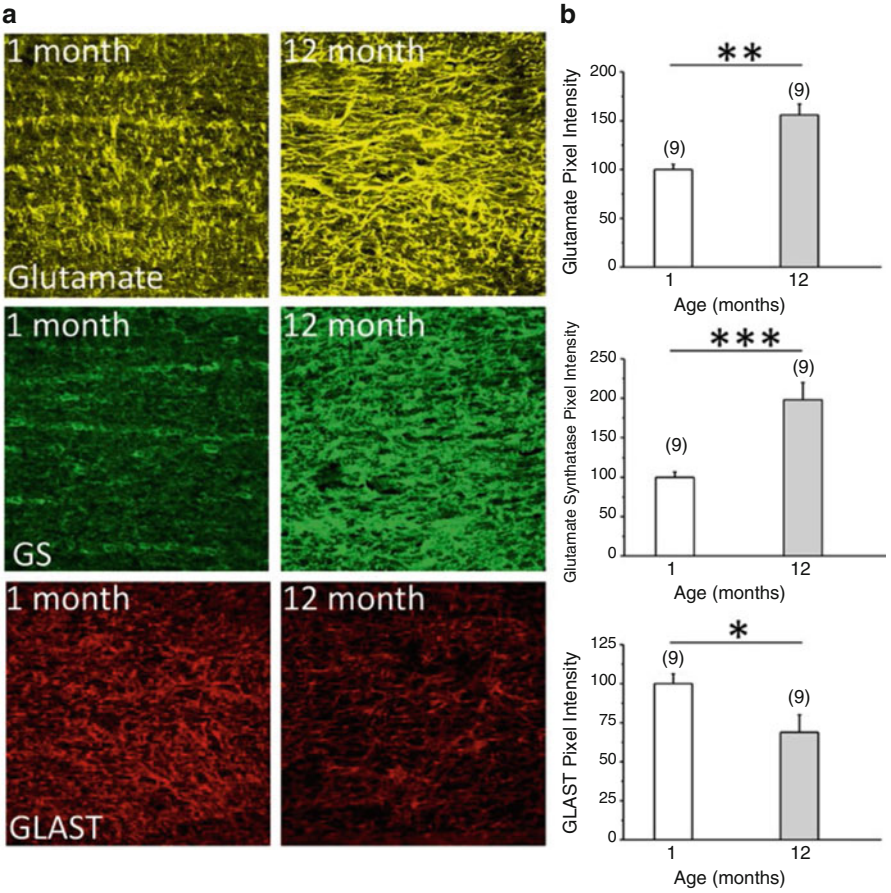

Fig. 6 Glutamate and glutamate synthetase (GS) expression are increased in 12-month-old MONs. (**a**) Immunolabeling and (**b**) quantification of glutamate, GS, and GLAST immunolabeling revealed that glutamate and GS labeling intensity is increased by 156 ± 11 % and 198 ± 22 %, respectively, in 12-month-old MONs. Note that GLAST-labeling intensity decreased to 69 ± 11 % in 12-month-old MONs. *$p = 0.0278$, **$p = 0.004$, ***$p = 0.0006$, two-tailed Student's *t*-test (Reproduced from Baltan (2014) [18])

glutamate levels remain elevated, suggesting that aging astrocytes cannot take up the excess glutamate and thereby extending the duration of excitotoxicity into the recovery period [3]. Despite the possibility that glutamate may be released from multiple sources in aging WM, astrocytes are expected to remove and store glutamate efficiently. Therefore, these results suggest a prominent change in aging astrocyte capacity to remove glutamate. On the other hand, GLAST expression in WM decreases with aging, raising the possibility that glutamate transporters can functionally substitute for one another with aging (Fig. 6).

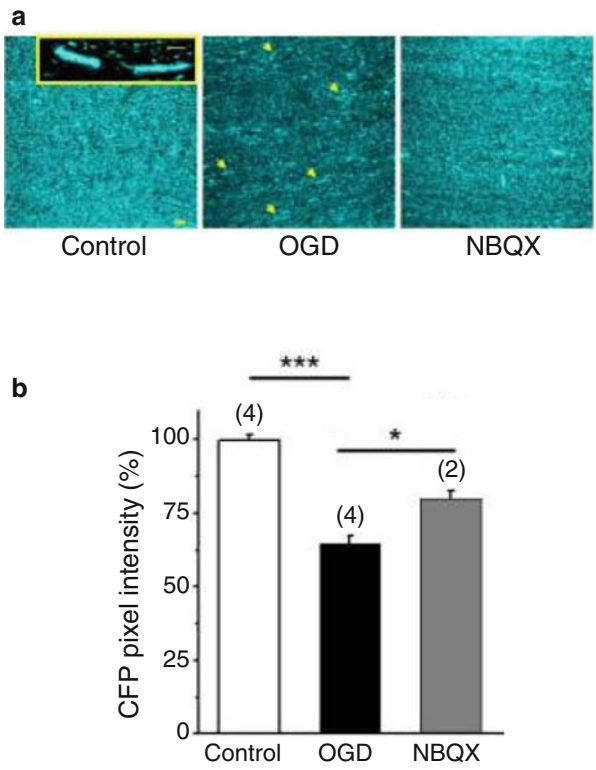

Fig. 7 In aged MONs, blockade of excitotoxicity preserves CFP (+) axonal mitochondria. (**a**) CFP (+) mitochondria were longer and thicker in MONs from 12-month-old compared to 1-month-old Thy-1 CFP mice (inset; scale bar = 2 μm). OGD reduced CFP pixel intensity in 12-month-old MONs despite longer and brighter CFP (+) mitochondria (*yellow arrows*). (**b**) Blockade of AMPA/KA receptors with NBQX (30 μM) preserved CFP pixel intensity during OGD. $*p < 0.05$, and $**p < 0.0001$, one-way ANOVA. Calibration bar = 10 μm (Reproduced in part from Baltan (2012) [4])

7 Mitochondria-Enhanced Excitotoxicity Underlies the Increased Vulnerability of Aging WM to Ischemic Injury

Mitochondria contribute to the enhanced excitotoxicity of aging WM and underlie the increased vulnerability of aging WM to ischemia. Mitochondria bioenergetics in neurons (gray matter) and their role in glutamate excitotoxicity are well-described [46]. Mitochondrial dysfunction and excitotoxicity share common features and are believed to act synergistically by potentiating one another [47–49]. Mitochondria are dynamic organelles that travel using axonal transport, in both the anterograde and retrograde directions, to reach peripheral locations and provide local energy supply [50, 51]. They constantly undergo fission and fusion events [52] and the relative rates of mitochondrial fusion and fission are implicated in the regulation of their size, number, and shape [53–55]. The balanced delivery of mitochondria to cell bodies, dendrites, axons, and axon terminals helps them serve multiple functions, including energy generation, regulation of Ca^{2+} homeostasis, cell death, and synaptic transmission and plasticity [56]. Expectedly, associations between many

neurological diseases, including aging, and defects in mitochondrial dynamics are emerging [55, 57].

In gray matter, aging neurons become more susceptible to glutamate excitotoxicity because of collapsed mitochondrial membrane potentials and increased generation of reactive oxygen and nitrogen species (ROS/RNS), leading to further reductions in mitochondrial function and energy production [58]. Mitochondrial function appears to decline in older animals, presumably causing reduced ATP production. This has been shown in the heart [59], liver [60], and brain [61]. Ion homeostasis accounts for ≈50 % of all ATP consumption, for which the Na^+/K^+ ATPase, the key enzyme to maintain ion homeostasis, is responsible for the majority of this consumption [62]. Therefore, the combined loss of ATP reserves and the high energy requirements of the Na^+/K^+ ATPase, diminishing the activity of this enzyme with aging, may heighten the sensitivity of aging WM to injury [63]. Consistent with this possibility, axon function in aging MONs, when transiently challenged with OGD, was slower to restore normal ion gradients, permitting pathological processes related to disruption of ion homeostasis to operate for a longer time (earlier reversal of the Na^+-dependent glutamate transporter, Fig. 7), thus producing more injury in aging MONs [3]. In addition, aging MONs showed greater recovery of WM function in older animals when OGD was applied at a lower temperature [3], supporting the hypothesis that ATP reserves are compromised in aged WM. Finally, axons with higher ATP requirements have many more mitochondria per unit length of process [64]; therefore, these axons would be preferentially targeted by low ATP reserve conditions.

In neurons, excitotoxicity and elevated Ca^{2+} induce marked changes in mitochondrial morphology, stopping their movement [56, 65, 66] and generating ROS [46]. In young WM, activation of either AMPA or kainate receptors [3] loads mitochondria with Ca^{2+} and fission is enhanced, associated with loss of fluorescence of mitochondria genetically tagged with CFP (Fig. 8) [67]. Ca^{2+} overload activates NOS to produce nitric oxide and ROS/RNS, which could act either directly on oligodendrocytes [68–70] to cause injury or as diffusible second messengers linking oligodendrocyte excitotoxicity to axonal injury [31, 32]. Axon function directly correlates with WM energy reserves, since Na^+–K^+ ATPase activity is dependent on ATP levels. As a result, OGD causes a reduction in ATP levels and CFP (+) mitochondria, which is prevented by AMPA/KA receptor blockade in young and old WM (Figs. 8 and 9). Despite the structural and functional changes in aging mitochondria that enhance oxidative stress and amplify glutamate-mediated excitotoxicity during OGD, blockade of AMPA/KA receptors proved to be a successful strategy in improving WM function after stroke.

In summary, we and others have identified WM as an important therapeutic target for stroke [3, 5, 13, 15, 16, 41]. We have proposed the optic nerve as an ideal model for the study of ischemic WM injury and have described, step-by-step, ischemic injury pathways and the glial cells that are impacted. We have identified that aging modifies these ischemic injury pathways to render WM more susceptible to injury, such that in aging WM, the removal of extracellular Ca^{2+} is injurious, while

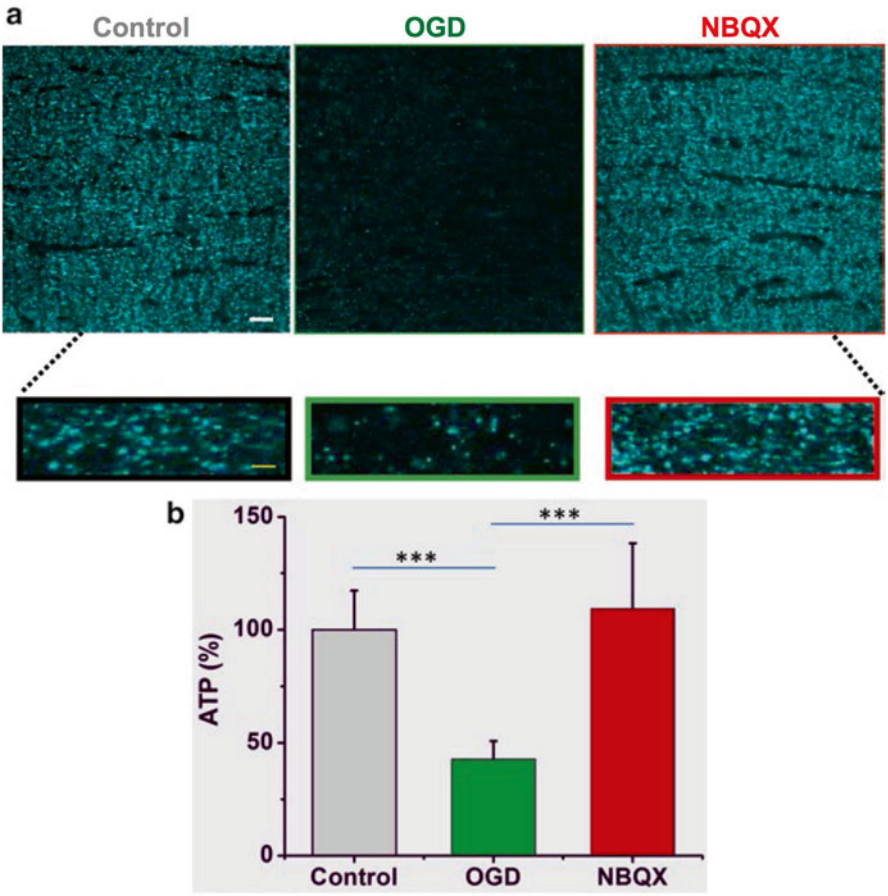

Fig. 8 In young MONs, Blockade of excitotoxicity preserves CFP (+) axonal mitochondria and ATP levels in response to OGD. (**a**) OGD severely reduced CFP fluorescence in MONs from mito CFP (+) mice and pretreatment with NBQX (30 μM) protected against this loss. Note the change in mitochondrial morphology from small and tubular under control conditions to tiny and punctate following OGD. Calibration bar = 10 μm (insets = 2 μM) (**b**) NBQX pretreatment conserved ATP levels in MONs. ***$p < 0.0001$, one-way ANOVA (Reproduced in part from Baltan et al. (2011) [36])

it was protective in young WM (Fig. 9). In addition, aging alters WM glutamate homeostasis and mitochondrial dynamics, which lead to an enhanced glutamate excitotoxicity period with ischemia, which starts earlier and extends into the recovery period. Furthermore, we have identified that AMPA/KA receptor blockade protects both young and old WM, whereas NMDA receptor blockade is not protective in neither young nor old WM. Unexpectedly, NMDA receptor blockade worsened OGD recovery in old WM. Our results suggest that NMDAR activation during OGD

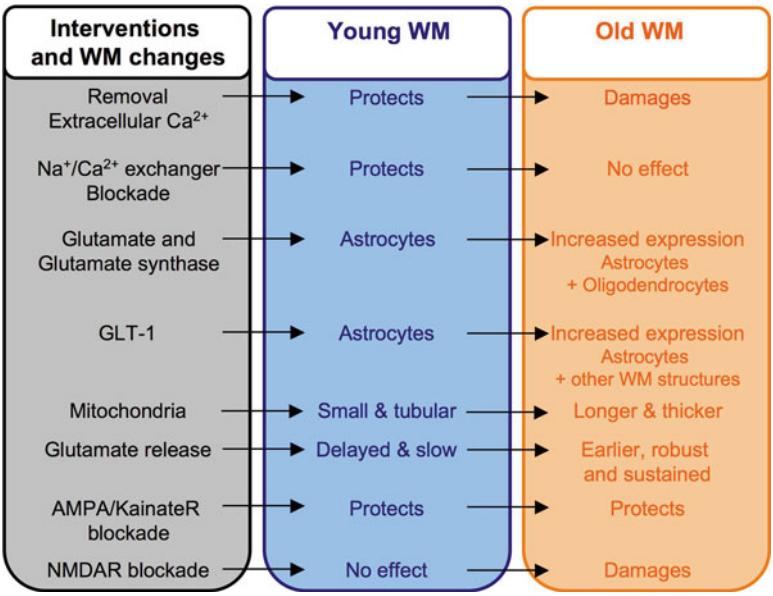

Fig. 9 Working molecular and cellular mechanisms responsible for ischemic WM injury. Age-dependent cellular remodeling of WM elements modifies the injury mechanisms and functional outcome to increase the sensitivity of aging WM to ischemic injury (Modified in part from Baltan 2014 [18])

does not contribute to failure of axon function and raises caution for the therapeutic use of NMDAR antagonists during ischemia, particularly in aging WM. Overall, our research suggests that for the development successful of future stroke therapies, we must tailor our approach to protect both gray matter and white matter as a function of age.

References

1. AHA. Every 40 seconds a stroke occurs in the United States. http://newsroom.heart.org/news/every-40-seconds-a-stroke-occurs-in-the-united-states (2013).
2. Baltan S. Ischemic injury to white matter: an age-dependent process. Neuroscientist. 2009;15(2):126–33.
3. Baltan S, Besancon EF, Mbow B, Ye Z, Hamner MA, Ransom BR. White matter vulnerability to ischemic injury increases with age because of enhanced excitotoxicity. J Neurosci. 2008;28(6):1479–89.
4. Baltan S. Histone deacetylase inhibitors preserve function in aging axons. J Neurochem. 2012;123 Suppl 2:108–15.
5. Rosenzweig S, Carmichael ST. Age-dependent exacerbation of white matter stroke outcomes: a role for oxidative damage and inflammatory mediators. Stroke. 2013;44(9):2579–86.

6. Tekkok SB, Brown AM, Ransom BR. Axon function persists during anoxia in mammalian white matter. J Cereb Blood Flow Metab. 2003;23(11):1340–7.
7. Baltan S. Surviving anoxia: a tale of two white matter tracts. Crit Rev Neurobiol. 2006;18(1–2):95–103.
8. Wrathall JR, Choiniere D, Teng YD. Dose-dependent reduction of tissue loss and functional impairment after spinal cord trauma with the AMPA/kainate antagonist NBQX. J Neurosci. 1994;14(11 Pt 1):6598–607.
9. Agrawal SK, Fehlings MG. Role of NMDA and non-NMDA ionotropic glutamate receptors in traumatic spinal cord axonal injury. J Neurosci. 1997;17(3):1055–63.
10. Fern R, Ransom BR. Ischemic injury of optic nerve axons: the nuts and bolts. Clin Neurosci. 1997;4(5):246–50.
11. McDonald JW, Althomsons SP, Hyrc KL, Choi DW, Goldberg MP. Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. Nat Med. 1998;4(3):291–7.
12. Sanchez-Gomez MV, Matute C. AMPA and kainate receptors each mediate excitotoxicity in oligodendroglial cultures. Neurobiol Dis. 1999;6(6):475–85.
13. Follett PL, Rosenberg PA, Volpe JJ, Jensen FE. NBQX attenuates excitotoxic injury in developing white matter. J Neurosci. 2000;20(24):9235–41.
14. Tekkok SB, Goldberg MP. Ampa/kainate receptor activation mediates hypoxic oligodendrocyte death and axonal injury in cerebral white matter. J Neurosci. 2001;21(12):4237–48.
15. Stys PK. White matter injury mechanisms. Curr Mol Med. 2004;4(2):113–30.
16. Tekkok SB, Ye Z, Ransom BR. Excitotoxic mechanisms of ischemic injury in myelinated white matter. J Cereb Blood Flow Metab. 2007;27(9):1540–52.
17. Fields RD. White matter in learning, cognition and psychiatric disorders. Trends Neurosci. 2008;31(7):361–70.
18. Baltan S. Age-dependent mechanisms of white matter injury after stroke. In: Baltan S, Carmichael ST, Matute C, Xi G, Zhang JH, editors. White matter injury in stroke and CNS disease. New York: Springer; 2014. p. 373–403.
19. Stys PK, Ransom BR, Waxman SG. Effects of polyvalent cations and dihydropyridine calcium channel blockers on recovery of CNS white matter from anoxia. Neurosci Lett. 1990;115(2–3):293–9.
20. Fern R, Ransom BR, Waxman SG. Voltage-gated calcium channels in CNS white matter: role in anoxic injury. J Neurophysiol. 1995;74(1):369–77.
21. Wolf JA, Stys PK, Lusardi T, Meaney D, Smith DH. Traumatic axonal injury induces calcium influx modulated by tetrodotoxin-sensitive sodium channels. J Neurosci. 2001;21(6):1923–30.
22. Ouardouz M, Nikolaeva MA, Coderre E, Zamponi GW, McRory JE, Trapp BD, et al. Depolarization-induced Ca^{2+} release in ischemic spinal cord white matter involves L-type Ca^{2+} channel activation of ryanodine receptors. Neuron. 2003;40(1):53–63.
23. Underhill SM, Goldberg MP. Hypoxic injury of isolated axons is independent of ionotropic glutamate receptors. Neurobiol Dis. 2007;25(2):284–90.
24. Matute C, Sanchez-Gomez MV, Martinez-Millan L, Miledi R. Glutamate receptor-mediated toxicity in optic nerve oligodendrocytes. Proc Natl Acad Sci U S A. 1997;94(16):8830–5.
25. Li S, Mealing GA, Morley P, Stys PK. Novel injury mechanism in anoxia and trauma of spinal cord white matter: glutamate release via reverse Na^{+}-dependent glutamate transport. J Neurosci. 1999;19(14):RC16.
26. Fern R, Moller T. Rapid ischemic cell death in immature oligodendrocytes: a fatal glutamate release feedback loop. J Neurosci. 2000;20(1):34–42.
27. Alberdi E, Sanchez-Gomez MV, Marino A, Matute C. Ca(2+) influx through AMPA or kainate receptors alone is sufficient to initiate excitotoxicity in cultured oligodendrocytes. Neurobiol Dis. 2002;9(2):234–43.
28. Rosenberg PA, Li Y, Ali S, Altiok N, Back SA, Volpe JJ. Intracellular redox state determines whether nitric oxide is toxic or protective to rat oligodendrocytes in culture. J Neurochem. 1999;73(2):476–84.

29. Oka A, Belliveau MJ, Rosenberg PA, Volpe JJ. Vulnerability of oligodendroglia to glutamate: pharmacology, mechanisms, and prevention. J Neurosci. 1993;13(4):1441–53.
30. Slemmer JE, Shacka JJ, Sweeney MI, Weber JT. Antioxidants and free radical scavengers for the treatment of stroke, traumatic brain injury and aging. Curr Med Chem. 2008;15(4):404–14.
31. Matute C, Alberdi E, Domercq M, Perez-Cerda F, Perez-Samartin A, Sanchez-Gomez MV. The link between excitotoxic oligodendroglial death and demyelinating diseases. Trends Neurosci. 2001;24(4):224–30.
32. Ouardouz M, Malek S, Coderre E, Stys PK. Complex interplay between glutamate receptors and intracellular Ca^{2+} stores during ischaemia in rat spinal cord white matter. J Physiol. 2006;577(Pt 1):191–204.
33. Cavallotti C, Pacella E, Pescosolido N, Tranquilli-Leali FM, Feher J. Age-related changes in the human optic nerve. Can J Ophthalmol. 2002;37(7):389–94.
34. Cavallotti C, Cavallotti D, Pescosolido N, Pacella E. Age-related changes in rat optic nerve: morphological studies. Anat Histol Embryol. 2003;32(1):12–6.
35. Brown AM, Tekkok SB, Ransom BR. Hypoglycemia and white matter: pathophysiology of axon injury and role of glycogen. Diabetes Nutr Metab. 2002;15(5):290–3. discussion 3–4.
36. Baltan S, Murphy SP, Danilov CA, Bachleda A, Morrison RS. Histone deacetylase inhibitors preserve white matter structure and function during ischemia by conserving ATP and reducing excitotoxicity. J Neurosci. 2011;31(11):3990–9.
37. Baltan S, Inman DM, Danilov CA, Morrison RS, Calkins DJ, Horner PJ. Metabolic vulnerability disposes retinal ganglion cell axons to dysfunction in a model of glaucomatous degeneration. J Neurosci. 2010;30(16):5644–52.
38. Tymianski M, Charlton MP, Carlen PL, Tator CH. Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons. J Neurosci. 1993;13(5):2085–104.
39. Rothman SM. The neurotoxicity of excitatory amino acids is produced by passive chloride influx. J Neurosci. 1985;5(6):1483–9.
40. Szatkowski M, Barbour B, Attwell D. Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. Nature. 1990;348(6300):443–6.
41. Karadottir R, Cavelier P, Bergersen LH, Attwell D. NMDA receptors are expressed in oligodendrocytes and activated in ischaemia. Nature. 2005;438(7071):1162–6.
42. Micu I, Jiang Q, Coderre E, Ridsdale A, Zhang L, Woulfe J, et al. NMDA receptors mediate calcium accumulation in myelin during chemical ischaemia. Nature. 2006;439(7079):988–92.
43. Salter MG, Fern R. NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury. Nature. 2005;438(7071):1167–71.
44. Rothstein JD, Dykes-Hoberg M, Pardo CA, Bristol LA, Jin L, Kuncl RW, et al. Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron. 1996;16(3):675–86.
45. Hazell AS, Rao KV, Danbolt NC, Pow DV, Butterworth RF. Selective down-regulation of the astrocyte glutamate transporters GLT-1 and GLAST within the medial thalamus in experimental Wernicke's encephalopathy. J Neurochem. 2001;78(3):560–8.
46. Nicholls DG, Johnson-Cadwell L, Vesce S, Jekabsons M, Yadava N. Bioenergetics of mitochondria in cultured neurons and their role in glutamate excitotoxicity. J Neurosci Res. 2007;85(15):3206–12.
47. Albin RL, Greenamyre JT. Alternative excitotoxic hypotheses. Neurology. 1992;42(4):733–8.
48. Jacquard C, Trioulier Y, Cosker F, Escartin C, Bizat N, Hantraye P, et al. Brain mitochondrial defects amplify intracellular [Ca^{2+}] rise and neurodegeneration but not Ca^{2+} entry during NMDA receptor activation. FASEB J. 2006;20(7):1021–3.
49. Silva-Adaya D, Perez-De La Cruz V, Herrera-Mundo MN, Mendoza-Macedo K, Villeda-Hernandez J, Binienda Z, et al. Excitotoxic damage, disrupted energy metabolism, and oxidative stress in the rat brain: antioxidant and neuroprotective effects of L-carnitine. J Neurochem. 2008;105(3):677–89.
50. Hollenbeck PJ, Saxton WM. The axonal transport of mitochondria. J Cell Sci. 2005;118(Pt 23):5411–9.

51. Hollenbeck PJ. Mitochondria and neurotransmission: evacuating the synapse. Neuron. 2005;47(3):331–3.
52. Karbowski M, Arnoult D, Chen H, Chan DC, Smith CL, Youle RJ. Quantitation of mitochondrial dynamics by photolabeling of individual organelles shows that mitochondrial fusion is blocked during the Bax activation phase of apoptosis. J Cell Biol. 2004;164(4):493–9.
53. Mozdy AD, Shaw JM. A fuzzy mitochondrial fusion apparatus comes into focus. Nat Rev Mol Cell Biol. 2003;4(6):468–78.
54. Scott SV, Cassidy-Stone A, Meeusen SL, Nunnari J. Staying in aerobic shape: how the structural integrity of mitochondria and mitochondrial DNA is maintained. Curr Opin Cell Biol. 2003;15(4):482–8.
55. Chen H, McCaffery JM, Chan DC. Mitochondrial fusion protects against neurodegeneration in the cerebellum. Cell. 2007;130(3):548–62.
56. Chang DT, Reynolds IJ. Mitochondrial trafficking and morphology in healthy and injured neurons. Prog Neurobiol. 2006;80(5):241–68.
57. Karbowski M, Youle RJ. Dynamics of mitochondrial morphology in healthy cells and during apoptosis. Cell Death Differ. 2003;10(8):870–80.
58. Parihar MS, Brewer GJ. Simultaneous age-related depolarization of mitochondrial membrane potential and increased mitochondrial reactive oxygen species production correlate with age-related glutamate excitotoxicity in rat hippocampal neurons. J Neurosci Res. 2007;85(5):1018–32.
59. Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, Hoppel CL. Mitochondrial dysfunction in cardiac disease: ischemia—reperfusion, aging, and heart failure. J Mol Cell Cardiol. 2001;33(6):1065–89.
60. Selzner M, Selzner N, Jochum W, Graf R, Clavien PA. Increased ischemic injury in old mouse liver: an ATP-dependent mechanism. Liver Transpl. 2007;13(3):382–90.
61. Toescu EC. Normal brain ageing: models and mechanisms. Philos Trans R Soc Lond B Biol Sci. 2005;360(1464):2347–54.
62. Erecinska M, Silver IA. Ions and energy in mammalian brain. Prog Neurobiol. 1994;43(1):37–71.
63. Scavone C, Munhoz CD, Kawamoto EM, Glezer I, de Sa Lima L, Marcourakis T, et al. Age-related changes in cyclic GMP and PKG-stimulated cerebellar Na,K-ATPase activity. Neurobiol Aging. 2005;26(6):907–16.
64. Bristow EA, Griffiths PG, Andrews RM, Johnson MA, Turnbull DM. The distribution of mitochondrial activity in relation to optic nerve structure. Arch Ophthalmol. 2002;120(6):791–6.
65. Rintoul GL, Filiano AJ, Brocard JB, Kress GJ, Reynolds IJ. Glutamate decreases mitochondrial size and movement in primary forebrain neurons. J Neurosci. 2003;23(21):7881–8.
66. Barsoum MJ, Yuan H, Gerencser AA, Liot G, Kushnareva Y, Graber S, et al. Nitric oxide-induced mitochondrial fission is regulated by dynamin-related GTPases in neurons. EMBO J. 2006;25(16):3900–11.
67. Misgeld T, Kerschensteiner M, Bareyre FM, Burgess RW, Lichtman JW. Imaging axonal transport of mitochondria in vivo. Nat Methods. 2007;4(7):559–61.
68. Baud O, Li J, Zhang Y, Neve RL, Volpe JJ, Rosenberg PA. Nitric oxide-induced cell death in developing oligodendrocytes is associated with mitochondrial dysfunction and apoptosis-inducing factor translocation. Eur J Neurosci. 2004;20(7):1713–26.
69. Merrill JE, Murphy SP, Mitrovic B, Mackenzie-Graham A, Dopp JC, Ding M, et al. Inducible nitric oxide synthase and nitric oxide production by oligodendrocytes. J Neurosci Res. 1997;48(4):372–84.
70. Yao S, Pandey P, Ljunggren-Rose A, Sriram S. LPS mediated injury to oligodendrocytes is mediated by the activation of nNOS: relevance to human demyelinating disease. Nitric Oxide. 2009;22(3):197–204.

Part V
Emerging Therapies to Target Non-neuronal Mechanisms After Stroke

Neurovascular Repair After Stroke

Sherrefa R. Burchell, Wing-Mann Ho, Jiping Tang, and John H. Zhang

Abbreviations

$[Ca^{2+}]_i$	Intracellular calcium concentration
BBB	Blood–brain barrier
CBF	Cerebral blood flow

S.R. Burchell, B.Sc. • J. Tang, M.D.
Department of Physiology, Loma Linda University School of Medicine,
11041 Campus Street, Risley Hall, Room 223, Loma Linda, CA 92350, USA

Center for Neuroscience Research, Loma Linda University School of Medicine,
Loma Linda, CA 92350, USA
e-mail: sburchell@llu.edu; jipingtang@yahoo.com

W.-M. Ho, M.D.
Department of Physiology, Loma Linda University School of Medicine,
11041 Campus Street, Risley Hall, Room 223, Loma Linda, CA 92350, USA

Center for Neuroscience Research, Loma Linda University School of Medicine,
Loma Linda, CA 92350, USA

Department of Neurosurgery, Medical University Innsbruck, Innsbruck, Tyrol 6020, Austria
e-mail: wing.ho@tirol-kliniken.at

J.H. Zhang, M.D., Ph.D. (✉)
Department of Anesthesiology, Loma Linda University School of Medicine,
Loma Linda, CA 92350, USA

Department of Pharmacology, Loma Linda University School of Medicine,
11041 Campus Street, Risley Hall, Room 223, Loma Linda, CA 92350, USA

Department of Physiology, Loma Linda University School of Medicine,
11041 Campus Street, Risley Hall, Room 223, Loma Linda, CA 92350, USA

Center for Neuroscience Research, Loma Linda University School of Medicine,
Loma Linda, CA 92350, USA
e-mail: johnzhang3910@yahoo.com; jhzhang@llu.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_17

CNS	Central nervous system
DCI	Delayed cerebral ischemia
EBI	Early brain injury
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelin 1
ICAM-1	Intercellular adhesion molecule 1
ICH	Intracerebral hemorrhage
ICP	Intracranial pressure
IL	Interleukin
iNOS	Inducible nitric oxide synthase
mGluR	Metabotropic glutamate receptors
MMP	Matrix metalloproteinase
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NSC	Neural stem cell
PDGF	Platelet-derived growth factor
PPAR-γ	Peroxisome proliferator-activated receptor gamma
ROS	Reactive oxygen species
SAH	Subarachnoid hemorrhage
SGZ	Subgranular zone
SVZ	Subventricular zone
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
tPA	Tissue plasminogen activator
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VNN	Vascular neural network
VSMC	Vascular smooth muscle cell
ZO-1	Zona occludens 1

1 Introduction: Hemorrhagic Stroke and the Vascular Neural Network

Hemorrhagic strokes (subarachnoid hemorrhage (SAH), intracerebral hemorrhage (ICH), germinal matrix hemorrhage (GMH)) result from weakened vessels that rupture and bleed into the surrounding brain, leading to the accumulation of blood, an increase in intracranial pressure (ICP), and compression of the surrounding tissue. Hemorrhagic strokes also result in an immediate or postponed reduction in cerebral blood flow (CBF) [1]. These phenomena have important effects on the vasculature, and successful recovery from stroke requires a functional cerebral circulation; thus, vascular cells play an important role in the sequence of events in hemorrhagic

stroke. A comprehensive model that can be used to evaluate the pathophysiology and repair of hemorrhagic stroke is the vascular neural network (VNN), as proposed by Zhang and colleagues [2]. The VNN includes all the structures that are required to maintain CBF under physiological and pathological conditions, and encompasses vascular smooth muscle cells (VSMCs), arterial endothelial cells, and perivascular nerves connected to arteries and arterioles upstream of the cerebral microcirculation [2, 3]. This network is an extension of the neurovascular unit, which is comprised of neurons, glial cells (astrocytes, microglia, perivascular macrophages), pericytes, endothelial cells, and extracellular matrix, whose primary function is maintaining the homeostasis of the brain's microenvironment by mediating communication between the central nervous system (CNS) and the vascular network [4]. While the neurovascular unit model focuses on the areas immediately surrounding the capillaries, the VNN includes both venous downstream vasculature and upstream arteries and arterioles, and is therefore more inclusive for the mechanisms of vascular injury and impaired reperfusion induced by stroke [2].

The blood–brain barrier (BBB), which is between the CNS and the vasculature, is a part of the neurovascular network and is a highly organized multicellular complex that serves to control many metabolic and signaling actions occurring between the CNS and the rest of the body. It was originally thought that the barrier was principally mediated by tight junctions in endothelial cells [5]. However, it is now known that astrocytes, glial cells, and pericytes play important roles in BBB function [6, 7]. Consequently, any disruption of signaling between these cells and the cerebral endothelium can interfere with the integrity of the BBB and the brain microvasculature [8]. Many current therapies for hemorrhagic stroke are targeted at BBB repair, as loss of BBB integrity is one of the hallmarks of vascular disruption.

An understanding of the mechanisms regulating interactions among cells in the VNN is thus essential for the development of effective therapies against hemorrhagic stroke. In fact, both the beneficial and adverse effects of tissue plasminogen activator (tPA), the only Food and Drug Administration-approved treatment for ischemic stroke, have been linked to its interaction with components of this network [9]. Treatment with tPA was shown to attenuate early brain injury (EBI) after SAH by improving CBF and cortical perfusion, which are reduced due to increased ICP [10]. However, tPA is known to increase the levels of matrix metalloproteinases (MMP)-9 and 3, via the lipoprotein receptor (LRP)-1 in endothelial cells and astrocytes, which in turn increases the occurrence of hemorrhagic transformation in ischemic stroke [11–13]. Niego et al. reported a concentration-dependent increase in BBB permeability with tPA administration in vitro; tPA also induced changes in the morphology of brain endothelial cells and astrocytes, and its modulation of the astrocytic skeleton via plasmin formation led to a decrease in BBB integrity [14]. Furthermore, the interaction of tPA with LRP has been shown to play a role in the permeability of the neurovascular unit by promoting the detachment of astrocyte endfoot processes [15, 16], and MMPs themselves are known to degrade components of the neurovascular matrix [17]. (Figure 1 outlines the pathophysiology of hemorrhagic stroke, illustrating the interactions of the various cell components).

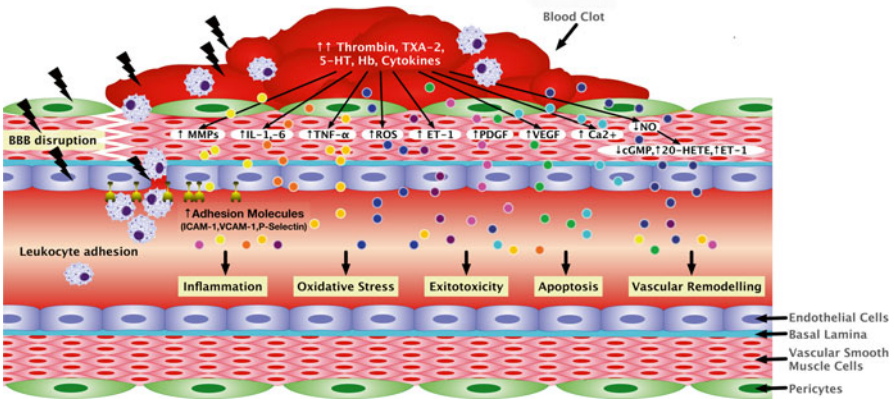

Fig. 1 Pathophysiology of hemorrhagic stroke. Vascular inflammation, oxidative stress, excitotoxicity, apoptosis, and vascular remodeling contribute to neurovascular injury. After stroke, thrombin, thromboxane-2 (TXA-2), serotonin (5-HT), hemoglobin, and cytokines are released. In response, signaling pathways are activated, leading to increased matrix metalloproteinases (MMPs), inflammatory cytokines (interleukin (IL)-1), IL-6, tumor necrosis factor alpha (TNF-α), reactive oxygen species (ROS), endothelin-1 (ET-1), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), intracellular calcium (Ca^{2+}), and decreased nitric oxide (NO), cyclic guanosine monophosphate (cGMP), and the eicosanoid hydroxyicosatetraenoic acid (HETE). These are the main factors inducing vascular injury. Additionally, the inflammatory response leads to increased expression of intraluminal adhesion molecules (intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1)), and P-Selectin, resulting in leukocyte adhesion, and eventually, blood–brain-barrier (BBB) disruption

2 Hemorrhagic Stroke-Induced Neurovascular Disruption: How VNN Interactions Are Altered During Stroke

2.1 Heme Breakdown Leads to Neurotoxicity

Due to the buildup of potentially neurotoxic iron-rich blood, and the products of heme breakdown, neuronal injury and severe cognitive impairment occur following a hemorrhagic stroke [18–24]. Additionally, thrombin, erythrocyte lysis, toll-like receptor (TLR)-4 activation, and other blood components induce activation of microglia and other inflammatory agents and lead to the recruitment of leukocytes into a normally immune privileged area [25–28]. Thus, limiting the effects of this toxicity by hematoma clearance, presents potential for protecting the neurovascular unit after hemorrhagic stroke [29, 30]. In fact, Zhao et al. reported that peroxisome proliferator-activated receptor (PPAR)-γ activation resulted in resolution of the hematoma following murine ICH, which further led to decreased neuronal damage and improved functional outcomes [31].

Heme is degraded into biliverdin, iron, and carbon monoxide by heme oxygenases (HO). HO-1 is the ubiquitously expressed isoform of the enzyme that possesses cytoprotective abilities. Schallner, et al. found that substituting for the absence of

microglial HO-1 by inhalation of carbon monoxide after SAH reduced injury by erythrophagocytosis, and that HO-1 is necessary to attenuate neuronal cell death, vasospasm, impaired cognitive function, and clearance of blood in the subarachnoid space [32]. HO-1 clears the hematoma and thus reduces the oxidation burden and neuronal injury via microglial phagocytosis, similar to the action of PPAR-γ.

2.2 Excitotoxicity and the Role of Astrocytes

Glutamate excitotoxicity is another mechanism that induces neuronal death after hemorrhagic stroke. As such, a recent study found that inhibition of glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors was neuroprotective in EBI after SAH [33]. Cerebral glutamate levels increase within minutes after SAH, and excitotoxicity through the glutamate receptors is thought to play an important role in SAH pathophysiology [34]. EBI involves vasogenic edema and apoptotic cell death, and is compounded by inflammation, oxidative stress, and ionic disturbances, which leads to BBB disruption, edema, and neuronal apoptosis [35]. These events, along with delayed cerebral ischemia (DCI), cortical spreading depression, and development of microthrombi, impact glutamate receptor physiology following SAH. Microthrombi accumulate in the parenchymal vessels after SAH, and platelet aggregation is critical to their formation [36]. Platelets are released into the brain following the breakdown of vessel walls in SAH, which they help to initiate, and are thought to release glutamate as part of their cell–cell signaling [37, 38]. Indeed, Bell et al. recently found that when platelets were activated with thrombin, glutamate release was significantly increased, which led to an increase in neuronal cell death in vitro due to the proximity of neurons to microthrombi. Interestingly, they also found that platelet-rich plasma downregulated glutamate receptor 2 surface expression (which was likely downstream of excessive receptor stimulation) [37]. This is significant as glutamate receptors play an integral role in memory consolidation and executive functioning.

Many cell types in the CNS express glutamate transporters: astrocytes, neurons, oligodendrocytes, microglia, and the endothelium. However, the role of astrocytes in the transporter-mediated uptake of the neurotransmitter is the most important for maintaining normal glutamate levels, and reducing excitotoxicity, particularly following an insult to the brain [39]. Neuronal vulnerability to glutamate excitotoxicity is 100 times greater in cultures low in astrocytes than in astrocyte-rich cultures [40]. Additionally, while neurons do play a role in synaptic glutamate uptake, the role of astrocytes in this process seems significantly more important. Glutamate can also move in the opposite direction through the transporters. Since membrane electrical potentials and transmembrane ion gradients are maintained by sodium/potassium adenosine triphosphatases, changes in adenosine triphosphate (ATP) concentrations influence glutamate transport. Thus, since ATP levels are severely depleted in the case of cerebral ischemia, which occurs secondary to SAH, glutamate uptake is then reversed, and astrocytes release the neurotransmitter. Neurons can also undergo glutamate uptake reversal, thus increasing extracellular glutamate concentrations, resulting in greater neurotoxicity.

2.3 Vascular Disruption and Constriction

The entire cerebral circulation includes large arteries and arterioles, capillaries, and downstream venules and veins. The increased heme and heme breakdown products from the rupturing of vessels and the expanding hematoma during a hemorrhagic stroke were found in a recent study to induce vasoconstriction of cerebral vessels [41]. After SAH, following the usual reduction in CBF, there is a DCI, beginning between day 2 and 4 post-SAH, up to 14 days in humans [42, 43]. Posthemorrhagic vasospasm, induced by heme and its breakdown products, is what leads to DCI, which is also associated with spreading depression and microthrombosis [42]. Several reports have described acute vasoconstriction within hours after SAH, particularly small arteries and arterioles. In guinea pigs, topical blood application to the exposed brain cortex resulted in vasoconstriction of pial vessels [44]. The occurrence of superficial pial vessel constriction was observed predominantly in arterioles, though the time of occurrence varied between the perforation model and the blood-injection model. Nevertheless, smaller arterioles were more constricted than larger vessels in both models [45, 46]. Decreased blood flow was observed in venules as well [47], other reports have shown that venule diameter was unchanged after SAH [48].

The venous compartment of cerebral circulation contains about 70–80 % of the circulating volume in the cranial cavity and is surrounded by adrenergic nerve fibers, while postcapillary venules are covered with pericytes [49, 50]. There are several differences between the venous and arterial systems, which affect the roles they play in hemorrhagic injuries. Small veins and venules lack smooth muscle cells; thus, they do not contract as extensively as arteries [51]. As a result, they do not function as much in the regulation of CBF, and only undergo small changes in diameter according to ICP under normal physiological conditions [52, 53]. However, in pathological conditions, the veins may be easily compressed by high ICP. Secondly, veins have thin walls and do not have valves to prevent the backflow of venous blood [54]. The severe brain edema that occurs due to hemorrhagic stroke leads to an increase in ICP that may compress or collapse the thin walls of the veins [55]. Swollen astrocyte endfeet and adherent leukocytes also induce compression of the venule walls. Deep cerebral vein vasospasm has been reported in a rabbit model of SAH [56]. This may be due to the presence of pericytes on the vessels, as contraction of pericytes is essential for active vein constriction [57]. Additionally, in a blood-injection SAH model, leukocyte-platelet aggregates were observed in the cerebral veins at 2 h after SAH [58]. The resultant oxidative stress and inflammation may induce damage to venous endothelial cells and trigger clot formation in the cerebral venous system [56]. This suggests that alterations to the venous system may play a significant role in hemorrhagic stroke-induced secondary brain injury (Fig. 2).

Beyond constriction, alteration in the reactivity and ultrastructural changes in the vessel walls also occur after SAH. For example, platelet activation releases vasoconstrictors, such as thromboxane-A2, endothelin (ET)-1, serotonin (5-HT), transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF) [59–61]. Furthermore, PDGF, TGF-β, and

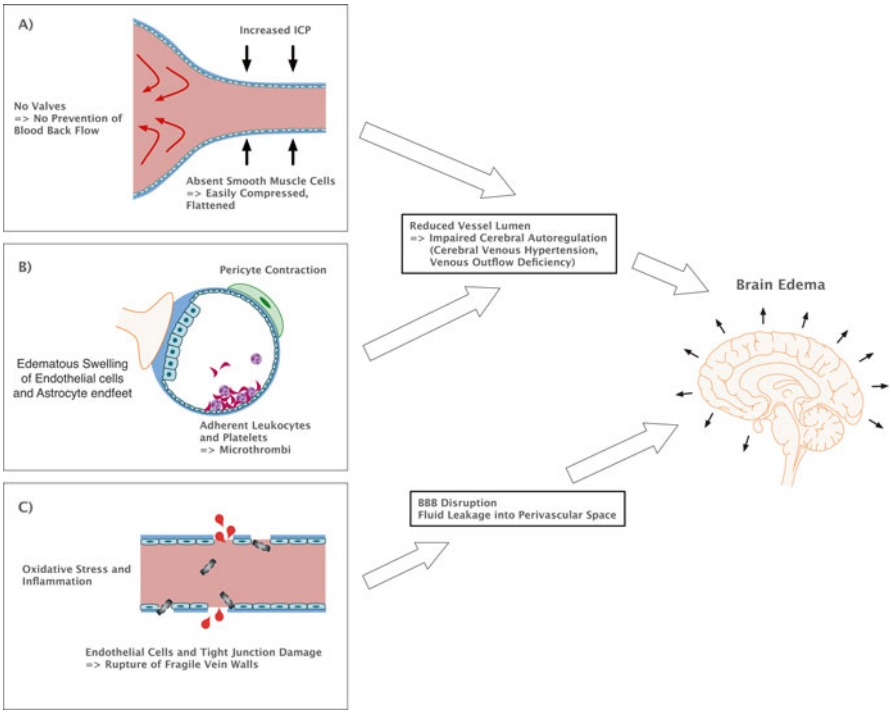

Fig. 2 Alterations in veins, venules, and capillaries after hemorrhagic stroke. (**a**) Increased ICP flattens and compresses veins easily compared to arteries due to the absence of smooth muscle cells. This leads to reduced vessel diameter and increased back flow of blood. (**b**) Edematous, swollen endothelial cells and astrocyte endfeet, active pericyte contraction, and adherent leukocytes and platelets also result in reduced vessel lumen. (**c**) Oxidative stress and inflammation induce endothelial cell and tight junction damage, leading to BBB disruption and brain edema

ET-1 promote proliferation and migration of smooth muscle cells located in the medial and adventitial fibroblasts. These changes may result in the synthesis of additional collagen, increasing the thickness and structural stiffness of the vessel wall [62]. ET-1, which is released from damaged endothelial cells, has also been shown to stimulate phospholipase C-induced extracellular calcium influx through calcium-permeable nonselective cation channels and store-operated calcium channels in vascular smooth-muscle cells, leading to contraction of arteries following SAH in a rabbit model [63]. Further, ET-1-induced vasoconstriction in SAH cells was blocked by inhibition of transient receptor potential canonical 1 and 4, which mediate calcium entry through nonselective cationic pathways, suggesting that ET-1 works by increasing calcium influx [64]. Moreover, ET-1 contributes to hemoglobin-mediated vascular injury via RhoA/Rho kinase and protein kinase C (PKC), which inhibits myosin phosphatase [65]. It was reported that inhibiting the ET-1-induced vasospasm after SAH led to reduced expression of VEGF, the most potent angiogenic factor; thus, the ischemic effect of ET-1-induced vasoconstriction enhances angiogenesis [66]. Figure 3 highlights some vasoconstrictors and vasodilators known to impact vessel diameter following hemorrhagic stroke.

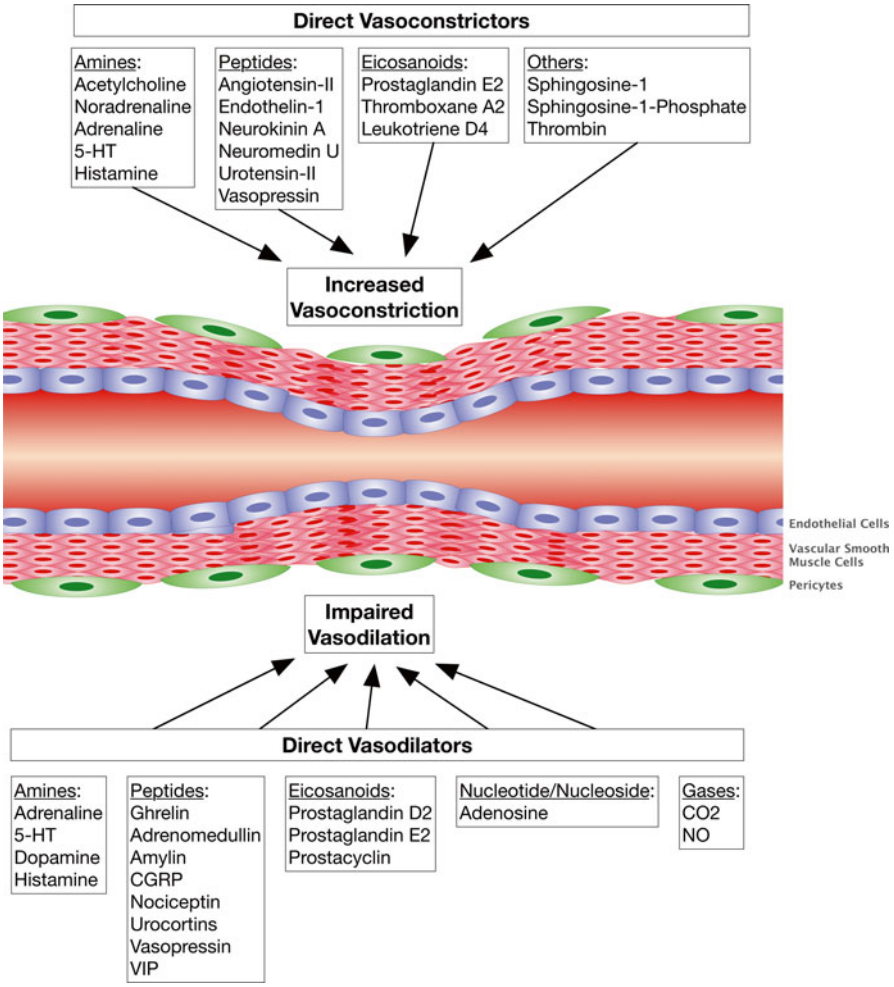

Fig. 3 Vasoconstriction and impaired vasodilation. Hemorrhagic stroke alters cerebral autoregulation, impairing vascular responses. After injury, there is an immediate or delayed reduction in cerebral blood flow due to constriction of cerebral vessels. Vasoconstricting mediators lead to significant contraction of the vessel lumen, decreasing blood flow, while the vasodilators, trying to restore perfusion to the injured area, can induce only restricted expansion of the cerebral vessels. *5-HT* serotonin, *CGRP* calcitonin gene-related peptide, *VIP* vasoactive intestinal peptide, CO_2 carbon dioxide, *NO* nitric oxide

2.4 Vascular Injury and Blood Flow

Intertwined with the vascular disruption after hemorrhagic stroke is an increase in the permeability of the BBB. This is further enhanced by the hemoglobin-induced increase in oxidative stress by reactive oxygen species (ROS) and reactive nitrogen

species (RNS), including superoxide radical and nitric oxide [18], which, along with their metabolites, such as peroxynitrite, activate MMP-9, and disrupt the vascular matrix [67–69]. MMPs are upregulated in neurons and reactive astrocytes in hemorrhagic stroke, and play a role in mediating BBB leakage and edema by degrading the neurovascular matrix. Brain edema is the clinical observation of BBB disruption, and leads to a greater increase in ICP [70]. The increased ICP interferes with the flow of blood after injury by inducing constriction of the blood vessels.

With the induced vasoconstriction and vascular disruption after hemorrhagic stroke, the metabolic demands of the brain cannot be sufficiently met, and an increase in neuronal death may result. This is becausethe brain utilizes approximately 20 % of cardiac output, and is extremely dependent on a constant supply of nutrients and oxygen via the blood, as evidenced by its extensive distribution of blood vessels. Furthermore, it is highly specialized to fulfill this function, such that, when neuronal activity increases in a specific brain region, blood flow increases to this area to meet the enhanced metabolic demand, a phenomenon referred to as functional hyperemia [71].

2.5 Astrocytes in the Cerebrovascular Response

Coupling of the neurovascular components to meet neuronal activity is regulated by various neurotransmitters [72], as well as astrocytes, which are known to have important effects on vessel diameter, as their endfeet are layered around over 99 % of cerebral capillaries [53]. Furthermore, receptors for the vasoactive mediators released by neurons are found on astrocytes and microvascular endothelial and/or smooth muscle cells, and can either dilate or constrict cortical microvessels upon activation [73]. Thus, though vasodilation of parenchymal arterioles in response to neuronal stimulation can be severely compromised after hemorrhagic stroke [74], an increase in the number of reactive astrocytes (astrogliosis) after injury can increase vasodilation by the release of vasoactive molecules from the astrocyte endfeet, thus helping to maintain blood flow. In demonstrating the essential role of astrocytes (neuron-astrocyte signaling) in mediating the cerebrovascular response to neurons, Zonta et al. found that when neuronal afferents were stimulated by an extracellular electrode placed in the cortex, arterioles showed a transient relaxation, and interestingly, this also triggered an increase in intracellular calcium ($[Ca^{2+}]_i$) in the astrocytic endfeet surrounding the arterioles; these events occurred in a temporally related manner and links synaptic activity to the control of vascular tone [75]. They also reported that when the metabotropic glutamate receptors (mGluR) (known to mediate the $[Ca^{2+}]_i$ increase in astrocytes) were blocked, there was reduced dilation of the arterioles in response to afferent stimulation of the neurons. Conversely, direct stimulation of the mGluR produced an increase in vasodilation, correlated to the timing of $[Ca^{2+}]_i$ increase in astrocytes, and direct stimulation of the astrocytes also triggered dilation of the arteriole region that was in contact with the astrocytes' processes [75, 76]. Interestingly, the effect of mGluR stimulation implicates the involvement of synaptically released glutamate, the most abundant

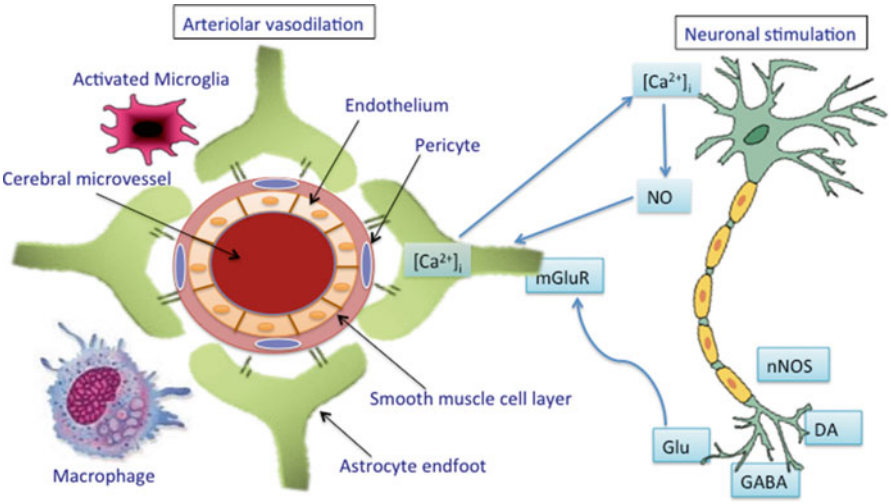

Fig. 4 Neurovascular coupling. Neuron-astrocyte crosstalk plays a crucial role in mediating neurovascular coupling and functional hyperemia. Astrocyte endfeet are layered around smooth muscle cells surrounding cortical microvessels, and upon afferent stimulation of neurons, there is an increase in intracellular calcium ($[Ca^{2+}]_i$) in the astrocyte endfeet, mediated by the metabotropic glutamate receptors (mGluR) upon binding of glutamate (Glu). The result is increased vasodilation of the arterioles ensheathed by the astrocyte endfeet processes. Glutamate release from neurons also induces calcium uptake, which activates neuronal nitric oxide synthase (nNOS), leading to release of nitric oxide (NO), a vasodilator. The NO released from neurons diffuses into the astrocytic endfeet where it modulates the release of vasoactive substances, and may also directly regulate smooth muscle cells. In addition, other neurotransmitters, such as dopamine (DA), and gamma aminobutyric acid (GABA) help to regulate cerebrovascular responses to neural activity. Microglia and macrophages are recruited to the damaged area following a stroke, inducing several inflammatory reactions. This can lead to microvascular injury. However, they also engulf the blood and damaged or dead tissues, providing an environment for tissue repair

excitatory transmitter in the CNS. The uptake of glutamate from the synapse by astrocytes, as discussed above, aids in reducing excitotoxicity by glutamate following a stroke, as the levels of the neurotransmitter are maintained by astrocyte activity [39, 77]. It also helps to drive glucose uptake from local blood vessels to fulfill the metabolic needs of the neurons via the astrocytic endfeet processes [78]. These data illustrate the unified action of cells of the VNN to ensure that the metabolic demand and blood flow requirements of the brain are met, and also that neuronal injury is reduced (Fig. 4).

Ionic imbalance is another major mechanism by which vascular smooth muscle constriction occurs after a hemorrhagic stroke. Intracellular calcium levels in VSMCs are elevated by either increased influx through voltage-gated calcium channels or impaired calcium reuptake in the sarcoplasmic reticulum [79–83]. After SAH, the reuptake is compromised by the lack of nitric oxide (NO), which, under normal conditions, would elevate cyclic guanosine monophosphate (cGMP) via calcium reuptake in smooth muscle cells [84]. Increased calcium levels induce opening of

calcium-activated potassium channels in astrocytes, which results in highly elevated extracellular potassium, leading to arteriolar vasoconstriction [46]. Additionally, increased expression of the voltage-gated potassium (Kv) channels in parenchymal arteriolar myocytes leads to depolarization and increased vascular tone via the heparin-binding epidermal growth factor-like growth factor [85]. Consequently, the Kv7 channels are common targets of vasoconstrictor spasmogens [86].

2.6 Pericyte-Endothelial Interactions and the Blood Brain Barrier

During injury, vascular function is regulated by various mediators that initiate the differentiation and migration of cerebrovascular pericytes, which are able to further modulate the structure and function of cerebral arteries and maintain BBB function [87]. These include VEGF, PDGF, nerve growth factor, angiopoietins, TGF-β, notch, etc. [4]. Extracellular TGF-β and intracellular Rho GTPase were previously reported to be involved in vasoconstriction. Several studies suggest that this contractile effect is due to pericytes [88–90]. Pericytes in the CNS have receptors for a large number of vasoactive mediators, thus they may play a role in cerebrovascular autoregulation. Electrical stimulation of cerebellar pericytes evoked localized capillary constriction in response to neural activity, indicating a role for pericytes in blood flow regulation during injury [91].

In a perforation-induced SAH model [92], electron microscopic evaluation revealed partially collapsed capillaries, which were associated with enlarged perivascular astrocyte foot processes and luminal endothelial cell protrusions, as well as defects in the basal lamina and endothelium [93]. Increased endothelial apoptosis has been shown in parenchymal vessels 10 min after SAH, up to 24 h [94, 95], and this can result in BBB disruption [96]. Several studies have reported decreased occludin and collagen IV in parenchymal vessels, associated with the disruption of endothelial tight junctions and widening of the inter-endothelial spaces [38, 97–99]. Additionally, BBB tight junction opening has been shown after hemorrhagic stroke, with the greatest opening occurring at 3 and 72 h, coinciding with the downregulation of the tight junction proteins zona occludens (ZO)-1 and occludin [100].

Pericyte–endothelial interactions are important for vascular integrity following a cerebral injury. Pericytes are located in precapillary arterioles, capillaries, and postcapillary venules, and they synthesize and deposit elements of the basal lamina [101]. In the mature capillary, they are located between endothelial cells and parenchymal astrocytes and neurons; thus, there is active crosstalk among these cells. The ratio of pericytes to endothelial cells is much higher in the cerebral microcirculation than in other tissues, and they play a role in microvascular blood flow as a final regulatory step after arterioles [102]. As such, pericytes on isolated retinal capillaries have been shown to constrict or dilate in response to neurotransmitters, due to an increase in intracellular calcium concentrations. Furthermore, pericyte numbers around endothelial cells have been found to inversely correlate with BBB function,

as loss of pericyte coverage leads to an increase in the gene expression of proteins known to increase vascular permeability (angiopoietin-2, intercellular adhesion molecule (ICAM)-1, etc.) [4]. Pericytes maintain BBB homeostasis by inducing the expression of occludin, maintaining the expression of other BBB-specific genes in endothelial cells, and by inducing polarization of astrocyte endfeet adjacent to the cerebral microvasculature [6, 103]. Antigen-presenting pericytes induce a local pro-inflammatory response mediated by the elevated expression of adhesion molecules (ICAM-1 and vascular cell adhesion molecule (VCAM-1)) near endothelial cells and circulating cytokines and chemokines, which are involved in neutrophil infiltration and tight junction degradation [104, 105].

2.7 Inflammation and Vascular Injury

Another contributor to microvascular injury after hemorrhagic stroke is inflammatory reactions, including leukocyte rolling and adherence to the walls of cerebral vessels [106, 107]. Coinciding with this, a progressive increase in leukocyte adhesion to venules was reported after SAH [58]. As a result, the adhesion molecule P-selectin was more expressed in the endothelial cell membrane following SAH, while no difference in cytosolic P-selectin expression was observed [47]. Other inflammatory mediators, apart from leukocytes, are also produced and elevated after a hemorrhagic stroke, including the cytokines interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and high-mobility group box protein 1 [108, 109]. These cytokines, combined with the induced oxidative stress, also contribute to vascular injury and BBB disruption [110].

3 Mechanisms of Neurovascular Repair

3.1 Glial Cells in Hemorrhage Clearance

Resident microglia and peripheral macrophages are rapidly mobilized to the site of injury (around the hematoma), and recruit other immune cells, as well as initiate the release of effector molecules [111–113]. Microglial activation within the perihematomal region has been seen as early as 1 h after collagenase-induced experimental ICH, and within 4 h in the autologous blood double injection model [114, 115]. By 72 h post ICH, microglia reach their peak numbers in the perihematoma, then levels begin to fall back down by 7 days post-injury, and return to basal levels within 3–4 weeks [112, 116, 117]. This time course coincides with the hematoma volume over time in ICH (peaking at 72 h, remaining elevated up to 7 days, and returning to basal levels within 2–4 weeks) [118], suggesting that activated microglia and macrophages play a significant role in hematoma resolution [119]. Activated microglia recruit hematogenous phagocytes to the area of the hemorrhage; the phagocytes in

turn engulf the deposited blood, damaged and dead tissue, and then provide an environment for tissue repair [24]. Zhao et al. found that PPAR-γ treatment led to more efficient microglia-induced phagocytosis [31]. PPAR-γ treatment was also found to reduce IL-1β, TNF-α, MMP-9, and inducible nitric oxide synthase (iNOS) expression, while increasing CD-36-mediated microglial phagocytosis of red blood cells [31, 118]. Another treatment targeted at microglial activation following ICH is minocycline, which was found to reduce microglia/macrophage numbers at 5 days when given 3 h post ictus [120]. It also reduced TNF-α, IL-1β, and MMP-12 levels both in vivo and in vitro [121, 122]. These studies indicate an important role for glial cells in neurovascular repair.

Yet, there lies a paradox in the activity of microglia and macrophages: while they are clearing up the hematoma, they generate large quantities of oxidant by-products, which increase oxidative stress to the microglia, and may limit their functioning [25], and could also exacerbate tissue damage. This double-sided action on brain repair and tissue damage may be due to the polarization of microglia and macrophages toward different phenotypes at different stages of injury [123]. M1 microglia/macrophages are the classically activated phenotype, and are considered to be pro-inflammatory, secreting TNF-α, iNOS, IL-1β, etc., while M2 microglia/macrophages represent the alternatively activated phenotype and are seen as healing cells involved in neuroprotection and repair via arginase activity and upregulation of neurotrophic factors [25, 124, 125]. Macrophages and microglia do not exist as terminally differentiated M1 or M2 states, but are able to switch phenotypes depending on the microenvironment [126, 127]. PPAR-γ plays an important role in modulating macrophage M2 polarization induced by IL-4 or IL-13 [128, 129].

3.2 Neurogenesis and Angiogenesis: The Neurovascular Niche

Neurovascular repair involves, among other mechanisms discussed, the synthesis of new neurons and vessels to replace the dead and damaged ones that result from cerebral injury. Neurogenesis is the process by which neurons are generated from the neural stem cells (NSCs) and progenitor cells, and angiogenesis is the process by which new blood vessels are formed from existing ones. Interestingly, the neural precursors, committed neuroblasts, glia, and endothelial precursors (angioblasts) are found in tight clusters grouped around small capillaries, referred to as the neurovascular niche, indicating a close association between neurogenesis and angiogenesis [130, 131].

Neurogenesis is mostly active in the prenatal period; however, contrary to initial beliefs, NSCs exist beyond embryonic development, into adulthood; thus, a large number of newly generated cells in the adult brain are neurons [132]. Under physiological conditions, adult NSCs differentiate predominantly to inhibitory granule/periglomerular interneurons in the olfactory bulb, and excitatory granule neurons in the dentate gyrus. Also, in adults, neurogenesis constitutively occurs only in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal

dentate gyrus and olfactory bulb [133, 134]. Insults to the CNS, such as hemorrhagic strokes, induce increased neurogenesis, which appear to also be generated in non-neurogenic sites, such as the cortex [135]. Immature neurons may be found in the tissue adjacent to the area of the stroke within the first 2–4 weeks after injury [131]. Neurogenesis induces the migration of neuroblasts into regions of degenerating striatum bordering the SVZ, the primary source of which are multipotent glial fibrillary acidic protein (GFAP)-expressing progenitors [132, 136]. The progenitor cells have the capacity to generate neurons, oligodendrocytes, and astrocytes.

After a hemorrhagic stroke, when neurogenesis is initiated, the formed neuroblasts migrate from the SVZ to the peri-infarct cortex, where there is also increased vascular remodeling, likely due to an increase in the metabolic demand following injury, again illustrating the presence of a neurovascular niche, as well as that neurogenesis may be dependent on environmental cues [137]. Additionally, the growth factors and chemokines secreted by vascular endothelial cells may support the survival of the newly formed cells. In line with this, Shen et al. found that coculturing neuroblasts with brain vascular endothelium significantly promoted neurogenesis, and that NSCs were in close proximity to the endothelial cells, where they interdigitated within the folds of the blood vessels and ensheathed the vascular endothelial cells [138]. They also observed that inhibiting angiogenesis resulted in a significant reduction in the number of new neural cells, and upon removal of the endothelial cells, the NSCs generate neurons, as well as astrocytes and oligodendrocytes, as the endothelial factors inhibited differentiation of the stem cells, and instead, promoted self-renewal [138]. Thus, it is suggested that growing NSCs with endothelial cells may be an effective approach to producing more NSCs and neurons for use in replacement therapies.

3.2.1 Astrocytes and Pericytes in Neurogenesis and Angiogenesis

The neural progenitors in the SVZ and SGZ are also in intimate contact with astrocytes, which helps to build a microenvironment that promotes neurogenesis and regulate fate specification [139]. The astrocytes produce signals, such as sonic hedgehog, which dictate the neurogenic potential of different CNS regions, such that neural progenitors are distributed beyond just the neurogenic SVZ and SGZ, into the cortex, cerebellum, and spinal cord. These cells, like those in the SVZ and SGZ, are GFAP-expressing cells [135]. This, therefore, indicates that the neurogenic and non-neurogenic areas of the brain differ not in the properties of their progenitor cells, but in their microenvironment, and this could therefore be therapeutically targeted in order to activate the regenerative potential in non-neurogenic regions of the injured adult brain [135].

Pericytes also play a role in these processes as they interact closely with endothelial cells during the early stages of angiogenesis and with neurons during the maturation of newly formed vessels [140, 141]. The recruitment of pericytes and astrocytes to the newly forming vessels is associated with the formation of tight

junctions, although the tight junctions can be formed without the ensheathment of astrocyte foot processes [101]. Pericytes are involved in the induction of endothelial activation and the augmentation of various proteases, adhesion molecules, and proteoglycans at the initiation of angiogenesis [142, 143], and are conversely involved in the silencing of MMP activities at maturation [144].

3.3 MMPs in Neurovascular Plasticity and Remodeling

While MMPs, which modulate the brain matrix, contribute to neurovascular disruption in the acute phase of hemorrhagic stroke, in the chronic phase, the neurovascular proteases play a role in repair and remodeling by regulating various factors such as VEGF and Kit-ligand [145]. Furthermore, inhibition of MMPs in the late stages results in reduced neuronal plasticity and vascular remodeling, and impaired functional recovery [146]. MMPs belong to a family of zinc-containing endopeptidases, most of which can be produced by endothelial cells. In the early stages after stroke, MMPs were found to be colocalized mainly with endothelial cells, but in the chronic stages, their expression is principally colocalized with neurons and astrocytes [146], which we know are involved in regulating blood flow and responding to increases in neural activity, a vital aspect of repair. MMP-2, MMP-9, and membrane type 1 MMP are known to play a role in the onset of angiogenesis, and matrix degradation is an important aspect of their ability to stimulate angiogenesis. The proteases are able to induce pro- and antiangiogenic effects, such as activating growth factors and cytokines, degrading inhibitors, and generating angiogenesis-inhibiting matrix-derived peptides [144]. For example, MMP-9 stimulates the onset of angiogenesis, but subsequently generates angiogenesis inhibitors, thus retarding the process [147].

It has also been shown that MMP-9 is colocalized with cells from the subventricular zone after stroke, and treatment with an MMP inhibitor significantly decreased migration of the cells to the striatum, indicating that MMPs play a role in neurogenesis after brain injury, and thus are important for the repair process [148]. Being able to modulate these endogenous matrix mechanisms may enhance neurovascular recovery following a hemorrhagic brain injury.

3.4 VSMCs and Neurovascular Coupling

Smooth muscle cells in the VNN, together with neurons, astrocytes, and endothelial cells, maintain homeostasis in the cerebral microcirculation (neurovascular coupling) [149, 150] by constricting or dilating the vessels when they contract or relax, respectively. VSMCs in arteries are able to migrate, proliferate, secrete extracellular matrix proteins, and contract, and they play an essential role in the cerebrovascular response to injury [87]. SMCs communicate with endothelial cells regarding the

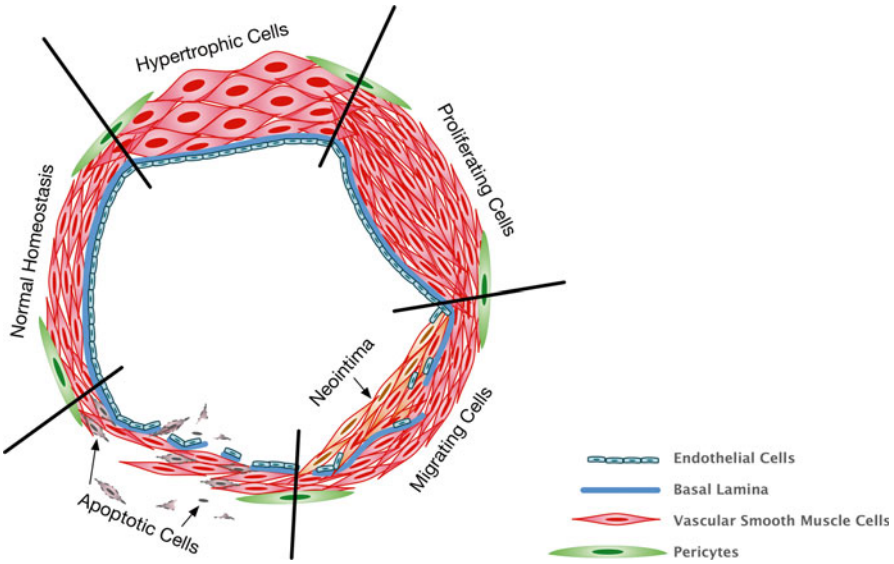

Fig. 5 Phenotypic changes of vascular smooth muscle cells in vascular remodeling after hemorrhagic stroke. Following hemorrhagic stroke, smooth muscle cells undergo phenotypic changes, leading to hypertrophy, proliferation, migration, and apoptosis. Hypertrophic and proliferating cells result in reduced vessel lumen, while apoptotic and migrating cells build a fragile neointima, leading to rupture and leakage. Inflammation, thrombin, and various growth factors may influence these changes, which affect autoregulation and neurovascular coupling

state of blood flow, via heterocellular connexin gap junctions, and also with astrocytes regarding neural activity; thus, they are considered to be the center of the neurovascular network [151]. After aneurysmal SAH, VSMCs undergo phenotypic transformation, proliferate, and secrete cytokines, which may result in loss of myogenic tone and autoregulation, compromising neurovascular coupling and the return of normal CBF (Fig. 5). This phenotypic change can be accelerated by inflammation, thrombin, and various growth factors [152, 153].

Originally, it was thought that neurovascular coupling was solely mediated by changes in the smooth muscle tone around the arterioles. However, it was discovered that pericytes, present at 50 μm intervals along the capillary, can markedly affect capillary diameter, and thus could possibly regulate CBF at the capillary level [150]. Perivascular nerve fibers are also able to promote vasoconstriction and vasodilation, as well as smooth muscle growth and differentiation [73]. SAH reduces both the density and cerebrovascular tone of the nerve fibers, which may lead to functional denervation of cerebral arteries, thus significantly impairing neurovascular coupling [154].

Longden et al. found that after a stress event, parenchymal arterioles did not dilate in response to neuronal stimulation as well as they did without the stress, and dilation of isolated arteries to external potassium was also reduced, suggesting that there was a defect in the functioning of smooth muscle inwardly rectifying potassium (Kir)

channels. As such, they reported that stress reduced Kir channel density, and that blocking the channels significantly inhibited neurovascular coupling in stressed, but not control, brain slices [74]. They conclude that the reduction in these potassium channels makes the arterioles less responsive to potassium released from the astrocytic endfeet, thus affecting neurovascular coupling. Moreover, Koide et al. found that, following SAH, neurovascular coupling was inverted, that is, parenchymal arterioles were dilated in response to elevation of calcium from astrocytic endfeet in control brain slices but were constricted in post-SAH brain slices, and that inhibiting the potassium efflux diminished the vessel response to neuronal activity [46]. These present targets for repair of neurovascular functioning after hemorrhagic stroke.

Another mechanism of neurovascular coupling is via nitric oxide. Neuronal nitric oxide synthase (nNOS) is activated upon calcium entry into neurons, which is induced by glutamate release [155]. This in turn triggers the release of NO, a potent vasodilator. NO can also be released by calcium-independent iNOS and from the endothelium via endothelial NOS (eNOS). In fact, all components of the neurovascular unit can produce NO. In the cortex, inhibition of nNOS reduces the increase in blood flow due to neural activity, indicating that NO plays an important role in neurovascular coupling [156]. Since NO is released upon neuronal activation, and it is known that CBF regulation is tightly bound to neuronal activity, it follows that NO plays a role in regulating CBF. The NO released from activated neurons diffuses through the cell membranes to the astrocytic endfeet (surrounding the arterioles) where it can modulate the release of various vasoactive metabolites; it may also be involved in vasodilation by acting directly on smooth muscle cells [157]. NO increases the release of ATP from astrocytes, leading to the formation of calcium waves via the 2PY purinergic receptors [158]. The calcium waves stimulate the production of arachidonic acid-derived vasodilation mediators such as epoxyeicosatrienoic acids (EETs) or activate large-conductance calcium-dependent potassium (BK) channels to induce arterial vasodilation. In line with this, epoxygenase inhibitors were reported to reduce cortical blood flow in response to vibrissal stimulation, indicating a role for EETs in neurovascular coupling [159]. Moreover, inhibiting potassium efflux via the BK channels in the astrocytic endfeet prevented both vasodilation and vasoconstriction in response to neuronal activity in SAH slices [46].

4 Current Therapies Targeted at Neurovascular Repair

The concepts of the neurovascular unit and VNN integrate neural and vascular cell types, which help to explain the failure of various neuroprotective strategies that target neurons only, without addressing vascular injury. As a result, over the last several years, there have been a lot of studies evaluating various therapies with the goal of improving neurovascular repair. Table 1 looks at some therapies that have been targeted at vascular repair after hemorrhagic stroke.

The BBB is the most effective deterrent to effective delivery of drugs to the CNS due to the tight junctions between the microvascular endothelial cells, as well as the support from surrounding astrocytes, microglia, and extracellular matrix [6]. This

Table 1 Experimental therapies for vascular repair

Therapy	Effects on vascular repair and blood flow	References
tPA	Improved cerebral blood flow and cortical perfusion, thus attenuating early brain injury after SAH	[10]
mGluR stimulation	Increased vasodilation, correlated to the timing of intracellular Ca^{2+} increase in astrocytes	[75, 76]
PPAR-γ agonist	Attenuated the vasoconstrictive response by upregulating caveolin-1; increased efficiency of microglia-induced phagocytosis; reduced interleukin-1β, TNF-α, MMP-9, and iNOS expression	[31, 118]
Nitric oxide donors	Dilated spasmic cerebral arteries, and reduced excitotoxicity and leukocyte migration	[90, 169]
Memantine	Attenuated delayed vasospasm by targeting eNOS	[167]
Dopamine D2-receptor agonist	Attenuated vasospasm by mediating increases in vascular and endothelial NOS activity	[168]
L-Arginine	Ameliorated secondary cerebral ischemic injury after SAH by enhancing cerebral blood perfusion, increasing nitric oxide, and decreasing endothelin-1	[171]
Kv7 channel openers	Attenuated basilar artery spasm	[172]
Soluble epoxide hydrolase gene deletion	Increased vasoprotective eicosanoids, and suppressed vascular inflammation and VCAM-1, leading to reduced hydrocephalus and edema	[177]
Sulforaphane	Activated Nrf2/ARE pathway in vascular smooth muscle cells—improved outcomes	[178]
Protein kinase C or Raf inhibitors	Reduced the vasospastic effect, and maintained neurovascular integrity	[179, 180]
PAR-1 inhibitor	Maintained microvascular integrity	[181]
Calcitonin gene-related peptide	Enhanced vasorelaxation by enhancing endothelial nitric oxide and cAMP-mediated endothelium-independent signaling	[182]
TNF-α proteinase inhibitor	Reversed the augmented myogenic tone in isolated vessels	[183]

Various therapies have been targeted at different components of neurovascular interactions. This table summarizes some current experimental treatments that are directed at vascular repair after a hemorrhagic stroke [177–183]

mGluR metabotropic glutamate receptor, *tPA* tissue plasminogen activator, *SAH* subarachnoid hemorrhage, *VCAM-1* vascular cell adhesion molecule 1, *Nrf2/ARE* nuclear factor erythroid 2-related factor 2/antioxidant responsive element, *PAR-1* protease-activated receptor, *Kv7* voltage-gated potassium channel, type 7, *NOS* nitric oxide synthase, *cAMP* cyclic adenosine monophosphate, *TNF* tumor necrosis factor, *PPAR* peroxisome proliferator-activated receptor, *MMP* matrix metalloproteinase

stringent barrier is necessary for normal physiological functioning: facilitating nutrient transport, regulating ion balance, and preventing toxic substances in the systemic circulation from entering the brain; restoring its function after stroke is the primary goal of many current therapies. The Src-family kinase inhibitor protein phosphatase-1 was found to decrease the phosphorylation of VEGF and mitogen-activated protein kinases (MAPK), thereby reducing BBB permeability, brain edema, and

mortality after hemorrhagic stroke [160]. Further, thrombomodulin preserved endothelial junction proteins and reduced cell apoptosis and inflammation in endothelial cells via blocking p38 and MAPK [161]. Norrin, a frizzled-4 receptor agonist that promotes β-catenin nuclear translocation, is also targeted at BBB preservation. Treatment with norrin attenuated BBB permeability by enhancing the expression of occludin, vascular endothelial (VE)-cadherin, and ZO-1 after SAH. [162].

Since MMPs and the by-products of reactive oxygen and nitrogen species are known to be involved in BBB breakdown during cerebral injury, several studies have also investigated interventions in these pathways in order to achieve neurovascular repair. A peroxynitrite inhibitor ameliorated neurovascular dysfunction and improved neurological function, in part by reducing MMP-9 after hemoglobin injection into the caudate nucleus [163]. Also, inhibition of c-Jun N-terminal kinases-induced MMP-9 activation was shown to protect the neurovascular unit and preserve the tight junction protein, ZO-1 [164]. Imatinib, a PDGF receptor alpha inhibitor, also preserved BBB integrity following experimental SAH by reducing MMP-9 [165].

Other therapies have been targeted at reducing vascular injury and the concomitant reduction in blood flow secondary to hemorrhagic stroke. For example, heparin, which blocks the activity of free radicals, including ROS, antagonizes endothelin-mediated vasoconstriction, smooth muscle depolarization, and inflammatory and fibrogenic responses via complex-formation with oxyhemoglobin [166]. Targeting eNOS, memantine attenuated delayed vasospasm after SAH [167]. Further, a dopamine D2-receptor agonist was found to mediate increases in vascular and endothelial NOS activity in vitro, and this was associated with attenuated vasospasm [168]. Notably, NO donors administered after experimental SAH dilated spasmic cerebral arteries, and reduced excitotoxicity and leukocyte migration [98, 169]. This proved consistent with the observation that acute ischemic injury can be reversed by the NO donor *S*-nitrosoglutathione [170]. Moreover, L-Arginine, which increases NO, and decreases ET-1 levels in blood, enhanced cerebral blood perfusion, and ameliorated secondary cerebral ischemic injury after experimental SAH [171]. Increases in intracellular calcium levels also induce vasoconstriction by activating potassium channels on astrocytes. As such, the voltage-gated potassium channel (type 7) openers (retigabine and celecoxib) were reported to significantly attenuate basilar artery spasm in rats with experimentally induced SAH [172].

Still other therapies have been targeted at oxidative stress and inflammation and their effects on neuronal survival and other outcomes after hemorrhagic stroke. Nuclear factor erythroid 2-related factor 2 (Nrf2) activation can induce antioxidation, reduce peroxide formation, and upregulate phagocytosis by inducing CD36 expression, thus promoting hematoma clearance after ICH [173]. Activating Nrf2 reduced the permeability of the BBB after ICH, and reduced microglial activation in the perihematoma, resulting in improved functional outcomes [174]. Nrf2 and other such therapies therefore protect the neurovascular components by reducing the toxicity induced by blood components and reducing vascular disruption. In fact, knockout of Nrf2 led to impaired hematoma clearance, suggesting that Nrf2 in microglia plays an important role in augmenting this process [175]. Notably, resolution of the

hematoma also leads to a reduction in inflammation, as blood components are able to promote the initiation of inflammation following injury. This in itself can limit injury to the neurovascular network by decreasing the disruption of the BBB following hemorrhagic stroke [24].

Other anti-inflammatory therapies investigated include a P-selectin antibody and the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, apocynin, which decreased rolling and adherent platelets and leukocytes in pial venules after SAH [58]. This is important as NADPH is one of the major sources of free radicals after subarachnoid bleeding. Vinblastine and anti-polymorphonuclear serum were also able to reduce the neutrophil adherence to parenchymal vessels [106]. Additionally, hyperbaric oxygen ameliorated neurovascular injury via several inhibitory signaling pathways with antioxidant, anti-inflammatory, and anti-apoptotic effects [176].

These are just a few of the many therapies being evaluated, and while advances have been made in the field, and these experimental drugs seem promising in experimental models, there is still the challenge of successful clinical translation. There may be a number of reasons for this, and of course, more studies are needed in this area. However, a closer look at combination therapies may be worthwhile, targeting neuronal and vascular injury simultaneously.

5 Conclusions

All the cells of the VNN play significant roles in brain injury and repair. Hemorrhagic stroke induces significant damage to neurons and the vasculature, impairing the functionality of the brain. Heme and heme breakdown products lead to an increase in neurotoxicity and vascular injury. Cerebral vessels and smooth muscle cells undergo phenotypic changes that alter the supply and demand system of the brain, whereby CBF is linked to neural activity. This can therefore result in significant damage to the VNN. Additionally, endothelial dysfunction leads to disruptions in the blood–brain barrier, vasoconstriction, and neuronal death. However, astrocytes help to protect neurons from excitotoxicity by modulating glutamate release and uptake, as well as in regulating CBF through calcium release; microglia and macrophages aid in repair by engaging in the clearance of toxic blood products; and pericytes and endothelial cells play vital roles in maintaining BBB function and in regulating inflammation. Additionally, there is increased neurogenesis and angiogenesis following a hemorrhagic stroke insult, and targeting these processes, within the neurovascular niche where they are codependent, could have significant impacts on neurovascular repair. Moreover, there is an increase in MMPs that initially enhances damage to the brain, but in chronic stroke, can be modulated to improve outcomes by interactions with the neurovascular matrix. Coordinately targeting the VNN, neuronal and vascular injury, may be the best approach for improving hemorrhagic stroke outcomes.

References

1. Chen S, Chen Y, Xu L, et al. Venous system in acute brain injury: mechanisms of pathophysiological change and function. Exp Neurol. 2015;272:4–10.
2. Zhang JH, Badaut J, Tang J, Obenaus A, Hartman R, Pearce WJ. The vascular neural network—a new paradigm in stroke pathophysiology. Nat Rev Neurol. 2012;8(12):711–6.
3. Zhang JH. Vascular neural network in subarachnoid hemorrhage. Transl Stroke Res. 2014;5(4):423–8.
4. Hill J, Rom S, Ramirez SH, Persidsky Y. Emerging roles of pericytes in the regulation of the neurovascular unit in health and disease. J Neuroimmune Pharmacol. 2014;9(5):591–605.
5. Huber JD, Egleton RD, Davis TP. Molecular physiology and pathophysiology of tight junctions in the blood–brain barrier. Trends Neurosci. 2001;24(12):719–25.
6. Ronaldson PT, Davis TP. Blood-brain barrier integrity and glial support: mechanisms that can be targeted for novel therapeutic approaches in stroke. Curr Pharm Des. 2012;18(25):3624–44.
7. Dohgu S, Takata F, Yamauchi A, et al. Brain pericytes contribute to the induction and up-regulation of blood-brain barrier functions through transforming growth factor-beta production. Brain Res. 2005;1038(2):208–15.
8. Zlokovic BV. The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron. 2008;57(2):178–201.
9. Lo EH, Broderick JP, Moskowitz MA. tPA and proteolysis in the neurovascular unit. Stroke. 2004;35(2):354–6.
10. Siler DA, Gonzalez JA, Wang RK, Cetas JS, Alkayed NJ. Intracisternal administration of tissue plasminogen activator improves cerebrospinal fluid flow and cortical perfusion after subarachnoid hemorrhage in mice. Transl Stroke Res. 2014;5(2):227–37.
11. Sumii T, Lo EH. Involvement of matrix metalloproteinase in thrombolysis-associated hemorrhagic transformation after embolic focal ischemia in rats. Stroke. 2002;33(3):831–6.
12. Castellanos M, Leira R, Serena J, et al. Plasma metalloproteinase-9 concentration predicts hemorrhagic transformation in acute ischemic stroke * Editorial comment. Stroke. 2002;34(1):40–6.
13. Docagne F, Parcq J, Lijnen R, Ali C, Vivien D. Understanding the functions of endogenous and exogenous tissue-type plasminogen activator during stroke. Stroke. 2015;46(1):314–20.
14. Niego B, Freeman R, Puschmann TB, Turnley AM, Medcalf RL. t-PA-specific modulation of a human blood-brain barrier model involves plasmin-mediated activation of the Rho kinase pathway in astrocytes. Blood. 2012;119(20):4752–61.
15. Yepes M, Sandkvist M, Moore EG, Bugge TH, Strickland DK, Lawrence DA. Tissue-type plasminogen activator induces opening of the blood-brain barrier via the LDL receptor–related protein. J Clin Invest. 2003;112(10):1533–40.
16. Polavarapu R, Gongora MC, Yi H, et al. Tissue-type plasminogen activator-mediated shedding of astrocytic low-density lipoprotein receptor-related protein increases the permeability of the neurovascular unit. Blood. 2007;109(8):3270–8.
17. Asahi M, Wang X, Mori T, et al. Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood–brain barrier and white matter components after cerebral ischemia. J Neurosci. 2001;21(19):7724–32.
18. Wang X, Mori T, Sumii T, Lo EH. Hemoglobin-induced cytotoxicity in rat cerebral cortical neurons: caspase activation and oxidative stress. Stroke. 2002;33(7):1882–8.
19. Wu J, Hua Y, Keep RF, Schallert T, Hoff JT, Xi G. Oxidative brain injury from extravasated erythrocytes after intracerebral hemorrhage. Brain Res. 2002;953(1–2):45–52.
20. Aronowski J, Zhao X. Molecular pathophysiology of cerebral hemorrhage: secondary brain injury. Stroke. 2011;42(6):1781–6.
21. Babu R, Bagley JH, Di C, Friedman AH, Adamson C. Thrombin and hemin as central factors in the mechanisms of intracerebral hemorrhage-induced secondary brain injury and as potential targets for intervention. Neurosurg Focus. 2012;32(4):E8.

22. Xiong XY, Wang J, Qian ZM, Yang QW. Iron and intracerebral hemorrhage: from mechanism to translation. Transl Stroke Res. 2014;5(4):429–41.
23. Chen Q, Zhang J, Guo J, et al. Chronic hydrocephalus and perihematomal tissue injury developed in a rat model of intracerebral hemorrhage with ventricular extension. Transl Stroke Res. 2015;6(2):125–32.
24. Chen S, Yang Q, Chen G, Zhang JH. An update on inflammation in the acute phase of intracerebral hemorrhage. Transl Stroke Res. 2015;6(1):4–8.
25. Taylor RA, Sansing LH. Microglial responses after ischemic stroke and intracerebral hemorrhage. Clin Dev Immunol. 2013;2013:746068.
26. Carson MJ, Doose JM, Melchior B, Schmid CD, Ploix CC. CNS immune privilege: hiding in plain sight. Immunol Rev. 2006;213(1):48–65.
27. Keep RF, Hua Y, Xi G. Intracerebral haemorrhage: mechanisms of injury and therapeutic targets. Lancet Neurol. 2012;11(8):720–31.
28. Xiong XY, Yang QW. Rethinking the roles of inflammation in the intracerebral hemorrhage. Transl Stroke Res. 2015;6(5):339–41.
29. Pandey AS, Xi G. Intracerebral hemorrhage: a multimodality approach to improving outcome. Transl Stroke Res. 2014;5(3):313–5.
30. Khanna A, Kahle KT, Walcott BP, Gerzanich V, Simard JM. Disruption of ion homeostasis in the neurogliovascular unit underlies the pathogenesis of ischemic cerebral edema. Transl Stroke Res. 2014;5(1):3–16.
31. Zhao X, Sun G, Zhang J, et al. Hematoma resolution as a target for intracerebral hemorrhage treatment: role for peroxisome proliferator-activated receptor gamma in microglia/macrophages. Ann Neurol. 2007;61(4):352–62.
32. Schallner N, Pandit R, LeBlanc III R, et al. Microglia regulate blood clearance in subarachnoid hemorrhage by heme oxygenase-1. J Clin Invest. 2015;125(7):2609–25.
33. Chen Y, Luo C, Zhao M, et al. Administration of a PTEN inhibitor BPV(pic) attenuates early brain injury via modulating AMPA receptor subunits after subarachnoid hemorrhage in rats. Neurosci Lett. 2015;588:131–6.
34. Huang CY, Wang LC, Wang HK, et al. Memantine alleviates brain injury and neurobehavioral deficits after experimental subarachnoid hemorrhage. Mol Neurobiol. 2015;51(3): 1038–52.
35. Fujii M, Yan J, Rolland WB, Soejima Y, Caner B, Zhang JH. Early brain injury, an evolving frontier in subarachnoid hemorrhage research. Transl Stroke Res. 2013;4(4):432–46.
36. Jeon H, Ai J, Sabri M, Tariq A, Macdonald RL. Learning deficits after experimental subarachnoid hemorrhage in rats. Neuroscience. 2010;169(4):1805–14.
37. Joshua DB, Theresa Currier T, Elliot L, et al. Platelet-mediated changes to neuronal glutamate receptor expression at sites of microthrombosis following experimental subarachnoid hemorrhage. J Neurosurg. 2014;121(6):1424–31.
38. Friedrich V, Flores R, Muller A, Sehba FA. Luminal platelet aggregates in functional deficits in parenchymal vessels after subarachnoid hemorrhage. Brain Res. 2010;1354:179–87.
39. Anderson CM, Swanson RA. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia. 2000;32(1):1–14.
40. Rosenberg PA, Aizenman E. Hundred-fold increase in neuronal vulnerability to glutamate toxicity in astrocyte-poor cultures of rat cerebral cortex. Neurosci Lett. 1989;103(2):162–8.
41. Joerk A, Seidel RA, Walter SG, et al. Impact of heme and heme degradation products on vascular diameter in mouse visual cortex. J Am Heart Assoc. 2014;3(4): pii: e001220.
42. Edvinsson L, Larsen SS, Maddahi A, Nielsen J. Plasticity of cerebrovascular smooth muscle cells after subarachnoid hemorrhage. Transl Stroke Res. 2014;5(3):365–76.
43. Suzuki H. What is early brain injury? Transl Stroke Res. 2015;6(1):1–3.
44. Herz DA, Baez S, Shulman K. Pial microcirculation in subarachnoid hemorrhage. Stroke. 1975;6(4):417–24.
45. Sun BL, Zheng CB, Yang MF, Yuan H, Zhang SM, Wang LX. Dynamic alterations of cerebral pial microcirculation during experimental subarachnoid hemorrhage. Cell Mol Neurobiol. 2009;29(2):235–41.

46. Koide M, Bonev AD, Nelson MT, Wellman GC. Inversion of neurovascular coupling by subarachnoid blood depends on large-conductance Ca^{2+}-activated K+ (BK) channels. Proc Natl Acad Sci U S A. 2012;109(21):E1387–95.
47. Sabri M, Ai J, Lakovic K, D'Abbondanza J, Ilodigwe D, Macdonald RL. Mechanisms of microthrombi formation after experimental subarachnoid hemorrhage. Neuroscience. 2012;224:26–37.
48. Friedrich B, Muller F, Feiler S, Scholler K, Plesnila N. Experimental subarachnoid hemorrhage causes early and long-lasting microarterial constriction and microthrombosis: an in-vivo microscopy study. J Cereb Blood Flow Metab. 2012;32(3):447–55.
49. Edvinsson L, Hogestatt ED, Uddman R, Auer LM. Cerebral veins: fluorescence histochemistry, electron microscopy, and in vitro reactivity. J Cereb Blood Flow Metab. 1983;3(2):226–30.
50. Allt G, Lawrenson JG. Pericytes: cell biology and pathology. Cells Tissues Organs. 2001; 169(1):1–11.
51. Takahashi A, Ushiki T, Abe K, Houkin K, Abe H. Cytoarchitecture of periendothelial cells in human cerebral venous vessels as compared with the scalp vein. A scanning electron microscopic study. Arch Histol Cytol. 1994;57(4):331–9.
52. Tso MK, Macdonald RL. Acute microvascular changes after subarachnoid hemorrhage and transient global cerebral ischemia. Stroke Res Treat. 2013;2013:425281.
53. Kulik T, Kusano Y, Aronhime S, Sandler AL, Winn HR. Regulation of cerebral vasculature in normal and ischemic brain. Neuropharmacology. 2008;55(3):281–8.
54. Kilic T, Akakin A. Anatomy of cerebral veins and sinuses. Front Neurol Neurosci. 2008;23:4–15.
55. Ostergaard L, Aamand R, Karabegovic S, et al. The role of the microcirculation in delayed cerebral ischemia and chronic degenerative changes after subarachnoid hemorrhage. J Cereb Blood Flow Metab. 2013;33(12):1825–37.
56. Li Q, Khatibi N, Zhang J. Vascular neural network: the importance of vein drainage in stroke. Transl Stroke Res. 2014;5(2):163–6.
57. Yemisci M, Gursoy-Ozdemir Y, Vural A, Can A, Topalkara K, Dalkara T. Pericyte contraction induced by oxidative-nitrative stress impairs capillary reflow despite successful opening of an occluded cerebral artery. Nat Med. 2009;15(9):1031–7.
58. Ishikawa M, Kusaka G, Yamaguchi N, et al. Platelet and leukocyte adhesion in the microvasculature at the cerebral surface immediately after subarachnoid hemorrhage. Neurosurgery. 2009;64(3):546–53. discussion 53–4.
59. Ansar S, Larsen C, Maddahi A, Edvinsson L. Subarachnoid hemorrhage induces enhanced expression of thromboxane A2 receptors in rat cerebral arteries. Brain Res. 2010;1316: 163–72.
60. Whiteside LA, McCarthy DS. Laboratory evaluation of alignment and kinematics in a unicompartmental knee arthroplasty inserted with intramedullary instrumentation. Clin Orthop Relat Res. 1992;274:238–47.
61. Maeda Y, Hirano K, Hirano M, et al. Enhanced contractile response of the basilar artery to platelet-derived growth factor in subarachnoid hemorrhage. Stroke. 2009;40(2):591–6.
62. Zhang ZD, Macdonald RL. Contribution of the remodeling response to cerebral vasospasm. Neurol Res. 2006;28(7):713–20.
63. Kawanabe Y, Masaki T, Hashimoto N. Involvement of phospholipase C in endothelin 1-induced stimulation of Ca++ channels and basilar artery contraction in rabbits. J Neurosurg. 2006;105(2):288–93.
64. Xie A, Aihara Y, Bouryi VA, et al. Novel mechanism of endothelin-1-induced vasospasm after subarachnoid hemorrhage. J Cereb Blood Flow Metab. 2007;27(10):1692–701.
65. Lan C, Das D, Wloskowicz A, Vollrath B. Endothelin-1 modulates hemoglobin-mediated signaling in cerebrovascular smooth muscle via RhoA/Rho kinase and protein kinase C. Am J Physiol Heart Circ Physiol. 2004;286(1):H165–73.
66. Josko J. Cerebral angiogenesis and expression of VEGF after subarachnoid hemorrhage (SAH) in rats. Brain Res. 2003;981(1–2):58–69.

67. Ding R, Chen Y, Yang S, et al. Blood-brain barrier disruption induced by hemoglobin in vivo: involvement of up-regulation of nitric oxide synthase and peroxynitrite formation. Brain Res. 2014;1571:25–38.
68. Keep RF, Zhou N, Xiang J, Andjelkovic AV, Hua Y, Xi G. Vascular disruption and blood-brain barrier dysfunction in intracerebral hemorrhage. Fluids Barriers CNS. 2014;11:18.
69. Katsu M, Niizuma K, Yoshioka H, Okami N, Sakata H, Chan PH. Hemoglobin-induced oxidative stress contributes to matrix metalloproteinase activation and blood-brain barrier dysfunction in vivo. J Cereb Blood Flow Metab. 2010;30(12):1939–50.
70. Hae Seo J, Guo S, Lok J, et al. Neurovascular matrix metalloproteinases and the blood-brain barrier. Curr Pharm Des. 2012;18(25):3645–8.
71. Roy CS, Sherrington CS. On the regulation of the blood-supply of the brain. J Physiol. 1890;11(1–2):85–158.17.
72. Lou HC, Edvinsson L, MacKenzie ET. The concept of coupling blood flow to brain function: revision required? Ann Neurol. 1987;22(3):289–97.
73. Hamel E. Perivascular nerves and the regulation of cerebrovascular tone. J Appl Physiol (1985). 2006;100(3):1059–64.
74. Longden TA, Dabertrand F, Hill-Eubanks DC, Hammack SE, Nelson MT. Stress-induced glucocorticoid signaling remodels neurovascular coupling through impairment of cerebrovascular inwardly rectifying K(+) channel function. Proc Natl Acad Sci U S A. 2014;111(20):7462–7.
75. Zonta M, Angulo MC, Gobbo S, et al. Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. Nat Neurosci. 2003;6(1):43–50.
76. Anderson CM, Nedergaard M. Astrocyte-mediated control of cerebral microcirculation. Trends Neurosci. 2003;26(7):340–4.
77. Besancon E, Guo S, Lok J, Tymianski M, Lo EH. Beyond NMDA and AMPA glutamate receptors: emerging mechanisms for ionic imbalance and cell death in stroke. Trends Pharmacol Sci. 2008;29(5):268–75.
78. Parri R, Crunelli V. An astrocyte bridge from synapse to blood flow. Nat Neurosci. 2003;6(1):5–6.
79. Bernard SA, Gray TW, Buist MD, et al. Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. N Engl J Med. 2002;346(8):557–63.
80. Sehba FA, Friedrich V. Early micro vascular changes after subarachnoid hemorrhage. Acta Neurochir Suppl. 2011;110(Pt 1):49–55.
81. Kamii H, Kato I, Kinouchi H, et al. Amelioration of vasospasm after subarachnoid hemorrhage in transgenic mice overexpressing CuZn-superoxide dismutase. Stroke. 1999;30(4):867–71. discussion 72.
82. Wellman GC, Koide M. Impact of subarachnoid hemorrhage on parenchymal arteriolar function. Acta Neurochir Suppl. 2013;115:173–7.
83. Nystoriak MA, O'Connor KP, Sonkusare SK, Brayden JE, Nelson MT, Wellman GC. Fundamental increase in pressure-dependent constriction of brain parenchymal arterioles from subarachnoid hemorrhage model rats due to membrane depolarization. Am J Physiol Heart Circ Physiol. 2011;300(3):H803–12.
84. Park IS, Meno JR, Witt CE, et al. Impairment of intracerebral arteriole dilation responses after subarachnoid hemorrhage. Laboratory investigation. J Neurosurg. 2009;111(5):1008–13.
85. Koide M, Bonev AD, Nelson MT, Wellman GC. Subarachnoid blood converts neurally evoked vasodilation to vasoconstriction in rat brain cortex. Acta Neurochir Suppl. 2013;115:167–71.
86. Jahromi BS, Aihara Y, Ai J, et al. Temporal profile of potassium channel dysfunction in cerebrovascular smooth muscle after experimental subarachnoid haemorrhage. Neurosci Lett. 2008;440(1):81–6.
87. Alexander MR, Owens GK. Epigenetic control of smooth muscle cell differentiation and phenotypic switching in vascular development and disease. Annu Rev Physiol. 2012;74:13–40.

88. Bertolino P, Deckers M, Lebrin F, ten Dijke P. Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders. Chest. 2005;128(6 Suppl):585s–90.
89. Kolyada AY, Riley KN, Herman IM. Rho GTPase signaling modulates cell shape and contractile phenotype in an isoactin-specific manner. Am J Physiol Cell Physiol. 2003;285(5):C1116–21.
90. Kutcher ME, Herman IM. The pericyte: cellular regulator of microvascular blood flow. Microvasc Res. 2009;77(3):235–46.
91. Hamilton NB, Attwell D, Hall CN. Pericyte-mediated regulation of capillary diameter: a component of neurovascular coupling in health and disease. Front Neuroenergetics. 2010;2: pii: 5.
92. Sehba FA. Rat endovascular perforation model. Transl Stroke Res. 2014;5(6):660–8.
93. Sehba FA, Friedrich V. Cerebral microvasculature is an early target of subarachnoid hemorrhage. Acta Neurochir Suppl. 2013;115:199–205.
94. Friedrich V, Flores R, Sehba FA. Cell death starts early after subarachnoid hemorrhage. Neurosci Lett. 2012;512(1):6–11.
95. Gules I, Satoh M, Nanda A, Zhang JH. Apoptosis, blood-brain barrier, and subarachnoid hemorrhage. Acta Neurochir Suppl. 2003;86:483–7.
96. Yan J, Li L, Khatibi NH, et al. Blood-brain barrier disruption following subarachnoid hemorrhage may be facilitated through PUMA induction of endothelial cell apoptosis from the endoplasmic reticulum. Exp Neurol. 2011;230(2):240–7.
97. Yan J, Chen C, Hu Q, et al. The role of p53 in brain edema after 24 h of experimental subarachnoid hemorrhage in a rat model. Exp Neurol. 2008;214(1):37–46.
98. Sehba FA, Friedrich Jr V, Makonnen G, Bederson JB. Acute cerebral vascular injury after subarachnoid hemorrhage and its prevention by administration of a nitric oxide donor. J Neurosurg. 2007;106(2):321–9.
99. Friedrich V, Flores R, Muller A, Sehba FA. Escape of intraluminal platelets into brain parenchyma after subarachnoid hemorrhage. Neuroscience. 2010;165(3):968–75.
100. Li Z, Liang G, Ma T, et al. Blood-brain barrier permeability change and regulation mechanism after subarachnoid hemorrhage. Metab Brain Dis. 2015;30(2):597–603.
101. Bonkowski D, Katyshev V, Balabanov RD, Borisov A, Dore-Duffy P. The CNS microvascular pericyte: pericyte-astrocyte crosstalk in the regulation of tissue survival. Fluids Barriers CNS. 2011;8(1):8.
102. Dalkara T, Alarcon-Martinez L. Cerebral microvascular pericytes and neurogliovascular signaling in health and disease. Brain Res. 2015;1623:3–17.
103. Armulik A, Genove G, Mae M, et al. Pericytes regulate the blood-brain barrier. Nature. 2010;468(7323):557–61.
104. Arima Y, Kamimura D, Sabharwal L, et al. Regulation of immune cell infiltration into the CNS by regional neural inputs explained by the gate theory. Mediators Inflamm. 2013;2013: 898165.
105. Kovac A, Erickson MA, Banks WA. Brain microvascular pericytes are immunoactive in culture: cytokine, chemokine, nitric oxide, and LRP-1 expression in response to lipopolysaccharide. J Neuroinflammation. 2011;8:139.
106. Friedrich V, Flores R, Muller A, Bi W, Peerschke EI, Sehba FA. Reduction of neutrophil activity decreases early microvascular injury after subarachnoid haemorrhage. J Neuroinflammation. 2011;8:103.
107. Wu L, Walas S, Leung W, et al. Neuregulin1-beta decreases IL-1beta-induced neutrophil adhesion to human brain microvascular endothelial cells. Transl Stroke Res. 2015;6(2): 116–24.
108. Chang CZ, Lin CL, Wu SC, Kwan AL. Purpurogallin, a natural phenol, attenuates high-mobility group box 1 in subarachnoid hemorrhage induced vasospasm in a rat model. Int J Vasc Med. 2014;2014:254270.
109. Merrill JE, Murphy SP. Inflammatory events at the blood brain barrier: regulation of adhesion molecules, cytokines, and chemokines by reactive nitrogen and oxygen species. Brain Behav Immun. 1997;11(4):245–63.

110. Harukuni I, Bhardwaj A. Mechanisms of brain injury after global cerebral ischemia. Neurol Clin. 2006;24(1):1–21.
111. Mracsko E, Javidi E, Na SY, Kahn A, Liesz A, Veltkamp R. Leukocyte invasion of the brain after experimental intracerebral hemorrhage in mice. Stroke. 2014;45(7):2107–14.
112. Zhou Y, Wang Y, Wang J, Anne Stetler R, Yang QW. Inflammation in intracerebral hemorrhage: from mechanisms to clinical translation. Prog Neurobiol. 2014;115:25–44.
113. Seifert HA, Pennypacker KR. Molecular and cellular immune responses to ischemic brain injury. Transl Stroke Res. 2014;5(5):543–53.
114. Wang J, Doré S. Heme oxygenase-1 exacerbates early brain injury after intracerebral haemorrhage. Brain. 2007;130(Pt 6):1643–52.
115. Xue M, Del Bigio MR. Intracerebral injection of autologous whole blood in rats: time course of inflammation and cell death. Neurosci Lett. 2000;283(3):230–2.
116. Wang J. Preclinical and clinical research on inflammation after intracerebral hemorrhage. Prog Neurobiol. 2010;92(4):463–77.
117. Yabluchanskiy A, Sawle P, Homer-Vanniasinkam S, Green CJ, Motterlini R. Relationship between leukocyte kinetics and behavioral tests changes in the inflammatory process of hemorrhagic stroke recovery. Int J Neurosci. 2010;120(12):765–73.
118. Zhao X, Grotta J, Gonzales N, Aronowski J. Hematoma resolution as a therapeutic target: the role of microglia/macrophages. Stroke. 2009;40(3 Suppl):S92–4.
119. Klebe D, McBride D, Flores JJ, Zhang JH, Tang J. Modulating the immune response towards a neuroregenerative peri-injury milieu after cerebral hemorrhage. J Neuroimmune Pharmacol. 2015;10(4):576–86.
120. Szymanska A, Biernaskie J, Laidley D, Granter-Button S, Corbett D. Minocycline and intracerebral hemorrhage: influence of injury severity and delay to treatment. Exp Neurol. 2006;197(1):189–96.
121. Wasserman JK, Schlichter LC. Minocycline protects the blood–brain barrier and reduces edema following intracerebral hemorrhage in the rat. Exp Neurol. 2007;207(2):227–37.
122. Jimin W, Shuxu Y, Guohua X, Guosheng F, Richard FK, Ya H. Minocycline reduces intracerebral hemorrhage-induced brain injury. Neurol Res. 2009;31(2):183–8.
123. Hu X, Leak RK, Shi Y, et al. Microglial and macrophage polarization—new prospects for brain repair. Nat Rev Neurol. 2015;11(1):56–64.
124. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol. 2011;11(11):723–37.
125. Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage M1–M2 polarization balance. Front Immunol. 2014;5:614.
126. Eggen BJL, Raj D, Hanisch UK, Boddeke HW. Microglial phenotype and adaptation. J Neuroimmune Pharmacol. 2013;8(4):807–23.
127. Stout RD, Jiang C, Matta B, Tietzel I, Watkins SK, Suttles J. Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. J Immunol. 2005;175(1):342–9.
128. Luzina IG, Keegan AD, Heller NM, Rook GA, Shea-Donohue T, Atamas SP. Regulation of inflammation by interleukin-4: a review of "alternatives". J Leukoc Biol. 2012;92(4):753–64.
129. Pisanu A, Lecca D, Mulas G, et al. Dynamic changes in pro- and anti-inflammatory cytokines in microglia after PPAR-gamma agonist neuroprotective treatment in the MPTPp mouse model of progressive Parkinson's disease. Neurobiol Dis. 2014;71:280–91.
130. Palmer TD, Willhoite AR, Gage FH. Vascular niche for adult hippocampal neurogenesis. J Comp Neurol. 2000;425(4):479–94.
131. Ohab JJ, Fleming S, Blesch A, Carmichael ST. A neurovascular niche for neurogenesis after stroke. J Neurosci. 2006;26(50):13007–16.
132. Ma DK, Bonaguidi MA, Ming GL, Song H. Adult neural stem cells in the mammalian central nervous system. Cell Res. 2009;19(6):672–82.
133. Alvarez-Buylla A, Garcia-Verdugo JM. Neurogenesis in adult subventricular zone. J Neurosci. 2002;22(3):629–34.

134. Lie DC, Song H, Colamarino SA, Ming GL, Gage FH. Neurogenesis in the adult brain: new strategies for central nervous system diseases. Annu Rev Pharmacol Toxicol. 2004;44:399–421.
135. Jiao J, Chen DF. Induction of neurogenesis in nonconventional neurogenic regions of the adult central nervous system by niche astrocyte-produced signals. Stem Cells. 2008;26(5): 1221–30.
136. Garcia ADR, Doan NB, Imura T, Bush TG, Sofroniew MV. GFAP-expressing progenitors are the principal source of constitutive neurogenesis in adult mouse forebrain. Nat Neurosci. 2004;7(11):1233–41.
137. Tsai PT, Ohab JJ, Kertesz N, et al. A critical role of erythropoietin receptor in neurogenesis and post-stroke recovery. J Neurosci. 2006;26(4):1269–74.
138. Shen Q, Goderie SK, Jin L, et al. Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science. 2004;304(5675):1338–40.
139. Ma DK, Ming GL, Song H. Glial influences on neural stem cell development: cellular niches for adult neurogenesis. Curr Opin Neurobiol. 2005;15(5):514–20.
140. Jojart I, Joo F, Siklos L, Laszlo FA. Immunoelectronhistochemical evidence for innervation of brain microvessels by vasopressin-immunoreactive neurons in the rat. Neurosci Lett. 1984;51(2):259–64.
141. Dore-Duffy P, LaManna JC. Physiologic angiodynamics in the brain. Antioxid Redox Signal. 2007;9(9):1363–71.
142. Xing C, Arai K, Park KP, Lo EH. Induction of vascular endothelial growth factor and matrix metalloproteinase-9 via CD47 signaling in neurovascular cells. Neurochem Res. 2010; 35(7):1092–7.
143. Fukushi J, Makagiansar IT, Stallcup WB. NG2 proteoglycan promotes endothelial cell motility and angiogenesis via engagement of galectin-3 and alpha3beta1 integrin. Mol Biol Cell. 2004;15(8):3580–90.
144. van Hinsbergh VW, Koolwijk P. Endothelial sprouting and angiogenesis: matrix metalloproteinases in the lead. Cardiovasc Res. 2008;78(2):203–12.
145. Zhao BQ, Tejima E, Lo EH. Neurovascular proteases in brain injury, hemorrhage and remodeling after stroke. Stroke. 2007;38(2 Suppl):748–52.
146. Zhao BQ, Wang S, Kim HY, et al. Role of matrix metalloproteinases in delayed cortical responses after stroke. Nat Med. 2006;12(4):441–5.
147. Hamano Y, Zeisberg M, Sugimoto H, et al. Physiological levels of tumstatin, a fragment of collagen IV α3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via αVβ3 integrin. Cancer Cell. 2003;3(6):589–601.
148. Lee SR, Kim HY, Rogowska J, et al. Involvement of matrix metalloproteinase in neuroblast cell migration from the subventricular zone after stroke. J Neurosci. 2006;26(13):3491–5.
149. Drewes LR. Making connexons in the neurovascular unit. J Cereb Blood Flow Metab. 2012;32(8):1455–6.
150. Attwell D, Buchan AM, Charpak S, Lauritzen M, MacVicar BA, Newman EA. Glial and neuronal control of brain blood flow. Nature. 2010;468(7321):232–43.
151. Dormanns K, van Disseldorp EMJ, Brown RG, David T. Neurovascular coupling and the influence of luminal agonists via the endothelium. J Theor Biol. 2015;364:49–70.
152. Shimamura N, Ohkuma H. Phenotypic transformation of smooth muscle in vasospasm after aneurysmal subarachnoid hemorrhage. Transl Stroke Res. 2014;5(3):357–64.
153. Hald ES, Alford PW. Smooth muscle phenotype switching in blast traumatic brain injury-induced cerebral vasospasm. Transl Stroke Res. 2014;5(3):385–93.
154. Alabadi JA, Torregrosa G, Salom JB, et al. Changes in the adrenergic mechanisms of cerebral arteries after subarachnoid hemorrhage in goats. Neurosurgery. 1994;34(6):1027–33. discussion 33–4.
155. Busija DW, Bari F, Domoki F, Louis T. Mechanisms involved in the cerebrovascular dilator effects of N-methyl-d-aspartate in cerebral cortex. Brain Res Rev. 2007;56(1):89–100.
156. Ma J, Ayata C, Huang PL, Fishman MC, Moskowitz MA. Regional cerebral blood flow response to vibrissal stimulation in mice lacking type I NOS gene expression. Am J Physiol. 1996;270(3 Pt 2):H1085–90.

157. Tabatabaei SN, Girouard H. Chapter 14—Nitric oxide and cerebrovascular regulation. In: Gerald L, editor. Vitamins & hormones, vol. 96. San Diego: Academic; 2014. p. 347–85.
158. Simard M, Arcuino G, Takano T, Liu QS, Nedergaard M. Signaling at the gliovascular interface. J Neurosci. 2003;23(27):9254–62.
159. Peng X, Carhuapoma JR, Bhardwaj A, et al. Suppression of cortical functional hyperemia to vibrissal stimulation in the rat by epoxygenase inhibitors. Am J Physiol Heart Circ Physiol. 2002;283(5):H2029–37.
160. Kusaka G, Ishikawa M, Nanda A, Granger DN, Zhang JH. Signaling pathways for early brain injury after subarachnoid hemorrhage. J Cereb Blood Flow Metab. 2004;24(8):916–25.
161. Xu T, Zhang WG, Sun J, et al. Protective effects of thrombomodulin on microvascular permeability after subarachnoid hemorrhage in mouse model. Neuroscience. 2015;299:18–27.
162. Chen Y, Zhang Y, Tang J, et al. Norrin protected blood-brain barrier via frizzled-4/beta-catenin pathway after subarachnoid hemorrhage in rats. Stroke. 2015;46(2):529–36.
163. Ding R, Feng L, He L, et al. Peroxynitrite decomposition catalyst prevents matrix metalloproteinase-9 activation and neurovascular injury after hemoglobin injection into the caudate nucleus of rats. Neuroscience. 2015;297:182–93.
164. Sozen T, Tsuchiyama R, Hasegawa Y, et al. Role of interleukin-1beta in early brain injury after subarachnoid hemorrhage in mice. Stroke. 2009;40(7):2519–25.
165. Zhan Y, Krafft PR, Lekic T, et al. Imatinib preserves blood-brain barrier integrity following experimental subarachnoid hemorrhage in rats. J Neurosci Res. 2015;93(1):94–103.
166. Simard JM, Schreibman D, Aldrich EF, et al. Unfractionated heparin: multitargeted therapy for delayed neurological deficits induced by subarachnoid hemorrhage. Neurocrit Care. 2010;13(3):439–49.
167. Huang CY, Wang LC, Shan YS, Pan CH, Tsai KJ. Memantine attenuates delayed vasospasm after experimental subarachnoid hemorrhage via modulating endothelial nitric oxide synthase. Int J Mol Sci. 2015;16(6):14171–80.
168. Pyne-Geithman GJ, Caudell DN, Cooper M, Clark JF, Shutter LA. Dopamine D2-receptor-mediated increase in vascular and endothelial NOS activity ameliorates cerebral vasospasm after subarachnoid hemorrhage in vitro. Neurocrit Care. 2009;10(2):225–31.
169. Momin EN, Schwab KE, Chaichana KL, Miller-Lotan R, Levy AP, Tamargo RJ. Controlled delivery of nitric oxide inhibits leukocyte migration and prevents vasospasm in haptoglobin 2-2 mice after subarachnoid hemorrhage. Neurosurgery. 2009;65(5):937–45. discussion 45.
170. Sehba FA, Ding WH, Chereshnev I, Bederson JB. Effects of S-nitrosoglutathione on acute vasoconstriction and glutamate release after subarachnoid hemorrhage. Stroke. 1999; 30(9):1955–61.
171. Sun BL, Zhang SM, Xia ZL, et al. L-arginine improves cerebral blood perfusion and vasomotion of microvessels following subarachnoid hemorrhage in rats. Clin Hemorheol Microcirc. 2003;29(3–4):391–400.
172. Mani BK, O'Dowd J, Kumar L, Brueggemann LI, Ross M, Byron KL. Vascular KCNQ (Kv7) potassium channels as common signaling intermediates and therapeutic targets in cerebral vasospasm. J Cardiovasc Pharmacol. 2013;61(1):51–62.
173. Zhao X, Sun G, Ting SM, et al. Cleaning up after ICH: the role of Nrf2 in modulating microglia function and hematoma clearance. J Neurochem. 2015;133(1):144–52.
174. Iniaghe LO, Krafft PR, Klebe DW, Omogbai EK, Zhang JH, Tang J. Dimethyl fumarate confers neuroprotection by casein kinase 2 phosphorylation of Nrf2 in murine intracerebral hemorrhage. Neurobiol Dis. 2015;82:349–58.
175. Zhao X, Aronowski J. Nrf2 to pre-condition the brain against injury caused by products of hemolysis after ICH. Transl Stroke Res. 2013;4(1):71–5.
176. Ostrowski RP, Zhang JH. Hyperbaric oxygen for cerebral vasospasm and brain injury following subarachnoid hemorrhage. Transl Stroke Res. 2011;2(3):316–27.
177. Siler DA, Berlow YA, Kukino A, et al. Soluble epoxide hydrolase in hydrocephalus, cerebral edema, and vascular inflammation after subarachnoid hemorrhage. Stroke. 2015;46(7): 1916–22.

178. Zhao XD, Zhou YT, Lu XJ. Sulforaphane enhances the activity of the Nrf2-ARE pathway and attenuates inflammation in OxyHb-induced rat vascular smooth muscle cells. Inflamm Res. 2013;62(9):857–63.
179. Maddahi A, Ansar S, Chen Q, Edvinsson L. Blockade of the MEK/ERK pathway with a raf inhibitor prevents activation of pro-inflammatory mediators in cerebral arteries and reduction in cerebral blood flow after subarachnoid hemorrhage in a rat model. J Cereb Blood Flow Metab. 2011;31(1):144–54.
180. Ansar S, Edvinsson L. Subtype activation and interaction of protein kinase C and mitogen-activated protein kinase controlling receptor expression in cerebral arteries and microvessels after subarachnoid hemorrhage. Stroke. 2008;39(1):185–90.
181. Yan J, Manaenko A, Chen S, et al. Role of SCH79797 in maintaining vascular integrity in rat model of subarachnoid hemorrhage. Stroke. 2013;44(5):1410–7.
182. Kokkoris S, Andrews P, Webb DJ. Role of calcitonin gene-related peptide in cerebral vasospasm, and as a therapeutic approach to subarachnoid hemorrhage. Front Endocrinol (Lausanne). 2012;3:135.
183. Scherer EQ, Yang J, Canis M, et al. Tumor necrosis factor-alpha enhances microvascular tone and reduces blood flow in the cochlea via enhanced sphingosine-1-phosphate signaling. Stroke. 2010;41(11):2618–24.

The Role of Nonneuronal Nrf2 Pathway in Ischemic Stroke: Damage Control and Potential Tissue Repair

Tuo Yang, Yang Sun, and Feng Zhang

Ischemic stroke is one of the leading causes of disability and mortality in the world. Multiple pathological processes participate in the progression of stroke, including excitotoxicity, oxidative stress, inflammation, mitochondrial dysfunction, etc. Excessive oxidative stress is one of the most important pathogenic mechanisms in stroke. Therefore, therapeutic strategies against oxidative stress may be feasible for the treatment of stroke. Given that nuclear factor erythroid 2-related factor 2 (Nrf2) pathway is the predominant antioxidant system, we will focus on the promising protective role of Nrf2 pathway against ischemic stroke.

1 Introduction of Nrf2 Pathway

Nrf2 is a member of the basic leucine zipper (bZIP) transcription factor family featuring a Cap 'n' collar structure [1, 2], and it is essential for the transcriptional induction of phase II drug-metabolizing and antioxidant enzymes, such as glutathione s-transferase (GST), heme oxygenase 1 (HO-1), and NADPH quinine oxidoreductase 1 (NQO-1) [3]. Nrf2 regulates gene transcription of these enzymes by binding the antioxidant response elements (AREs) on their promoters.

T. Yang, M.D. • Y. Sun, M.D. • F. Zhang, M.D., Ph.D. (✉)
Department of Neurology, University of Pittsburgh School of Medicine,
200 Lothrop Street, Pittsburgh, PA 15213, USA
e-mail: tuo.yang@pitt.edu; suny3@upmc.edu; zhanfx2@upmc.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_18

1.1 Regulation of Nrf2/ARE Pathway

Nrf2 has six functional domains known as Nrf2-ECH homologies (Neh), designated as Neh1-6. Each Neh domain has its own function [4]. Among them, the Neh1 domain is the CNC-bZIP domain, enabling Nrf2 to form heterodimer with the ZIP domain of small musculoaponeurotic fibrosarcoma (Maf) proteins [5]; and the Neh2 domain mediates binding with the cytosolic repressor of Nrf2. Keap1 encompasses three functional domains: a bric-a-brac (BTB) domain, an intervening region (IVR), and a Kelch domain (also named DGR domain). Under non-stressed conditions, Nrf2 forms a complex with Kelch-like ECH-associated protein 1 (Keap1), which promotes rapid proteasomal degradation of Nrf2 by proteasome, giving Nrf2 a half-life time of about 20 min [3]. As a result, Keap1 functions to inhibit Nrf2.

It has been reported that alterations in the structure of Keap1 leads to dissociation of the Nrf2–Keap1 complex, and this is essential for Nrf2/ARE activation [5]. Three cysteine residues (Cys151, Cys273, and Cys288) in Keap1 are essential for Keap1 repression of Nrf2 activity under unstressed conditions [6]. The oxidation of these cysteine residues causes conformational change of Keap1 and leads to dissociation of the Nrf2-Keap1 complex. Some oxidative products can free Nrf2 from the complex and increase the expression of phase II genes [7].

Transcription factors need to translocate to the nucleus in order to transactivate. As for Nrf2, this process is quite rapid: Nrf2 can accumulate in the nucleus within 15 min after tert-Butylhydroquinone (t-BHQ) treatment [8]. The key mediators that regulate nuclear import and export of transcription factors are the nuclear localization signals (NLS) and nuclear export sequences (NES). A number of such nuclear shuttling signals have been identified on Nrf2, including three NLS motifs and two NES motifs. The direction of Nrf2 movement is determined by a homeostatic balance between import and export driving forces. Under normal condition, Nrf2 stays in the cytosol because the import force is less than the export force while NES1 is functional. Under oxidative stress, Cys183 of NES1 is adducted and NES1 loses function. Then the import force is more than the export force, resulting in Nrf2 nuclear translocation [9]. Additionally, Nrf2 nuclear translocation can also be triggered when phosphorylated by kinases. The phosphorylation sites of protein kinase C (PKC), glycogen synthase kinase-3 and Fyn have been identified. The phosphorylation site of PKC is Ser40 in Neh2 domain [10], but its role in Nrf2 nuclear shuttling is controversial. Fyn phosphorylates Tyr568 in the Neh3 domain, controlling nuclear export of Nrf2 [11, 12].

After translocating into the nucleus, Nrf2 can form heterodimer with Maf proteins [13], which further enhances the specificity of Nrf2 to bind to a *cis*-acting enhancer ARE [14] located at the promoter of phase II genes [1, 15]. Subsequently, the transcription of phase II gene is initiated.

Recent studies have also proposed a Keap1-independent ubiquitination model of Nrf2 degradation [16]; in which, GSK3β is a key player because it can phosphorylate Nrf2 at Ser342 and Ser347 located at Neh6. Phosphorylated Neh6 can bind with an ubiquitin ligase adaptor beta-transducin repeat-containing protein (β-TrCP). β-TrCP is a scaffolding protein that directly links Nrf2 to the Cullin1/Rbx1 ubiqui-

tination complex. Therefore, GSK-3β-mediated phosphorylation of Neh6 causes the ubiquitination and degradation of Nrf2 via β-TrCP. This model is supported by the stabilization of Nrf2 by GSK-3β inhibitors in Keap1$^{-/-}$ mouse embryo fibroblasts (MEFs) [12]. Additionally, cancer-chemo preventive agent nordihydroguaiaretic acid can activate Nrf2 and increase HO-1 protein levels through inhibiting GSK-3β phosphorylation in Keap1$^{-/-}$ MEFs [17].

Additionally, there are some other regulatory mechanisms to prevent excessive activation of Nrf2. For example, AREs are located in the promoter region of Cul3, Rbx1, and Keap1 genes; while activation of Nrf2/ARE pathway boots the expression of phase II genes, it also upregulates these inhibitory proteins at the same time. This negative feedback loop is known as an autoregulatory arm of the Nrf2/ARE pathway [18]. In addition, the Keap1-Cul3-Rbx1 complex can enter the nucleus, mediated by prothymosin α (ProTα), a Keap1-binding protein with a NLS. As a result, 10–15% of Keap1-Cul3-Rbx1 complex is localized in the nucleus. Once entering the nucleus, the Keap1-Cul3-Rbx1 complex drops ProTα and binds Nrf2, leading to the ubiquitination and degradation of nuclear Nrf2 [19].

1.2 Inducers and Effectors of Nrf2 Pathway

It is important to identify inducers and effectors of Nrf2 pathway, because they could be promising therapeutic targets. As discussed above, Keap1 is the major inhibitor of Nrf2 activation; changes in its structure and/or its dissociation from Nrf2 results in the release and activation of Nrf2, which is recognized as the target mechanisms of nearly all Nrf2 inducers. There are several ways to classify Nrf2 inducers. Based on their origin, inducers are divided into two classes, the exogenous and the endogenous; based on their chemical structure, inducers are divided into ten groups [20]; based on the Keap1 domains that the inducers react with, they can be divided into four categories [21].

Currently, over 200 Nrf2/ARE-driven proteins are described for detoxification and antioxidant defense. Among which, phase 2 enzymes initially caught the interest of scientists for their preventative action against carcinogens [22]. Later, their function has been expanded to neuroprotection [23, 24]. Table 1 lists the major cytoprotective enzymes that participate in protection against neuronal injury in common neurological diseases.

2 Nrf2 Provides Neuroprotection Against Ischemic Stroke Via Self-Defense and Cell–Cell Interaction

In ischemic stroke, several pathological mechanisms known as the ischemic cascade are triggered, and consequently cause rapid and irreversible neuronal death within the ischemic core due to severe and rapid loss of energy. In the surrounding hypoperfused brain tissues, known as the penumbral areas, the neurons may be

Table 1 Effectors of Nrf2 pathway

	Enzymes	Action mechanisms
Thioredoxin enzyme system	Thioredoxins	Reduction oxidizing proteins via the exchange between cysteine thiol and protein disulfides
	Thioredoxin reductase	Reduction of oxidized thioredoxins by consuming NADPH
	Peroxiredoxins	Catalyzing the reduction of peroxides by utilizing thioredoxins
	Sulfiredoxins	Reduction of the sulfinic acid phosphoric ester on oxidize peroxiredoxins
Glutathione system	GSH	Scavenging multiple oxidative species; Serving as a reservoir for cysteine
Transferase	GSH-S-transferase	Transference of GSH
	N-Acetyltransferase	Transfer of an acetyl group to an arylamine
	Sulfotransferase	Transferring a sulfo group to an alcohol or amine
Detoxifying enzymes	HO-1	Breakdown of heme; Generation of antioxidants
	NQO1	Catalyzing the two-electron reduction of quinone to the redox stable hydroquinone

potentially salvageable due to relatively moderate or mild energy loss. Therefore, salvaging the neurons in penumbra is of great translational significance. To do so, multiple protection and supports may be needed, because neuronal death in penumbra is not a stand-along event, instead, it is the consequence of neuronal injury itself plus dysfunction of all kinds of cells within the neurovascular unit (NVU), including endothelial cells, astrocytes, and others [25–27].

2.1 Oxidative Stress and Nrf2 Activation in Neurons After Brain Ischemia

Following ischemia and reperfusion, the cascade of events that leads to neuronal deaths can be summarized as below [28–30]. Energy failure is the first event of the cascade caused by decreased ATP production, the latter then disrupts Na^+/K^+-ATPase, Ca^{2+}/H^+-ATPase, and Na^+/Ca^{2+}-transporter, leading to depolarization of cellular membrane. After depolarization, excitotoxicity amino acids, especially glutamate, are released into the synaptic cleft, which consequently activates *N*-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and metabotropic glutamate receptors increasing intracellular Ca^{2+} load. A variety of Ca^{2+} dependent enzymes are then activated and hence induce protein phosphorylation, proteolysis, mitochondrial damage, and the generation of free radical, i.e., reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS eventually lead to oxidative stress, which cause lipid peroxidation, membrane injury, DNA damage and moreover, allowing the infiltration of

leukocytes and activating inflammation [31]. Multiple neuroinflammatory cascades are also activated, inducing secondary brain injury that leads to cell death [32].

During the procedure of neuronal death in the penumbra, oxidative stress triggered by ROS and RNS generation is considered the "key" event. The predominant ROS and RNS produced are superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), nitric oxide ($^{\bullet}NO$), peroxynitrite anion ($ONOO^-$), and nitrogen dioxide ($^{\bullet}NO_2$) [28]. Ischemic insults produce an excessive amount of free radicals especially in the reperfused regions, and the imbalance in the formation and clearance of ROS and RNS leads to oxidative stress, and subsequently alters the cell dynamics [33] and impacts major cellular components [26]. Complex signaling network involving numerous survival/death pathways have been reported to be redox mediated [34]. ROS and perhaps RNS, may act directly as executioners of cell death [31]. ROS leads to increase of mitochondrial permeability transition pore which subsequently causes mitochondrial swelling and neuronal death [35]. ROS also initiates apoptosis through activating p53 and p38 MAPK [36]. Another damage effect acted by ROS to ischemic brain is that ROS can directly increase the permeability of the blood–brain barrier (BBB) [37]. Focal ischemia induces a potent inflammatory response within a few hours after onset of ischemia, and BBB disruption allows various blood immune cells infiltrate into ischemic area [38–40]. These migrated immune cells, along with injured brain cells, produce inflammatory mediators, enhancing neuronal death [41].

The brain is sensitive to oxidative stress. Constituting only 2 % of the total body weight, the brain uses 20 % of the total oxygen consumed by the whole body, making the brain the major oxygen user and probably a major source of ROS [28]. In addition, a high level of unsaturated lipid content, multiple chemical reactions in dopamine oxidation and high concentrations of iron also contribute ROS generation in the brain [42, 43]. To defense against oxidative stress, both direct and indirect antioxidants are needed [44–46]. Direct antioxidants denote a group of low molecular weight compounds that can directly undergo redox reactions and scavenge reactive oxidation products, ROS and RNS. This group includes glutathione (GSH), ascorbate, tocopherols, lipoid acid, carotenes, and ubiquinones. Direct antioxidants are consumed in the process, therefore need replenishment or regeneration. Indirect antioxidants, however, may not present the redox activity themselves. They exert their antioxidant effects through the upregulation phase 2 and antioxidant enzymes (Table 1), or so-called "phase 2 response" [5, 47, 48], which display a wide variety of antioxidant activities.

Several studies suggest that ROS is generated in the brain after ischemia. Peters et al. reported that rats subjected to middle cerebral artery occlusion (MCAO) first exhibited a short period (10–30 min) of decreased ROS level after the occlusion, followed by a significant increase (to 162 ± 51 %; baseline = 100 %) from 100-min ischemia to permanent MCAO onward [49]. Moreover, reperfusion after 1 h of MCAO led to a burst-like pattern of ROS production (about fivefold), and lasted at least to 3 h post MCAO. Liu et al. studied the spatial distribution of ROS in ischemic rat brain and found that ROS was significantly increased in the infarct core during

both ischemia and reperfusion, but in penumbra ROS was only increased during reperfusion [50, 51].

Nrf2 pathway is activated in post-ischemic stroke. Tanaka et al. [52] explored the expression of Nrf2 and Keap1 in mice subjected to MCAO using immunohistochemistry and immunofluorescence. Their results showed that, Keap1 was significantly decreased from 24 to 72 h post MCAO, paralleled with increased expression of Nrf2. In consistency with previous results, rats subjected to MCAO also exhibited significantly upregulated Nrf2 expression 24 h later, predominantly located in the penumbra compared to the core [53]. Li et al. [54] detected Nrf2 levels in rat brain by immunohistochemistry and western blotting analyses after MCAO and reperfusion, and found that, Nrf2 and hemeoxygenase-1 (HO-1) were upregulated in ischemic cortex, beginning at 6 h, peaking at 48 h and declining at 72 h after reperfusion. With a novel immunohistochemical technique, Srivastava et al. [55] examined the temporal and spatial distribution of Nrf2 in rat brain following ischemia-reperfusion injury in a detailed way. They used the nuclear/cytoplasmic Nrf2 ratio to indicate the level of Nrf2 activation of Nrf2 pathway, and demonstrated that nuclear to cytoplasmic Nrf2 ratios in stroke-affected regions were increased after 24 h and then declined after 72 h reperfusion. Moreover, Nrf2 expression was significantly higher in the penumbra than the infarct core. Oligemia is another model used to study the stroke penumbra, in which lipid peroxidation induces an increased oxidative stress and an augmented $^{\bullet}OH$ production during the reperfusion phase. Liverman et al. [56] adopted this model in mice by lowering the mean arterial pressure to 30–40 mmHg, and reported a significant elevation of Nrf2 level in neurons in the Purkinje cells of the cerebellar cortex and pyramidal neurons of the cingulate cortex.

In short, piles of evidence verify the indispensable role of Nrf2 pathway as a neuronal endogenous protective mechanism through the induction of its downstream antioxidants, including the direct (small molecular antioxidants) and indirect (predominant phase 2 enzyme) ones. These antioxidants then provide a cytoprotective role against ischemia-induced neuronal death.

2.2 Nrf2 Activations in Nonneuronal Cells: The Contribution of Cell–Cell Interaction

As indicated previously, the neuronal death in ischemic stroke is a not a separate procedure; instead, it is caused by additional dysfunction of other types of cells within and beyond the NVU due to complicated cell–cell interactions. Diverse types of cells in the brain work as a unit to maintain the central nervous system (CNS) homeostasis. This can partially explain the reason why neuroprotection trials have mostly failed, as any disruption within the complicated system might lead to NVU dysfunction after brain ischemia [57]. In this regard, therapeutic strategies should not only aim to neurons, but also to salvage other cells and hence restore function of the NVU [26, 29, 57].

Dual-direction responses have been reported between neurons and endothelial cells in the brain. During brain development, microvessel and neuron are already arranged to grow together along the extracellular matrix paths [58–60]. In adult brain, the regulation of regional cerebral blood flow depends on the activity of neurons [61]. Zonta et al. reported that, in a rat hyperemia model, neuronal afferent stimulation mediates the dilation of cerebral arterioles, which is dependent on the glutamate-mediated $[Ca^{2+}]_i$ oscillations in astrocytes [25, 62]. On the other hand, some novel discoveries focusing on microvessel structure, endothelial cells, and astrocyte endothelial adhesion indicated an opposite regulatory direct, from microvessel to the neurons they supply [63, 64]. An interesting research by Mabuchi et al. showed that an ordered and sequential microvessel–neuron relationship existed in contralateral basal ganglia; in ischemic area, neurons more distant from their nearest microvessel are more sensitive to ischemia, indicating that neuronal survival is dependent on the microvascular function [65]. Besides, microglias and macrophages play specific roles in the ischemic brain, functioning as a "double-edged sword" by either cleaning up or inducing local inflammation [66, 67]. In the following context, we will discuss the contribution of non-neuronal Nrf2 pathways to neuronal survival.

2.2.1 Nrf2 Pathway in Astrocyte

Comparing to neurons, astrocytes produce large amount of antioxidants [45, 68], and several lines of evidence have demonstrated that astrocytic Nrf2 contributes to neuronal survival following brain ischemia. Kraft and colleagues reported that the activation of astrocytic Nrf2 protected neurons from hydrogen peroxide and glutamate in cell cultures [69]. This protection disappeared in cultures from Nrf2 knockout animals; and the protection could be restored by infecting cells with a replication-deficient adenovirus that carried Nrf2, indicating an important role of astrocytic Nrf2 in neuronal protection. Shih et al. also studied the astrocyte–neuron interaction using a co-culture model of neurons, naïve astrocytes, and infected astrocytes (Nrf2 overexpression cells) [70]. They reported that astrocytes have higher basal Nrf2 expression and ARE activity than neurons, with an increased expression of GSH. When stimulated, the co-culture with oxidative glutamate toxicity, they found that Nrf2 activation led to an increase in both media and intracellular GSH in astrocytes; experiments on selective inhibition of glial GSH synthesis indicated that an Nrf2-dependent increase in glial GSH synthesis was both necessary and sufficient for the protection of neurons [70]. It is not fully understood how astrocytic GSH protects neurons [71]. It has been reported that GSH can scavenge free radicals in the extracellular area [72]. In addition, GSH can be hydrolyzed on the external surface of astrocytes and thus provide high yield of cysteine and glycine, which can be taken up by neurons and used for intracellular GSH synthesis [73].

Astrocytic Nrf2 also contributes to neuronal protection in a mitochondria-dependent manner [74, 75]. 3-nitropropionic acid (3NP) and malonate are two mitochondrial

complex II inhibitors, and they can kill neurons probably by inducing ROS generation in mitochondria. Calkins et al. reported that Nrf2-deficient astrocytes and mice were more vulnerable to 3NP or malonate; and they also found that astrocytes showed increased ARE-regulated transcription [76]. If Nrf2-overexpressing astrocytes were transplanted into the brain before 3NP or malonate treatments, a dramatic protection was noticed against complex II inhibition [76]. To extend these findings, they developed a line of transgenic mice with astrocyte-specific overexpression of Nrf2, and found the transgenic mice was resistant to malonate insults, which was associated with elevation of Nrf2-driven genes such as NQO1, GCLM, and HO-1. Furthermore, they showed that malonate toxicity could also be reduced by striatal transplantation of neuroprogenitor cells overexpressing Nrf2, which differentiated into astrocytes after grafting [77].

Astrocytic Nrf2 pathway was also reported to play a positive role in brain protection following ischemic preconditioning [78]. Bell et al. reported that Nrf2 could be activated by mild oxidative stress in both rodent and human astrocytes, and that transient ischemic conditions in vitro and in vivo cause an increase in the expression of Nrf2 target genes, especially the GSH system [78]. Astrocytic Nrf2 also contributes to chemical preconditioning induced by resveratrol [79]. Nrf2 pathway was found activated in astrocytes by resveratrol, and loss of Nrf2 reduced resveratrol -mediated neuroprotection in mice. After resveratrol treatment, both wild-type and $Nrf2^{-/-}$ cortical mitochondria produced ROS, and $Nrf2^{-/-}$ cells showed decreased mitochondrial antioxidant expression and failed to elevate cellular antioxidants after preconditioning. These data suggest that astrocytic Nrf2 pathway is critical in preconditioning-induced neuroprotection [79].

It has also been reported that astrocytes can protect endothelial cells in a GSH-dependent manner. Schroeter et al. reported that astrocytic cultures showed higher antioxidative activity than endothelial cultures, indicated by increased levels of SOD, catalase, and glutathione peroxidase [80]. When cultured these types of cells together, they found an increased antioxidative capacity in endothelial cells. In another report, Laird et al. reported that hemin could induce apoptosis in mouse and human endothelial cells; and that astrocyte-conditioned media could rescue the apoptotic endothelia death, in which GSH played a key role as the protection was aborted by prior treatment of astrocyte with DL-buthionine (S, R)-sulfoximine, a GSH-depleting agent [81].

2.2.2 Nrf2 Pathway in Endothelial Cell

In term of structure, endothelial cells are the center of NVU, as they play important roles in nourishing surrounding cells and keeping the integrity of BBB and NVU. Under pathological conditions, however, endothelial cells may generate ROS that may induce neuronal cell death [82]. When their ROS reacts with nitric oxide (NO), peroxynitrite, a much more powerful oxidant than ROS, will form [83, 84]. In addition to damaged surrounding structures, those ROS and RNS will cause the

disruption of BBB integrity following ischemia, in the forms of increased passive diffusion or massive cellular infiltration [85, 86].

As a way of self-defense, endothelial cells equip themselves with Nrf2 system, and several studies suggest that Nrf2 and its target enzymes protect endothelial cells from oxidative insults and sustain BBB integrity following brain ischemia. Alfieri et al. [87] pretreated rats with sulforaphane and then subject them to MCAO followed by reperfusion. They found sulforaphane significantly increased HO-1 expression in brain microvessel and that BBB disruption was attenuated after stroke. Bénardais et al. detected the protective effects of dimethylfumarate (DMF), an Nrf2 activator, on BBB integrity and found that both DMF and its primary metabolite monomethylfumarate (MMF) activated Nrf2 pathway and upregulated NQO1 in brain endothelial cells [88]. Moreover, DMF can partially attenuate TNF-α-induced downregulation of tight junction (TJ) protein [88]. DMF may also protect endothelia cells by stabilizing the BBB via preventing TJ disruption and suppressing the activity of matrix metallopeptidase (MMP) [89]. A similar protective effect was reported by Wu et al. [90] with an oral administration of procyanidin B2, a dietary Nrf2 inducer, 3 h after MCAO. They found that procyanidin B2 significantly reduced the infarct volume, brain edema, and neurological deficits; moreover, they also noticed increased levels of TJ proteins in the brain microvessel and sustained BBB integrity indicated by Evans blue leakage. Nrf2 activation may underlie the observed protections as these protections are associated with increased expression of HO-1, NQO1, and GSTα [90].

In addition to ischemic stroke, BBB protection by Nrf2 pathway is also reported in other CNS disorders, such as subarachnoid hemorrhage (SAH) and traumatic injuries to brain and spinal cord.

SAH is another form of stroke with noticeable vasospasm and subsequent ischemia for several days, and BBB disruption is common after SAH that can result in further brain injury. It has been reported that Nrf2-ARE pathway was activated after SAH, and administration of Nrf2 inducers, such as sulforaphane [91] or t-BHQ [92, 93] can significantly attenuate BBB disruption induced by SAH. In addition to these Nrf2 inducers, some other neuroprotective agents can ameliorate BBB disruption through activating Nrf2 and upregulating HO-1, NAD(P)H, NQO-1; such agents include astaxanthin [94], propofol [95], melatonin [96], and recombinant human erythropoietin [97]. On the other hand, Nrf2 knockout exacerbated brain injury after SAH with increased brain edema, BBB deficits, and decreased GSH [98].

In traumatic brain injury, sulforaphane was reported to preserve BBB function in association with reduced loss of TJ protein and endothelial markers [99]. This protection disappeared in Nrf2 knockout mice or when the animals were pretreated with decoy oligonucleotides containing the binding site of Nrf2 [99]. Similar phenomenon was reported in animals subjected to spinal cord injury. For instance, t-BHQ could alleviate spinal cord edema and suppress the expression of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) [100]. $Nrf2^{-/-}$ mice showed more severe edema after spinal cord injury, which is associated with increased levels of MMP-9 and TNF-α probably caused by an

increase in BBB disruption [101]. These studies suggest that Nrf2 pathway contributes to endothelial protection in broad-spectrum conditions.

2.2.3 Nrf2 Pathway in Microglia/Macrophage

For a long time, the brain has been regarded as an "immune privileged" organ for its lack of classical immune response [102, 103]. However, it has become increasingly clear that the brain is actually an immune organ, especially with the discovery of lymphatic vascular system in the brain [104–106], suggesting that complicated interactions may exist between CNS and the immune system.

Microglias are CNS residential immune cells which share a lot of features with monocytes and microphages and provide innate immune responses. They need to consume energy for a broad range of activities, and are thus highly sensitive to energy deficits and local changes in blood perfusion [67]. It is not surprising that microglias are among the first cells to respond to CNS injuries. For example, they are mobilized within 1 h after ischemic stroke, start to proliferate within 72 h, and continue to accumulate for >1 month. The function of microglias in stroke is recognized as a "double-edged sword" [66, 104]; they release cytotoxic cytokines that can kill the other cells including neurons at acute stage after ischemia, but they clear cellular debris via phagocytosis and autophagy and restrict the inflammation at late stage. Therefore, it may be critical to fine-tune the function of microglias and macrophages in order to limit brain damage and enhance restoration functionally and partially structurally following stroke. We will briefly discuss the perspectives of activating microglial Nrf2 pathway in the modulation of inflammatory response, autophagy, and phagocytosis after ischemic stroke.

Nrf2 Suppresses Microglial Inflammatory Response

It is widely accepted that inflammatory processes play a pivotal role in neuronal death, especially the delayed one that may last for days and even weeks after ischemic stroke [107]. Microglias and macrophages mediate inflammatory response by releasing pro-inflammatory cytokines such as IL-1β and TNF-α and by releasing other factors such as cyclooxygenase-2 (COX-2), NO and MMPs [107, 108].

Foresti et al. screened 56 small molecules that are potent Nrf2 activators, including t-BHQ, and carnosol, and showed that activation of Nrf2 could significantly inhibit LPS-induced inflammation in mice microglia-like cell line BV2 cells, which could be abrogated by transfection of Nrf2 shRNA and HO-1 shRNA [109]. Dilshara and coworkers reported that α-viniferin could reduce LPS-induced NO and COX-2 production in BV-2 cells, which could also abrogated by Nrf2-siRNA transfection [110]. In addition, several other Nrf2 activators, including adenosine [111], β-lapachone [112], KCHO-1 [113] and tissue inhibitor of metalloproteinase-2 [114], could also reduce inflammatory responses in association with Nrf2 activation and HO-1 upregulation. It is not clear how Nrf2 activation can inhibit inflammatory

response. While Foresti [109] and Dilshara's findings [110] may suggest a direct link between Nrf2 pathway and the inhibition of inflammation, findings of other studies may need to be further investigated.

Nrf2 Pathway in Microglial Autophagy

Autophagy is a lysosome-mediated degradation process for non-essential or damaged cellular constituents, which involves more than 30 autophagy-related proteins (ATGs) and 50 lysosomal hydrolases [115]. Physiologically, autophagy also contributes to preserve homeostasis through the removal of unwanted or damaged mitochondria as well as other organelles. Three classes of autophagy have been described based on how protein substrates for degradation reach the lumen of lysosome [115]. (i) Macroautophagy, which is generally referred to as autophagy, is mediated by the formation of double-membrane vesicles known as autophagosomes. When autophagosomes mature, they fuse with and deliver their contents for degradation. (ii) Microautophagy is a process in which lysosomes directly engulf the cytoplasmic material to degrade. (iii) Chaperone-mediated autophagy is more complex and specific, which involves the recognition by the heat shock cognate protein 70 (HSC70)-containing complex. In brief, the target protein must contain the recognition site for HSC70 complex, which will allow it to bind to this chaperone, leading to the formation of the substrate/chaperone complex. The complex will then move to the lysosomal membrane and enter the cell, whereby get degraded by the lysosome. Among the three classes mentioned above, macroautophagy is the most common one and will be our focus in this section.

The overall mechanism of autophagic regulation is almost clear now. Generation of the autophagosomal structure requires the beclin-1-class III PI3K complex and the generation and insertion of light chain 3 (LC3, also known as Atg8)-II complex into the autophagosomal membrane. The Atg genes control autophagosome formation through Atg12-Atg5 and LC3-II complex [116]. Atg12 is conjugated to Agt5 in a ubiquitin-like reaction, which requires Atg7 and Atg10 as enzymes of the reaction. The Atg12-Atg5 conjugate then interacts with Atg16, and together form a large complex with beclin-1-class III PI3K. The generation of LC3-II complex requires Atg3, Agt4, and Atg7 as enzymes of a ubiquitin-like reaction. Sequestosome 1 (SQSTM1, also known as p62), an "adaptor" molecule in selective autophagy, helps the attachment of LC3-II complex to the autophagosome membrane.

There are at least two mechanisms via which Nrf2 pathway regulates autophagy. First, the Atg proteins mentioned above are sensitive to redox signaling, thus redox status regulates autophagy [117]. It is not surprised that Nrf2 can regulate autophagy through regulating the redox status. In addition, Nrf2 has also been reported to upregulate the expression of Sestrin 2 which acts to scavenge ROS and promote autophagy, through enhancement of Sestrin 2 promoter activity [118]. The other mechanisms involve the interaction of Nrf2 with p62. As a matter of fact, positive feedback loop exists between p62 and Nrf2 pathway. It is reported that Nrf2 pathway directly enhances macrophage autophagy through promoting p62 expression

[119, 120], because p62 is a target gene of Nrf2 [121, 122]. Nrf2 is also necessary in p62 aggregation. Using mouse RAW264.7 macrophages, Fujita et al. reported that treatment with LPS or *E. coli* could induce LC3-II and p62 expression, as well as the formation of selective autophagy of aggresome-like induced structures; and this effect is aborted in Nrf2-deficient microphages, indicating the necessity of Nrf2 in p62 aggregation and p62-mediated autophagy [122]. Similar phenomenon is also shown in liver cells [123] and in the septic lung [124]. In this positive feedback pattern, p62 also contributes to Nrf2 activation. There is a Keap1-interacting region (KIR) in p62, and various stresses can induce the phosphorylation of the serine residue in the KIR, which markedly increases its binding affinity to Keap1. Therefore, phosphorylated p62 can completely abrogate the interaction between Nrf2 and Keap1, thus activating Nrf2 [125]. In addition, Keap1 in the complex with phosphorylated p62 is degraded by selective autophagy [121, 126], contributing to the continuous activation of Nrf2 pathway.

So far, there is little information about the role of Nrf2 in regulating microglial autophagy in stroke, although two reports showed that hypoxia promote both Nrf2 activation and microglial autophagy [127, 128], without knowing the potential link between these two events. Nevertheless, given that phagocytosing microglia produce large amount of ROS, and that ROS is a strong inducer of Nrf2 activation and autophagy, it is reasonable to speculate that the activation of Nrf2 in microglia may promote its autophagic capacity. It is worth to note that autophagy may also be a "double-edged sword," with side effects of further induction of inflammation and delayed cell death, etc. The degree and time of autophagy would determine the overall prognosis [129]. In this regard, further research could be helpful and focus on how Nrf2 regulates microglial autophagy after stroke, and how to precisely control the degree and time of Nrf2 activation to achieve protective effects and avoid detrimental ones.

Nrf2 Pathway Promotes Microglial Phagocytosis

Phagocytosis is a Greek-derived term. Literally, it means the cellular processes of eating, which includes the recognition, engulfment, and degradation of large (>0.5 μm) particulated organisms or structures [130]. Using a co-culture system and live imaging technique, it has been determined that microglia can eliminate an apoptotic cell in 25–95 min in vivo [131]. In vitro under physiological conditions, the microglial clearance time of apoptotic cells has been estimated from 70–90 min to 1–2 h [132, 133]. Besides clearance of apoptotic cells and debris, which is critical for the CNS homeostasis [133], phagocytosis has other functions [134], which includes: (1) antigen presentation, (2) activation of respiratory burst, which can be triggered by hypoxia/reoxygenation, inducing ROS generation that contributes to killing engulfed microorganisms and degradation of other cargo, and (3) modulation of inflammatory responses.

In stroke, phagocytosis by microglias and macrophages plays a critical role in the clearance of cellular bodies and debris, thus limiting ischemic injury [135, 136] and

promoting tissue repair [137]. As demonstrated by Lalancette-Hébert et al., selective ablation of proliferating microglias/macrophages 72 h after MCAO led to a 2.7-fold increase in the number of apoptotic cells, predominantly neurons, and exacerbated ischemic brain injury [136]. In another research carried out by Faustino et al., they reported that depletion of microglia significantly enhanced focal inflammatory responses and ROS expression [135].

Few researches focus on the role of Nrf2 pathway in regulating microglial phagocytosis in the acute or sub-acute phase post the onset of stroke, though Nrf2 pathway has been shown to promote the phagocytosis of macrophages and microglia in several other circumstances. For example, curcumin could enhance the phagocytosis of parasitic protozoans by microphage, playing an antimalarial role; and this process was mediated by the activation of Nrf2 signal pathway but not PPAR-γ [138]. Sulforaphane and benzyl isothiocyanate, two Nrf2 activators, could increase the uptake of 2-μm diameter polystyrene beads by RAW 264.7, a line of murine macrophage-like cells, and this effect was aborted in peritoneal macrophages from $Nrf2^{-/-}$ mice [139]. In the lung, alveolar macrophages demonstrated a decreased ability to recognize and phagocytose bacteria in chronic obstructive pulmonary disease; sulforaphane treatment could restore bacteria recognition and phagocytosis of alveolar macrophages, which was only observed in wild-type mice but not in Nrf2-deficient mice [140]. Gene expression and promoter analysis revealed that Nrf2 increased the phagocytic ability of macrophages by direct transcriptional upregulation of the scavenger receptor MARCO (macrophage receptor with collagenous structure) [140]. Within the CNS, microglial phagocytosis is also under the regulation of Nrf2 pathway. Using an in vitro model of amyloid β-induced toxicity in microglia, Li et al. reported that milk fat globule-EGF factor 8 (MFG-E8) accelerated microglial phagocytosis, associated with enhanced Nrf2 and HO-1 expression [141]. In murine intracranial hemorrhage model, activating Nrf2 pathway with sulforaphane could induce the increased expression of CD36, a phagocytosis-mediating scavenger receptor, and increased hematoma clearance, which is abrogated in $Nrf2^{-/-}$ mice [142]. Adopted primary microglia and red blood cells in a phagocytic study, Zhao and colleges also found that activating Nrf2 pathway could upregulate CD36 expression and enhance red blood cell phagocytosis [142]. It is worth noting that the harmful condition of self-producing ROS and pro-inflammatory mediators makes microglias hard to survive; Nrf2 pathway might enhance the tolerance and survival of microglia under this detrimental milieu. As mentioned above, phagocytosis of microglia could regulate inflammation through releasing anti-inflammatory cytokines, one of which is transforming growth factor (TGF)-β, and reduced production of pro-inflammatory cytokines such as TNF-α [143]. It has been reported that TGF-β is critical for survival of phagocytizing microglia through autocrine suppression of TNF-α and ROS [144], and thus enhance microglial phagocytosis. Therefore, enhancing microglial Nrf2 may promote its phagocytosis through downregulating ROS and prolong the lifetime of phagocytizing microglia, expediting debris cleanup in ischemic brain.

Like autophagy being a “double-edged sword” in microglia/macrophage, phagocytosis in microglia/macrophage could also be recognized as such a “double-edged

sword." Under physiological and regulated conditions, the phagocytosis helps to scavenge the apoptotic and necrotic cells, to clear the debris and thus restrict the inflammation; under pathophysiological circumstances, however, dysregulated microglial phagocytosis may contribute to excess neuronal death after acute or chronic ischemia, leading to delayed neuronal loss, brain atrophy even vascular dementia. In a MCAO research carried out by Neher et al. [145], for example, genetic deficiency of phagocytic protein MFG-E8 or Mer receptor tyrosine kinase (MerTK) could completely prevent long-term functional deficits of motor neurons, and the phagocytic deficiency strongly reduced brain atrophy as a result of inhibiting phagocytosis of neurons. The mechanism might be associated with suppressed phagocytosis of neurons, as neurons reversibly exposed the "eat-me" signal phosphatidylserine (PS) to microglias or macrophages after stroke [145].

Taken together, several lines of evidence suggest that activation of Nrf2 pathway may contribute to the inhibition of inflammatory responses after stroke. Considering that cell death, either in the form of apoptosis or necrosis, is an inevitable event in ischemic stroke, enhancing phagocytic capacity of microglia and macrophage will expedite the clean-up process and enhance tissue repair.

3 Conclusion

In summary, Nrf2 is sequestered under physiological conditions and degraded through ubiquitination. Ischemic stroke induces severe oxidative stress that activate Nrf2 pathway by disassociating Nrf2 from Keap1. After nuclear translocation, Nrf2 induces the expression of antioxidants, mainly phase 2 enzymes, which then protect neurons against oxidative injury. It is worth noting that Nrf2 may protect not only through cellular self-defense, but also through cell–cell interaction. Therefore, Nrf2 pathway could be a promising therapeutic target to treat ischemic stroke.

References

1. Motohashi H, O'Connor T, Katsuoka F, et al. Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. Gene. 2002;294(1–2):1–12.
2. Jain AK, Jaiswal AK. GSK-3beta acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2. J Biol Chem. 2007;282(22):16502–10.
3. Itoh K, Chiba T, Takahashi S, et al. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun. 1997;236(2):313–22.
4. Baird L, Dinkova-Kostova AT. The cytoprotective role of the Keap1–Nrf2 pathway. Arch Toxicol. 2011;85(4):241–72.
5. Motohashi H, Yamamoto M. Nrf2–Keap1 defines a physiologically important stress response mechanism. Trends Mol Med. 2004;10(11):549–57.

6. Yamamoto T, Suzuki T, Kobayashi A, et al. Physiological significance of reactive cysteine residues of Keap1 in determining Nrf2 activity. Mol Cell Biol. 2008;28(8):2758–70. Pubmed Central PMCID: 2293100.
7. McMahon M, Thomas N, Itoh K, et al. Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex. J Biol Chem. 2006;281(34):24756–68.
8. Jain AK, Bloom DA, Jaiswal AK. Nuclear import and export signals in control of Nrf2. J Biol Chem. 2005;280(32):29158–68.
9. Li W, Kong AN. Molecular mechanisms of Nrf2-mediated antioxidant response. Mol Carcinog. 2009;48(2):91–104. Pubmed Central PMCID: 2631094.
10. Huang HC, Nguyen T, Pickett CB. Phosphorylation of Nrf2 at Ser-40 by protein kinase C regulates antioxidant response element-mediated transcription. J Biol Chem. 2002;277(45):42769–74. PubMed PMID: WOS:000179081200046. English.
11. Jain AK, Jaiswal AK. Phosphorylation of tyrosine 568 controls nuclear export of Nrf2. J Biol Chem. 2006;281(17):12132–42.
12. Rada P, Rojo AI, Chowdhry S, et al. SCF/{beta}-TrCP promotes glycogen synthase kinase 3-dependent degradation of the Nrf2 transcription factor in a Keap1-independent manner. Mol Cell Biol. 2011;31(6):1121–33. Pubmed Central PMCID: 3067901.
13. Itoh K, Igarashi K, Hayashi N, et al. Cloning and characterization of a novel erythroid cell-derived CNC family transcription factor heterodimerizing with the small Maf family proteins. Mol Cell Biol. 1995;15(8):4184–93. Pubmed Central PMCID: 230657.
14. Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J Biol Chem. 1991;266(18):11632–9.
15. Nguyen T, Sherratt PJ, Pickett CB. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu Rev Pharmacol Toxicol. 2003;43:233–60.
16. Rada P, Rojo AI, Evrard-Todeschi N, et al. Structural and functional characterization of Nrf2 degradation by the glycogen synthase kinase 3/beta-TrCP axis. Mol Cell Biol. 2012;32(17):3486–99. Pubmed Central PMCID: 3422007.
17. Rojo AI, Medina-Campos ON, Rada P, et al. Signaling pathways activated by the phytochemical nordihydroguaiaretic acid contribute to a Keap1-independent regulation of Nrf2 stability: role of glycogen synthase kinase-3. Free Radic Biol Med. 2012;52(2):473–87.
18. Lee OH, Jain AK, Papusha V, et al. An auto-regulatory loop between stress sensors INrf2 and Nrf2 controls their cellular abundance. J Biol Chem. 2007;282(50):36412–20.
19. Niture SK, Jaiswal AK. Prothymosin-alpha mediates nuclear import of the INrf2/Cul3 Rbx1 complex to degrade nuclear Nrf2. J Biol Chem. 2009;284(20):13856–68. Pubmed Central PMCID: 2679486.
20. Holtzclaw WD, Dinkova-Kostova AT, Talalay P. Protection against electrophile and oxidative stress by induction of phase 2 genes: the quest for the elusive sensor that responds to inducers. Adv Enzyme Regul. 2004;44(1):335–67.
21. Zhang M, An C, Gao Y, et al. Emerging roles of Nrf2 and phase II antioxidant enzymes in neuroprotection. Prog Neurobiol. 2013;100:30–47.
22. Zhang Y, Talalay P, Cho C-G, et al. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci U S A. 1992;89(6):2399–403.
23. Shih AY, Li P, Murphy TH. A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. J Neurosci. 2005;25(44):10321–35.
24. Kraft AD, Lee JM, Johnson DA, et al. Neuronal sensitivity to kainic acid is dependent on the Nrf2-mediated actions of the antioxidant response element. J Neurochem. 2006;98(6):1852–65.
25. del Zoppo GJ. The neurovascular unit in the setting of stroke. J Intern Med. 2010;267(2):156–71. Pubmed Central PMCID: Pmc3001328, Epub 2010/02/24. eng.

26. Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. Nat Rev Neurosci. 2003;4(5):399–414.
27. Zhang JH, Badaut J, Tang J, et al. The vascular neural network—a new paradigm in stroke pathophysiology. Nat Rev Neurol. 2012;8(12):711–6. Pubmed Central PMCID: Pmc3595043, Epub 2012/10/17. eng.
28. Silva-Islas C, Colín-González AL, Maldonado PD, et al. Nrf2 activation, an innovative therapeutic alternative in cerebral ischemia. INTECH Open Access; 2012.
29. Moskowitz MA, Lo EH, Iadecola C. The science of stroke: mechanisms in search of treatments. Neuron. 2010;67(2):181–98.
30. Terasaki Y, Liu Y, Hayakawa K, et al. Mechanisms of neurovascular dysfunction in acute ischemic brain. Curr Med Chem. 2014;21(18):2035.
31. Chan PH. Reactive oxygen radicals in signaling and damage in the ischemic brain. J Cereb Blood Flow Metab. 2001;21(1):2–14.
32. Danton GH, Dietrich WD. Inflammatory mechanisms after ischemia and stroke. J Neuropathol Exp Neurol. 2003;62(2):127–36.
33. Margaill I, Plotkine M, Lerouet D. Antioxidant strategies in the treatment of stroke. Free Radic Biol Med. 2005;39(4):429–43.
34. Crack PJ, Taylor JM. Reactive oxygen species and the modulation of stroke. Free Radic Biol Med. 2005;38(11):1433–44.
35. Gouriou Y, Demaurex N, Bijlenga P, et al. Mitochondrial calcium handling during ischemia-induced cell death in neurons. Biochimie. 2011;93(12):2060–7.
36. Zhang Y, Wang H, Li J, et al. Peroxynitrite-induced neuronal apoptosis is mediated by intracellular zinc release and 12-lipoxygenase activation. J Neurosci. 2004;24(47):10616–27.
37. Moro MA, Almeida A, Bolaños JP, et al. Mitochondrial respiratory chain and free radical generation in stroke. Free Radic Biol Med. 2005;39(10):1291–304.
38. Hallenbeck JM, Dutka AJ, Tanishima T, et al. Polymorphonuclear leukocyte accumulation in brain regions with low blood flow during the early postischemic period. Stroke. 1986;17(2):246–53.
39. Clark R, Lee E, Fish C, et al. Development of tissue damage, inflammation and resolution following stroke: an immunohistochemical and quantitative planimetric study. Brain Res Bull. 1993;31(5):565–72.
40. Campanella M, Sciorati C, Tarozzo G, et al. Flow cytometric analysis of inflammatory cells in ischemic rat brain. Stroke. 2002;33(2):586–92.
41. Shichita T, Ago T, Kamouchi M, et al. Novel therapeutic strategies targeting innate immune responses and early inflammation after stroke. J Neurochem. 2012;123(s2):29–38.
42. Heiss W-D. Stroke—acute interventions. Berlin: Springer; 2002.
43. Hou ST, MacManus JP. Molecular mechanisms of cerebral ischemia-induced neuronal death. Int Rev Cytol. 2002;221:93–148.
44. Dinkova-Kostova A, Cheah J, Samouilov A, et al. Phenolic Michael reaction acceptors: combined direct and indirect antioxidant defenses against electrophiles and oxidants. Med Chem. 2007;3(3):261–8.
45. Lindenau J, Noack H, Possel H, et al. Cellular distribution of superoxide dismutases in the rat CNS. Glia. 2000;29(1):25–34.
46. Dinkova-Kostova AT, Talalay P. Direct and indirect antioxidant properties of inducers of cytoprotective proteins. Mol Nutr Food Res. 2008;52(S1):S128–38.
47. Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu Rev Pharmacol Toxicol. 2007;47:89–116.
48. Kobayashi A, Kang M-I, Okawa H, et al. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol. 2004;24(16):7130–9.
49. Peters O, Back T, Lindauer U, et al. Increased formation of reactive oxygen species after permanent and reversible middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab. 1998;18(2):196–205.

50. Christensen T, Bruhn T, Balchen T, et al. Evidence for formation of hydroxyl radicals during reperfusion after global cerebral ischaemia in rats using salicylate trapping and microdialysis. Neurobiol Dis. 1994;1(3):131–8.
51. Liu S, Liu M, Peterson S, et al. Hydroxyl radical formation is greater in striatal core than in penumbra in a rat model of ischemic stroke. J Neurosci Res. 2003;71(6):882–8.
52. Tanaka N, Ikeda Y, Ohta Y, et al. Expression of Keap1–Nrf2 system and antioxidative proteins in mouse brain after transient middle cerebral artery occlusion. Brain Res. 2011;1370:246–53.
53. Dang J, Brandenburg L-O, Rosen C, et al. Nrf2 expression by neurons, astroglia, and microglia in the cerebral cortical penumbra of ischemic rats. J Mol Neurosci. 2012;46(3):578–84.
54. Li M, Zhang X, Cui L, et al. The neuroprotection of oxymatrine in cerebral ischemia/reperfusion is related to nuclear factor erythroid 2-related factor 2 (nrf2)-mediated antioxidant response: role of nrf2 and hemeoxygenase-1 expression. Biol Pharm Bull. 2011;34(5):595–601.
55. Srivastava S, Alfieri A, Siow R, et al. Temporal and spatial distribution of Nrf2 in rat brain following stroke: quantification of nuclear to cytoplasmic Nrf2 content using a novel immunohistochemical technique. J Physiol. 2013;591(14):3525–38.
56. Liverman CS, Cui L, Yong C, et al. Response of the brain to oligemia: gene expression, c-Fos, and Nrf2 localization. Mol Brain Res. 2004;126(1):57–66.
57. Gladstone DJ, Black SE, Hakim AM. Toward wisdom from failure lessons from neuroprotective stroke trials and new therapeutic directions. Stroke. 2002;33(8):2123–36.
58. Herken R, Götz W, Thies M. Appearance of laminin, heparan sulphate proteoglycan and collagen type IV during initial stages of vascularisation of the neuroepithelium of the mouse embryo. J Anat. 1990;169:189.
59. Engvall E, Davis GE, Dickerson K, et al. Mapping of domains in human laminin using monoclonal antibodies: localization of the neurite-promoting site. J Cell Biol. 1986;103(6):2457–65.
60. Grant D, Kleinman H. Regulation of capillary formation by laminin and other components of the extracellular matrix. Regulation of angiogenesis. Berlin: Springer; 1997. p. 317–33.
61. Iadecola C. Neurovascular regulation in the normal brain and in Alzheimer's disease. Nat Rev Neurosci. 2004;5(5):347–60.
62. Zonta M, Angulo MC, Gobbo S, et al. Neuron-to-astrocyte signaling is central to the dynamic control of brain microcirculation. Nat Neurosci. 2003;6(1):43–50.
63. del Zoppo GJ, Mabuchi T. Cerebral microvessel responses to focal ischemia. J Cereb Blood Flow Metab. 2003;23(8):879–94.
64. del Zoppo GJ, Milner R. Integrin–matrix interactions in the cerebral microvasculature. Arterioscler Thromb Vasc Biol. 2006;26(9):1966–75.
65. Mabuchi T, Lucero J, Feng A, et al. Focal cerebral ischemia preferentially affects neurons distant from their neighboring microvessels. J Cereb Blood Flow Metab. 2005;25(2):257–66.
66. Hu X, Leak RK, Shi Y, et al. Microglial and macrophage polarization—new prospects for brain repair. Nat Rev Neurol. 2015;11(1):56–64.
67. Fumagalli S, Perego C, Pischiutta F, et al. The ischemic environment drives microglia and macrophage function. Front Neurol. 2015;6:81.
68. Dringen R, Gutterer JM, Hirrlinger J. Glutathione metabolism in brain. Eur J Biochem. 2000;267(16):4912–6.
69. Kraft AD, Johnson DA, Johnson JA. Nuclear factor E2-related factor 2-dependent antioxidant response element activation by tert-butylhydroquinone and sulforaphane occurring preferentially in astrocytes conditions neurons against oxidative insult. J Neurosci. 2004;24(5):1101–12.
70. Shih AY, Johnson DA, Wong G, et al. Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. J Neurosci. 2003;23(8):3394–406.

71. Chen Y, Vartiainen NE, Ying W, et al. Astrocytes protect neurons from nitric oxide toxicity by a glutathione-dependent mechanism. J Neurochem. 2001;77(6):1601–10.
72. Halliwell B. Role of free radicals in the neurodegenerative diseases. Drugs Aging. 2001;18(9):685–716.
73. Vargas MR, Johnson JA. The Nrf2–ARE cytoprotective pathway in astrocytes. Expert Rev Mol Med. 2009;11:e17.
74. Bambrick L, Kristian T, Fiskum G. Astrocyte mitochondrial mechanisms of ischemic brain injury and neuroprotection. Neurochem Res. 2004;29(3):601–8.
75. Swanson RA, Ying W, Kauppinen TM. Astrocyte influences on ischemic neuronal death. Curr Mol Med. 2004;4(2):193–205.
76. Calkins MJ, Jakel RJ, Johnson DA, et al. Protection from mitochondrial complex II inhibition in vitro and in vivo by Nrf2-mediated transcription. Proc Natl Acad Sci U S A. 2005;102(1):244–9.
77. Calkins MJ, Vargas MR, Johnson DA, et al. Astrocyte-specific overexpression of Nrf2 protects striatal neurons from mitochondrial complex II inhibition. Toxicol Sci. 2010;115(2):557–68.
78. Bell KF, Fowler JH, Al-Mubarak B, et al. Activation of Nrf2-regulated glutathione pathway genes by ischemic preconditioning. Oxid Med Cell Longev. 2011;2011:689524.
79. Narayanan SV, Dave KR, Saul I, et al. Resveratrol preconditioning protects against cerebral ischemic injury via nuclear erythroid 2–related factor 2. Stroke. 2015;46(6):1626–32.
80. Schroeter ML, Mertsch K, Giese H, et al. Astrocytes enhance radical defence in capillary endothelial cells constituting the blood-brain barrier. FEBS Lett. 1999;449(2):241–4.
81. Laird MD, Ramesh SS, Alleyne CH, et al. Astrocyte-derived glutathione attenuates hemin-induced cytotoxicity in murine cerebral microvessel. FASEB J. 2009;23(1_MeetingAbstracts):614.12.
82. Lee BJ, Egi Y, van Leyen K, et al. Edaravone, a free radical scavenger, protects components of the neurovascular unit against oxidative stress in vitro. Brain Res. 2010;1307:22–7.
83. Chrissobolis S, Banfi B, Sobey CG, et al. Role of Nox isoforms in angiotensin II-induced oxidative stress and endothelial dysfunction in brain. J Appl Physiol. 2012;113(2):184–91.
84. Pacher P, Szabo C. Role of the peroxynitrite-poly (ADP-ribose) polymerase pathway in human disease. Am J Pathol. 2008;173(1):2–13.
85. Weiss N, Miller F, Cazaubon S, et al. The blood-brain barrier in brain homeostasis and neurological diseases. Biochim Biophys Acta. 2009;1788(4):842–57.
86. Posada-Duque RA, Barreto GE, Cardona-Gomez GP. Protection after stroke: cellular effectors of neurovascular unit integrity. Front Cell Neurosci. 2014;8:231.
87. Alfieri A, Srivastava S, Siow RC, et al. Sulforaphane preconditioning of the Nrf2/HO-1 defense pathway protects the cerebral vasculature against blood–brain barrier disruption and neurological deficits in stroke. Free Radic Biol Med. 2013;65:1012–22.
88. Bénardais K, Pul R, Singh V, et al. Effects of fumaric acid esters on blood–brain barrier tight junction proteins. Neurosci Lett. 2013;555:165–70.
89. Kunze R, Urrutia A, Hoffmann A, et al. Dimethyl fumarate attenuates cerebral edema formation by protecting the blood–brain barrier integrity. Exp Neurol. 2015;266:99–111.
90. Wu S, Yue Y, Li J, et al. Procyanidin B2 attenuates neurological deficits and blood–brain barrier disruption in a rat model of cerebral ischemia. Mol Nutr Food Res. 2015;59(10):1930–41.
91. Chen G, Fang Q, Zhang J, et al. Role of the Nrf2-ARE pathway in early brain injury after experimental subarachnoid hemorrhage. J Neurosci Res. 2011;89(4):515–23.
92. Li T, Sun K-J, Wang H-D, et al. Tert-butylhydroquinone ameliorates early brain injury after experimental subarachnoid hemorrhage in mice by enhancing Nrf2-independent autophagy. Neurochem Res. 2015;40(9):1829–38.
93. Wang Z, Ji C, Wu L, et al. Tert-butylhydroquinone alleviates early brain injury and cognitive dysfunction after experimental subarachnoid hemorrhage: role of Keap1/Nrf2/ARE pathway. PLoS One. 2014;9(5):e97685.
94. Wu Q, Zhang X-S, Wang H-D, et al. Astaxanthin activates nuclear factor erythroid-related factor 2 and the antioxidant responsive element (Nrf2-ARE) pathway in the brain after sub-

arachnoid hemorrhage in rats and attenuates early brain injury. Mar Drugs. 2014;12(12):6125–41.
95. Shi S-S, Zhang H-B, Wang C-H, et al. Propofol attenuates early brain injury after subarachnoid hemorrhage in rats. J Mol Neurosci. 2015;7(4):538–45.
96. Wang Z, Ma C, Meng CJ, et al. Melatonin activates the Nrf2-ARE pathway when it protects against early brain injury in a subarachnoid hemorrhage model. J Pineal Res. 2012;53(2):129–37.
97. Zhang J, Zhu Y, Zhou D, et al. Recombinant human erythropoietin (rhEPO) alleviates early brain injury following subarachnoid hemorrhage in rats: possible involvement of Nrf2–ARE pathway. Cytokine. 2010;52(3):252–7.
98. Li T, Wang H, Ding Y, et al. Genetic elimination of Nrf2 aggravates secondary complications except for vasospasm after experimental subarachnoid hemorrhage in mice. Brain Res. 2014;1558:90–9.
99. Zhao J, Moore AN, Redell JB, et al. Enhancing expression of Nrf2-driven genes protects the blood–brain barrier after brain injury. J Neurosci. 2007;27(38):10240–8.
100. Jin W, Ni H, Hou X, et al. Tert-butylhydroquinone protects the spinal cord against inflammatory response produced by spinal cord injury. Ann Clin Lab Sci. 2014;44(2):151–7.
101. Mao L, Wang H, Qiao L, et al. Disruption of Nrf2 enhances the upregulation of nuclear factor-kappaB activity, tumor necrosis factor-, and matrix metalloproteinase-9 after spinal cord injury in mice. Mediators Inflamm. 2010;2010:238321.
102. Allan SM, Rothwell NJ. Inflammation in central nervous system injury. Philos Trans R Soc Lond B Biol Sci. 2003;358(1438):1669–77.
103. Bechmann I, Galea I, Perry VH. What is the blood–brain barrier (not)? Trends Immunol. 2007;28(1):5–11.
104. Molina-Holgado E, Molina-Holgado F. Mending the broken brain: neuroimmune interactions in neurogenesis. J Neurochem. 2010;114(5):1277–90.
105. Aspelund A, Antila S, Proulx ST, et al. A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. J Exp Med. 2015;212(7):991–9. PubMed PMID: WOS:000357117200004. English.
106. Louveau A, Smirnov I, Keyes TJ, et al. Structural and functional features of central nervous system lymphatic vessels. Nature. 2015;523(7560):337–41. Pubmed Central PMCID: 4506234.
107. Stoll G, Jander S, Schroeter M. Inflammation and glial responses in ischemic brain lesions. Prog Neurobiol. 1998;56(2):149–71.
108. Amantea D, Nappi G, Bernardi G, et al. Post-ischemic brain damage: pathophysiology and role of inflammatory mediators. FEBS J. 2009;276(1):13–26.
109. Foresti R, Bains SK, Pitchumony TS, et al. Small molecule activators of the Nrf2-HO-1 antioxidant axis modulate heme metabolism and inflammation in BV2 microglia cells. Pharmacol Res. 2013;76:132–48.
110. Dilshara MG, Lee K-T, Kim HJ, et al. Anti-inflammatory mechanism of α-viniferin regulates lipopolysaccharide-induced release of proinflammatory mediators in BV2 microglial cells. Cell Immunol. 2014;290(1):21–9.
111. Min KJ, Kim JH, Jou I, et al. Adenosine induces heme oxygenase-1 expression in microglia through the activation of phosphatidylinositol-3-kinase and nuclear factor E2-related factor 2. Glia. 2008;56(9):1028–37.
112. Lee EJ, Ko HM, Jeong YH, et al. beta-Lapachone suppresses neuroinflammation by modulating the expression of cytokines and matrix metalloproteinases in activated microglia. J Neuroinflammation. 2015;12:133. Pubmed Central PMCID: Pmc4502557. Epub 2015/07/16. eng.
113. Lee D-S, Ko W, Yoon C-S, et al. KCHO-1, a novel antineuroinflammatory agent, inhibits lipopolysaccharide-induced neuroinflammatory responses through Nrf2-mediated heme oxygenase-1 expression in mouse BV2 microglia cells. Evid Based Complement Alternat Med. 2014;2014:357154.

114. Lee E-J, Kim H-S. The anti-inflammatory role of tissue inhibitor of metalloproteinase-2 in lipopolysaccharide-stimulated microglia. J Neuroinflammation. 2014;11:116.
115. Jisun L, Samantha G, Jianhua Z. Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. Biochem J. 2012;441(2):523–40.
116. Chen Y, Klionsky DJ. The regulation of autophagy–unanswered questions. J Cell Sci. 2011;124(2):161–70.
117. Cooper CE, Patel RP, Brookes PS, et al. Nanotransducers in cellular redox signaling: modification of thiols by reactive oxygen and nitrogen species. Trends Biochem Sci. 2002;27(10):489–92.
118. Bae SH, Sung SH, Oh SY, et al. Sestrins activate Nrf2 by promoting p62-dependent autophagic degradation of Keap1 and prevent oxidative liver damage. Cell Metab. 2013;17(1):73–84.
119. Vadlamudi RK, Joung I, Strominger JL, et al. p62, a phosphotyrosine-independent ligand of the SH2 domain of p56lck, belongs to a new class of ubiquitin-binding proteins. J Biol Chem. 1996;271(34):20235–7.
120. Bjørkøy G, Lamark T, Brech A, et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J Cell Biol. 2005;171(4):603–14.
121. Jain A, Lamark T, Sjøttem E, et al. p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. J Biol Chem. 2010;285(29):22576–91.
122. Fujita K-I, Maeda D, Xiao Q, et al. Nrf2-mediated induction of p62 controls Toll-like receptor-4–driven aggresome-like induced structure formation and autophagic degradation. Proc Natl Acad Sci U S A. 2011;108(4):1427–32.
123. Riley BE, Kaiser SE, Shaler TA, et al. Ubiquitin accumulation in autophagy-deficient mice is dependent on the Nrf2-mediated stress response pathway: a potential role for protein aggregation in autophagic substrate selection. J Cell Biol. 2010;191(3):537–52.
124. Chang AL, Ulrich A, Suliman HB, et al. Redox regulation of mitophagy in the lung during murine Staphylococcus aureus sepsis. Free Radic Biol Med. 2015;78:179–89.
125. Ichimura Y, Waguri S, Sou Y-S, et al. Phosphorylation of p62 activates the Keap1-Nrf2 pathway during selective autophagy. Mol Cell. 2013;51(5):618–31.
126. Taguchi K, Fujikawa N, Komatsu M, et al. Keap1 degradation by autophagy for the maintenance of redox homeostasis. Proc Natl Acad Sci U S A. 2012;109(34):13561–6.
127. Yang Z, Zhao T-Z, Zou Y-J, et al. Hypoxia induces autophagic cell death through hypoxia-inducible factor 1α in microglia. PLoS One. 2014;9(5):e96509.
128. Yang Z, Zhong L, Zhong S, et al. Hypoxia induces microglia autophagy and neural inflammation injury in focal cerebral ischemia model. Exp Mol Pathol. 2015;98(2):219–24.
129. Chen W, Sun Y, Liu K, et al. Autophagy: a double-edged sword for neuronal survival after cerebral ischemia. Neural Regen Res. 2014;9(12):1210.
130. Mukherjee S, Ghosh RN, Maxfield FR. Endocytosis. Physiol Rev. 1997;77(3):759–803.
131. Parnaik R, Raff MC, Scholes J. Differences between the clearance of apoptotic cells by professional and non-professional phagocytes. Curr Biol. 2000;10(14):857–60.
132. Sierra A, Encinas JM, Deudero JJ, et al. Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. Cell Stem Cell. 2010;7(4):483–95.
133. Henson PM, Hume DA. Apoptotic cell removal in development and tissue homeostasis. Trends Immunol. 2006;27(5):244–50.
134. Sierra A, Abiega O, Shahraz A, et al. Janus-faced microglia: beneficial and detrimental consequences of microglial phagocytosis. Front Cell Neurosci. 2013;7:6.
135. Faustino JV, Wang X, Johnson CE, et al. Microglial cells contribute to endogenous brain defenses after acute neonatal focal stroke. J Neurosci. 2011;31(36):12992–3001.
136. Lalancette-Hébert M, Gowing G, Simard A, et al. Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. J Neurosci. 2007;27(10):2596–605.

137. Manoonkitiwongsa PS, Jackson-Friedman C, McMillan PJ, et al. Angiogenesis after stroke is correlated with increased numbers of macrophages: the clean-up hypothesis. J Cereb Blood Flow Metab. 2001;21(10):1223–31.
138. Mimche PN, Thompson E, Taramelli D, et al. Curcumin enhances non-opsonic phagocytosis of Plasmodium falciparum through up-regulation of CD36 surface expression on monocytes/macrophages. J Antimicrob Chemother. 2012;67(8):1895–904.
139. Suganuma H, Fahey JW, Bryan KE, et al. Stimulation of phagocytosis by sulforaphane. Biochem Biophys Res Commun. 2011;405(1):146–51.
140. Harvey CJ, Thimmulappa RK, Sethi S, et al. Targeting Nrf2 signaling improves bacterial clearance by alveolar macrophages in patients with COPD and in a mouse model. Sci Transl Med. 2011;3(78):78ra32.
141. Li E, Noda M, Doi Y, et al. The neuroprotective effects of milk fat globule-EGF factor 8 against oligomeric amyloid β toxicity. J Neuroinflammation. 2012;9(148):2657–66.
142. Zhao X, Sun G, Ting SM, et al. Cleaning up after ICH: the role of Nrf2 in modulating microglia function and hematoma clearance. J Neurochem. 2015;133(1):144–52.
143. De Simone R, Ajmone-Cat MA, Tirassa P, et al. Apoptotic PC12 cells exposing phosphatidylserine promote the production of anti-inflammatory and neuroprotective molecules by microglial cells. J Neuropathol Exp Neurol. 2003;62(2):208–16.
144. Ryu K-Y, Cho G-S, Piao HZ, et al. Role of TGF-β in survival of phagocytizing microglia: autocrine suppression of TNF-α production and oxidative stress. Exp Neurobiol. 2012;21(4):151–7.
145. Neher JJ, Emmrich JV, Fricker M, et al. Phagocytosis executes delayed neuronal death after focal brain ischemia. Proc Natl Acad Sci U S A. 2013;110(43):E4098–107.

Stem Cell Therapy for Ischemic Stroke

Hung Nguyen, Naoki Tajiri, and Cesar V. Borlongan

1 Therapeutic Action of Stem Cell Transplantation in Stroke

The therapeutic utility of stem cells for treating neurological disorders has been demonstrated either by stimulating these cells endogenously [1–4] as well as by transplanting them exogenously within injured brain tissues [5–11]. Stem cell transplantation after a brain insult, such as stroke, is a major tenet of regenerative medicine [1–13]. Stem cell therapy may offer a glimpse of hope for stroke patients because very scarce successful clinical trials have emerged from preclinical laboratory research in this area [14–16]. Traditionally, the postulated therapeutic mechanism underlying stem cell repair for brain diseases implicates that the ensuing cellular regeneration occurs due to either the direct replacement of necrotizing cells with transplanted stem cells or the indirect repair of damaged tissue through the secretion of trophic factors by the stem cells [17, 18]. That stem cells act via cell replacement and growth factor release regenerative processes have accompanied the translation of stem cell therapy for stroke from the laboratory to the clinic over the last 25 years. Nonetheless, these two major mechanisms behind stem cells' ability to restore the stroke brain only partially explain the paradoxical robust functional recovery in stroke animals despite low graft survival of transplanted stem cells. Recent evidence from a relevant cerebrovascular disease, traumatic brain injury (TBI), suggests that transplanted stem cells are capable of forming a cellular migratory pathway as an active process for recruiting endogenous stem cells from the

H. Nguyen • N. Tajiri • C.V. Borlongan (✉)
Department of Neurosurgery and Brain Repair, University of South Florida Morsani College of Medicine, 12901 Bruce B. Downs Blvd, Tampa, FL 33612, USA
e-mail: cborlong@health.usf.edu

J. Chen et al. (eds.), *Non-Neuronal Mechanisms of Brain Damage and Repair After Stroke*, Springer Series in Translational Stroke Research,
DOI 10.1007/978-3-319-32337-4_19

neurogenic niche to the damaged area within the cortex [19]. This transplanted stem cell-generated "biobridge" has been visualized immunohistochemically and by laser capture microdissection to reside in the region between the neurogenic subventricular zone (SVZ) and the largely non-neurogenic TBI-damaged cortex. Because endogenous stem cells have limited capacity to migrate long-distances, as in the case of traversing from the SVZ to the injured cortex of non-transplanted TBI animals, the recognition of this biobridge should facilitate the migration of endogenous stem cells to damaged brain areas that are remotely inaccessible from the neurogenic niche [19, 20].

The overlapping pathologies between TBI and stroke indicate the potential of biobridge formation in stroke following stem cell transplantation. We discuss below the progress of stem cell therapy for stroke, highlighting this possibility of extending the concept of transplanted stem cell-mediated biobridge as a therapeutic mechanism underlying stroke recovery.

2 Secondary Cell Death in Stroke as Therapeutic Target for Stem Cell Grafts

Stroke is the consequence of blood flow restriction to a region of the brain. Its signature pathological characteristic is a region of dead neuronal cells known as the infarct core, and this region is encased within a zone of injured and dying tissue called the penumbra. This cardinal feature of vascular disablement associated with ischemic stroke results in a regional deficiency of glucose and oxygen, which then results in the stimulation of intricate secondary cell death processes [21]. Such blood flow interruption to the brain is commonly referred to as ischemic stroke, whereas blood vessel bleeding is termed hemorrhagic stroke. We focus our discussion of stem cell transplantation on ischemic stroke as this pathological condition, especially the secondary cell death, offers a far wide-ranging application of stem cell therapy than hemorrhagic stroke.

The wider therapeutic window, which can last for days, weeks, months, and even years, associated with the secondary cell death of ischemic stroke has been the target of stem cell therapy. Within a matter of minutes after stroke onset, the primary necrotic core becomes fixed and unsalvageable. In contrast, the secondary cell death that ensues in the tissue surrounding the core corresponds to the evolving ischemic penumbra [22], and may be resuscitated with proper treatment intervention such as stem cell therapy.

In view of the supracute window in the formation of the ischemic core, as opposed to the subacute and even chronic progression of the penumbra, a much better prognosis for arresting the secondary cell death seems to be indicated. Sufficient circulation must be re-established in a very short amount of time, preferably less than 3 h, in order to minimize the necrotic core and to limit the evolution of the penumbra. In this regard, tPA needs to be initiated during the initial 4.5 h after

stroke onset, but beyond this acute window, the drug carries significant adverse effects in particular bleeding [23]. Conversely, when targeting the evolving penumbra, treatment interventions over a much protracted window post-stroke should allow sequestration of secondary cell death events. Indeed, stimulation of endogenous neurogenesis, angiogenesis, and neuroplasticity has been shown to minimize and even reverse penumbra-associated functional and neurostructural deficits.

3 Engaging the Endogenous Regenerative Process in Stroke

The adult mammalian brain has long been considered to lack regenerative capacity, but scientific evidence has shown that neurogenesis occurs in discreet regions of the adult brain, namely in the SVZ of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus [24, 25]. Such endogenous regeneration even in adulthood [21], which has been documented to be further stimulated during injury, suggests that the adult brain is able to mount a reparative process against stroke. However, the proliferation level of newly formed cells, their neural lineage commitment, and their ability to migrate towards the injured brain areas may be limited, altogether indicating the insufficient regeneration by the host brain to exert clinically meaningful improvement after stroke. Current investigations into finding strategies to enhance this endogenous neurogenesis following stroke has been a major research area of interest in the field of regenerative medicine. Treatment regimens designed to boost proliferation, migration, and differentiation of neural progenitor cells by modifying the host microenvironment to be more conducive for synaptic integration of the newly generated neurons have been explored, but to date, compelling evidence suggests that the host neurogenesis remains relatively confined within the SVZ and SGZ, relegating most of the applications of this endogenous repair mechanism within these neurogenic niches or only to their neighboring tissues [25, 26]. Because cerebrovascular diseases, such as stroke and TBI, present with major tissue damage to brain areas remote to the neurogenic niches, utilizing endogenous neurogenesis against these neurological disorders will need consideration.

Along the lines of mobilizing endogenous neurogenesis, ischemic stroke has been demonstrated to upregulate specific molecules that stimulate neurogenic niches, in particular increasing neural progenitor cell survival, proliferation, migration, and differentiation [21]. Moreover, these mobilized neural progenitor cells (albeit a small population) have been shown to migrate from the SVZ to the post-ischemic striatum in rat stroke models [27, 28]. In tandem, resident stem cells which remain quiescent during homeostasis, but are awakened after stroke, have been detected in the hippocampus and the cerebral cortex. In the clinic, however, post-mortem analysis of the stroke brain has not offered evidence of newly generated cortical neurons during the time period of 3 days to 13 years after stroke onset [29]. A rough estimate of host neurogenesis secondary to stroke has been calculated at

0.1 %, or 1 out of every 1000 neurons [29], suggesting that relying merely on the scarcity of newly born cells and their capacity to migrate from the neurogenic niche to the injured area may not be feasible when contemplating endogenous neurogenesis as a regenerative process against stroke.

4 Biobridge: A Migratory Pathway Between Neurogenic Niche and Non-neurogenic Brain Areas

As noted in the preceding section, the two generally accepted mechanisms of action of stem cell transplantation which provides the therapeutic effects are cell replacement and modification of the microenvironment through trophic factors [17, 18]. The new biobridge concept was first described in a study of transplanting modified mesenchymal stromal cells (MSCs) in TBI model [19]. Adult rats were exposed to controlled cortical impact (CCI) TBI and were treated with MSCs. The MSCs formed a biobridge between the neurogenic niche of the host and the injured site, and served as a highway for the host endogenous stem cells to travel to the injured area [19]. The biobridge which spanned from the SVZ to the damaged cortex was visualized using immunohistochemistry and laser capture assay. Upon closer examination of the biobridge, the levels of extracellular matrix metalloproteinases (MMPs) were significantly increased. In particular, MMP-9 was found significantly increased within the biobridge, suggesting that this MMP may play an important role in facilitating the migration of neural progenitor cells in response to a brain insult, which likely released specific chemoattractant signals from the injured area. This study further suggests that an inflammation-enriched (albeit cytokines, chemokines, MMPs) mechanism closely mediates that formation of biobridge. Additional experiments are needed to better understand the effects of various inflammatory cues on the formation of biobridge. Interestingly, the grafted MSCs are eventually replaced by the host cells after a period of time [19], which is a welcomed translational safety outcome because the prolonged and unregulated survival and proliferation of stem cells may be associated with tumor formation. Moreover, that long-term graft survival per se is not a prerequisite robust and stable stroke recovery addresses the paradoxical dilemma on the reported studies demonstrating solid functional improvements despite mediocre engraftment.

A series of analyses to further characterize the biobridge reveals that MMPs assisted the grafted MSCs to form the biobridge. Once the biobridge was formed, the transplanted cells were replaced by the endogenous neurogenic niche-derived stem cells [19]. The biobridge guided the endogenous stem cells to successfully migrate from the SVZ and to home at the TBI injured site. The stem cells then populated the peri-impacted area and exerted their effects in this area. This novel concept of biobridge formation after transplantation is not limited to TBI but also can be extended to other neurological disorders, such as stroke. The damage areas in stroke are generally larger compared to TBI thus it may be more challenging for endogenous stem cells to migrate across tissues toward the injured site. The biobridge by

providing an extracellular matrix on which the endogenous stem cells could attach and migrate should improve in directed transport of neurogenic niche-derived cells to the remote injured areas [19].

5 The Molecular Composition of the Biobridge

The formation of the biobridge provides an alternative and complementary repair mechanism in stem cell therapy, in addition to the cell replacement and growth factor release regenerative processes. The biobridge allows a favorable microenvironment for cell migration and axonal growth. Three months after transplantation in the TBI brain, an increased level of proliferation and differentiation was observed at the peri-impacted area and a stream of cells labeled with nestin and DCX, markers for maturing neuron was maintained [19]. In contrast, control animals which received vehicle did not express significantly, the level of migration nor differentiation. In addition, the level of expression and activity of MMP-9 was upregulated ninefold which directly correlated with the endogenous stem cell migration compared with the animals that received the vehicle infusion [19]. Recent studies have demonstrated the significance of MMPs and extracellular matrices (ECMs) in stroke pathology [2, 12] which further validates the importance of biobridge formation in stem cell therapy for stroke. In the same vein, we envision that the grafted stem cells via a similar biobridge may promote the migration of stem cells from endogenous niche to the stroke core and infarct area. Stem cells, such as peripheral blood, umbilical cord blood, and adult brain stem cells, are able to modulate the levels and functions of MMPs and ECMs [30–32]. With MMPs and ECMs involved in stroke pathology, the ability of stem cells to generate a biobridge in stroke warrants investigations. A study has shown that inhibition of MMPs sequestered the migration of endogenous stem cell from the niche to the damaged site [33], likely due to the depletion of neurovascular network remodeling. On the other hand, the formation of a biobridge may stimulate the production of MMPs, which in turn can enhance the restoration of the neurovascular unit and the migration of host endogenous stem cells. It is worth noting that the physiochemical homeostasis between cell deformation, tissue barrier restriction, and migration rates correspond to limiting factors that determine the capacity of cell migration. In turn, this physiochemical homeostasis is regulated by the ability to degrade the ECM by proteolytic enzymes such as MMPs, and integrin- and actomyosin-mediated mechanocoupling [34].

Based on the concept of biobridge in the TBI model [19], we hypothesize that a similar biobridge will be realized after an intracerebral stem cell transplantation in the stroke brain. The biobridge will similarly guide the endogenous stem cells to migrate from the neurogenic niches, such as the SVZ and the SGZ, to the damaged areas in stroke brain, and eventually contributing to functional improvement [35]. Of note, laboratory studies have reported that transplantation of MSCs aids in stroke recovery, with modulation of inflammation and immune response as a postulated

therapeutic mechanism [36]. Indeed, it has been shown that transplanted stem cells can secrete transforming growth factor-beta (TGF-β) that can dampen the spreading of the inflammatory MPC-1 cells [36]. To this end, transplanted MSCs via formation of the biobridge can also secrete anti-inflammatory or immunomodulatory cytokines, termed as MSC secretome, which can mediate the therapeutic benefits in the stroke brain [37]. The MSC secretome may consist of insulin-like growth factor (IGF)-1, stromal cell-derived factor (SDF-1α), and glial cell-line-derived neurotrophic factor (GDNF) [38], which are growth factors that are elevated in the stroke brain after intracerebral transplantation of MSCs [38].

6 Translational Caveats of Biobridge Application to the Clinic

Despite the demonstration of the biobridge in mediating stem cell therapy TBI animal models, there are limitations in translating this concept to the clinic. Additional studies are needed to further characterize the biobridge facilitation of endogenous stem cell migration. In particular, the formation of such biobridge after peripheral transplantation of stem cells is a missing endeavor that will be of practical use in the clinic, as a minimally invasive procedure may be more preferred instead of a direct intracerebral transplantation approach when targeting the subacute phase of TBI and stroke models.

Another limitation of the biobridge is that the formation itself depends on the severity of the injury. Severe TBI or chronic stage of stroke presumably presents with more extensive damage tissue which may influence the biobridge formation. The pathology of TBI and stroke are also complicated and varied from patient to patient based on the severity of the insult, requiring tailoring of stem cell regimen, e.g., timing of cell transplant initiation, number of transplantations over the progressive period of secondary cell death, among others that may affect the creation of a biobridge. Moreover, the formation of biobridge might be influenced by the non-conductive nature of the microenvironment in the brain, necessitating the need to treat the severe cases of TBI and stroke in the early and acute stage to immediately halt the progression of the disease and arrest the evolution of a non-favorable host microenvironment.

The inflammatory responses accompanying TBI and stroke also warrant investigations, in order to assess the effects of this secondary cell death on biobridge formation. While prolonged inflammation has detrimental effect, acute inflammation may support the brain (i.e., biobridge). The timeline when inflammation produces beneficial effects then switches into a destructive mode remains debatable. Arguably, transplanting at an earlier period post-insult may aid in the biobridge formation and may enhance the pro-survival over the pro-death properties of inflammation. Additional studies are needed to understand the critical time point to maximize the therapeutic effect of inflammation on stem cell transplantation and its modulation of biobridge formation.

7 Towards a Safe and Effective Stem Cell Therapy for Stroke

According to the American Heart Association, approximately 800,000 Americans have stroke every year, with about 17 % mortality or 1 in every 18 deaths in the US is a result of stroke [39]. Stroke survivors also experience disability ranging from moderate to severe. The total estimated healthcare costs due to disability related to stroke are $18.8 billion annually. The number even approaches $34.3 billion if an additional $15.5 billion is added for loss of productivity and premature deaths [40]. Despite the fact that the mortality rate of stroke has fallen 33.5 % in 10 years period from 1996 to 2006, the cost of healthcare associated with stroke increases significantly for the past decades [39].

As the cost continues to increase, there is an urgent need for more effective treatment for neurological disorders such as TBI and stroke. Regenerative medicine has put stem cell therapy as a promising treatment for CNS disorders. The traditional mechanisms of action of stem cell therapy include cell replacement and bystander effects [41–44]. Our recent study has shown another novel mechanism of stem cell transplantation therapy, the formation of biobridge, which provides a cellular pathway for the exogenous transplanted cells to attract endogenous stem cells to migrate from the neurogenic niche towards remote damaged brain areas. This concept of biobridge may be extended in transplant studies to other CNS disorder, such as Parkinson's disease and spinal cord injury, whereby endogenous stem cells may require long-distance migration from the neurogenic niche to the injured tissues.

In conclusion, the multi-pronged therapeutic actions of stem cell therapy, such as cell replacement, secretion of growth factors, and biobridge formation may work in concert to provide beneficial outcomes in cerebrovascular diseases [19]. The formation of biobridge provides a cellular link between the exogenous repair mechanism and the endogenous regenerative processes. That stem cell transplantation may involve different biological pathways in affording its therapeutic effects caters to the multiple cell death events associated with stroke. In the end, an understanding of the mechanism of action that mediates stem cell transplantation may aid in optimizing the stem cell dose, timing of administration, and route of cell delivery, and the eventual laboratory-to-clinic translation of a safe and effective stem cell therapy for stroke.

Sources of Funding C.V.B. is funded by NIH NINDS RO1 1R01NS071956-01, NIH NINDS 1R21NS089851-01, Department of Defense TATRC W811XWH-11-1-0634, Veterans Affairs BX001407-01, James and Esther King Biomedical Research Program 09KB-01-23123, and 1KG01-33966.

References

1. Barha CK, Ishrat T, Epp JR, Galea LAM, Stein DG. Progesterone treatment normalizes the levels of cell proliferation and cell death in the dentate gyrus of the hippocampus after traumatic brain injury. Exp Neurol. 2011;231(1):72–81. doi:10.1016/j.expneurol.2011.05.016.

2. Borlongan CV. Bone marrow stem cell mobilization in stroke: a 'bonehead' may be good after all! Leukemia. 2011;25(11):1674–86. doi:10.1038/leu.2011.167.
3. Jaskelioff M, Muller FL, Paik J-H, Thomas E, Jiang S, Adams AC, et al. Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice. Nature. 2011;469(7328):102–6. doi:10.1038/nature09603.
4. Wang L, Chopp M, Teng H, Bolz M, Francisco M-A, et al. Tumor necrosis factor α primes cerebral endothelial cells for erythropoietin-induced angiogenesis. J Cereb Blood Flow Metab. 2011;31(2):640–7. doi:10.1038/jcbfm.2010.138.
5. Andres RH, Horie N, Slikker W, Keren-Gill H, Zhan K, Sun G, et al. Human neural stem cells enhance structural plasticity and axonal transport in the ischaemic brain. Brain. 2011;134(Pt 6):1777–89. doi:10.1093/brain/awr094.
6. Hargus G, Cooper O, Deleidi M, Levy A, Lee K, Marlow E, et al. Differentiated Parkinson patient-derived induced pluripotent stem cells grow in the adult rodent brain and reduce motor asymmetry in Parkinsonian rats. Proc Natl Acad Sci U S A. 2010;107(36):15921–6. doi:10.1073/pnas.1010209107.
7. Lee H-S, Bae E-J, Yi S-H, Shim J-W, Jo A-Y, Kang J-S, et al. Foxa2 and Nurr1 synergistically yield A9 nigral dopamine neurons exhibiting improved differentiation, function, and cell survival. Stem Cells. 2010;28(3):501–12. doi:10.1002/stem.294.
8. Liu Z, Li Y, Zhang RL, Cui Y, Chopp M. Bone marrow stromal cells promote skilled motor recovery and enhance contralesional axonal connections after ischemic stroke in adult mice. Stroke. 2011;42(3):740–4. doi:10.1161/strokeaha.110.607226.
9. Mazzocchi-Jones D, Döbrössy M, Dunnett SB. Embryonic striatal grafts restore bi-directional synaptic plasticity in a rodent model of Huntington's disease. Eur J Neurosci. 2009;30(11):2134–42. doi:10.1111/j.1460-9568.2009.07006.x.
10. Mezey E. The therapeutic potential of bone marrow-derived stromal cells. J Cell Biochem. 2011;112(10):2683–7. doi:10.1002/jcb.23216.
11. Yasuda A, Tsuji O, Shibata S, Nori S, Takano M, Kobayashi Y, et al. Significance of remyelination by neural stem/progenitor cells transplanted into the injured spinal cord. Stem Cells. 2011;29(12):1983–94. doi:10.1002/stem.767.
12. Yasuhara T, Hara K, Maki M, Mays RW, Deans RJ, Hess DC, et al. Intravenous grafts recapitulate the neurorestoration afforded by intracerebrally delivered multipotent adult progenitor cells in neonatal hypoxic-ischemic rats. J Cereb Blood Flow Metab. 2008;28(11):1804–10. doi:10.1038/jcbfm.2008.68.
13. Yasuhara T, Matsukawa N, Hara K, Yu G, Xu L, Maki M, et al. Transplantation of human neural stem cells exerts neuroprotection in a rat model of Parkinson's disease. J Neurosci. 2006;26(48):12497–511. doi:10.1523/jneurosci.3719-06.2006.
14. Pollock K, Stroemer P, Patel S, Stevanato L, Hope A, Miljan E, et al. A conditionally immortal clonal stem cell line from human cortical neuroepithelium for the treatment of ischemic stroke. Exp Neurol. 2006;199(1):143–55. doi:10.1016/j.expneurol.2005.12.011.
15. Seol HJ, Jin J, Seong D-H, Joo KM, Kang W, Yang H, et al. Genetically engineered human neural stem cells with rabbit carboxyl esterase can target brain metastasis from breast cancer. Cancer Lett. 2011;311(2):152–9. doi:10.1016/j.canlet.2011.07.001.
16. Yasuhara T, Matsukawa N, Hara K, Maki M, Ali MM, Yu SJ, et al. Notch-induced rat and human bone marrow stromal cell grafts reduce ischemic cell loss and ameliorate behavioral deficits in chronic stroke animals. Stem Cells Dev. 2009;18(10):1501–14. doi:10.1089/scd.2009.0011.
17. Lee J-P, Jeyakumar M, Gonzalez R, Takahashi H, Lee P-J, Baek RC, et al. Stem cells act through multiple mechanisms to benefit mice with neurodegenerative metabolic disease. Nat Med. 2007;13(4):439–47. doi:10.1038/nm1548.
18. Redmond DE, Bjugstad KB, Teng YD, Ourednik V, Ourednik J, Wakeman DR, et al. Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells. Proc Natl Acad Sci U S A. 2007;104(29):12175–80. doi:10.1073/pnas.0704091104.

19. Tajiri N, Kaneko Y, Shinozuka K, Ishikawa H, Yankee E, McGrogan M, et al. Stem cell recruitment of newly formed host cells via a successful seduction? Filling the gap between neurogenic niche and injured brain site. PLoS One. 2013;8(9):e74857. doi:10.1371/journal.pone.0074857.
20. Sanai N, Nguyen T, Ihrie RA, Mirzadeh Z, Tsai H-H, Wong M, et al. Corridors of migrating neurons in the human brain and their decline during infancy. Nature. 2011;478(7369):382–6. doi:10.1038/nature10487.
21. Merson TD, Bourne JA. Endogenous neurogenesis following ischaemic brain injury: insights for therapeutic strategies. Int J Biochem Cell Biol. 2014;56:4–19. doi:10.1016/j.biocel.2014.08.003.
22. Broughton BRS, Reutens DC, Sobey CG. Apoptotic mechanisms after cerebral ischemia. Stroke. 2009;40(5):e331–9. doi:10.1161/strokeaha.108.531632.
23. Graham GD. Tissue plasminogen activator for acute ischemic stroke in clinical practice: a meta-analysis of safety data. Stroke. 2003;34(12):2847–50. doi:10.1161/01.str.0000101752.23813.c3.
24. Gage FH. Mammalian neural stem cells. Science. 2000;287(5457):1433–8.
25. Ming G-L, Song H. Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron. 2011;70(4):687–702. doi:10.1016/j.neuron.2011.05.001.
26. Gould E. How widespread is adult neurogenesis in mammals? Nat Rev Neurosci. 2007;8(6):481–8. doi:10.1038/nrn2147.
27. Yamashita T, Ninomiya M, Hernández Acosta P, García-Verdugo JM, Sunabori T, Sakaguchi M, et al. Subventricular zone-derived neuroblasts migrate and differentiate into mature neurons in the post-stroke adult striatum. J Neurosci. 2006;26(24):6627–36. doi:10.1523/jneurosci.0149-06.2006.
28. Zhang RL, Chopp M, Gregg SR, Toh Y, Roberts C, Letourneau Y, et al. Patterns and dynamics of subventricular zone neuroblast migration in the ischemic striatum of the adult mouse. J Cereb Blood Flow Metab. 2009;29(7):1240–50. doi:10.1038/jcbfm.2009.55.
29. Huttner HB, Bergmann O, Salehpour M, Rácz A, Tatarishvili J, Lindgren E, et al. The age and genomic integrity of neurons after cortical stroke in humans. Nat Neurosci. 2014;17(6):801–3. doi:10.1038/nn.3706.
30. Barkho BZ, Munoz AE, Li X, Li L, Cunningham LA, Zhao X. Endogenous matrix metalloproteinase (MMP)-3 and MMP-9 promote the differentiation and migration of adult neural progenitor cells in response to chemokines. Stem Cells. 2008;26(12):3139–49. doi:10.1634/stemcells.2008-0519.
31. Lin C-H, Lee H-T, Lee S-D, Lee W, Cho C-WC, Lin S-Z, et al. Role of HIF-1α-activated Epac1 on HSC-mediated neuroplasticity in stroke model. Neurobiol Dis. 2013;58:76–91. doi:10.1016/j.nbd.2013.05.006.
32. Sobrino T, Pérez-Mato M, Brea D, Rodríguez-Yáñez M, Blanco M, Castillo J. Temporal profile of molecular signatures associated with circulating endothelial progenitor cells in human ischemic stroke. J Neurosci Res. 2012;90(9):1788–93. doi:10.1002/jnr.23068.
33. Zhao B-Q, Wang S, Kim H-Y, Storrie H, Rosen BR, Mooney DJ, et al. Role of matrix metalloproteinases in delayed cortical responses after stroke. Nat Med. 2006;12(4):441–5. doi:10.1038/nm1387.
34. Wolf K, Te Lindert M, Krause M, Alexander S, Te Riet J, Willis AL, et al. Physical limits of cell migration: control by ECM space and nuclear deformation and tuning by proteolysis and traction force. J Cell Biol. 2013;201(7):1069–84. doi:10.1083/jcb.201210152.
35. Stone LL, Grande A, Low WC. Neural repair and neuroprotection with stem cells in ischemic stroke. Brain Sci. 2013;3(2):599–614. doi:10.3390/brainsci3020599.
36. Yoo S-W, Chang D-Y, Lee H-S, Kim G-H, Park J-S, Ryu B-Y, et al. Immune following suppression mesenchymal stem cell transplantation in the ischemic brain is mediated by TGF-β. Neurobiol Dis. 2013;58:249–57. doi:10.1016/j.nbd.2013.06.001.
37. Hsieh J-Y, Wang H-W, Chang S-J, Liao K-H, Lee I-H, Lin W-S, et al. Mesenchymal stem cells from human umbilical cord express preferentially secreted factors related to neuroprotection, neurogenesis, and angiogenesis. PLoS One. 2013;8(8):e72604. doi:10.1371/journal.pone.0072604.

38. Drago D, Cossetti C, Iraci N, Gaude E, Musco G, Bachi A, et al. The stem cell secretome and its role in brain repair. Biochimie. 2013;95(12):2271–85. doi:10.1016/j.biochi.2013.06.020.
39. Writing Group Members, Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, et al. Heart disease and stroke statistics—2010 update: a report from the American Heart Association. Circulation. 2010;121(7):e46–215. doi:10.1161/circulationaha.109.192667.
40. Centers for Disease Control and Prevention. Prevalence of stroke—United States, 2006-2010. MMWR Morb Mortal Wkly Rep. 2012;61(20):379–82.
41. Acosta SA, Tajiri N, Shinozuka K, Ishikawa H, Sanberg PR, Sanchez-Ramos J, et al. Combination therapy of human umbilical cord blood cells and granulocyte colony stimulating factor reduces histopathological and motor impairments in an experimental model of chronic traumatic brain injury. PLoS One. 2014;9(3):e90953.
42. Chiang Y, Morales M, Zhou FC, Borlongan C, Hoffer BJ, Wang Y. Fetal intra-nigral ventral mesencephalon and kidney tissue bridge transplantation restores the nigrostriatal dopamine pathway in hemi-Parkinsonian rats. Brain Res. 2001;889(1–2):200–7.
43. Pastori C, Librizzi L, Breschi GL, Regondi C, Frassoni C, Panzica F, et al. Arterially perfused neurosphere-derived cells distribute outside the ischemic core in a model of transient focal ischemia and reperfusion in vitro. PLoS One. 2008;3(7):e2754. doi:10.1371/journal.pone.0002754.
44. Snyder EY, Yoon C, Flax JD, Macklis JD. Multipotent neural precursors can differentiate toward replacement of neurons undergoing targeted apoptotic degeneration in adult mouse neocortex. Proc Natl Acad Sci U S A. 1997;94(21):11663–8.