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Akio Suzumura
Kazuhiro Ikenaka
Editors

Neuron-Glia Interaction in Neuro-inflammation

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Akio Suzumura • Kazuhiro Ikenaka
Editors

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Editors
Akio Suzumura
Department of Neuroimmunology
Research Institute of Environmental Medicine
Nagoya University
Nagoya, Japan

Kazuhiro Ikenaka
Division of Neurobiology and Neuroinformatics
National Institute for Physiological Science
Aichi, Japan

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Preface

Accumulation of glia, gliosis, in various neurological disorders is not a static scar, but actively involved in pathogenesis of various neurological and psychiatric disorders. There, glial cells produce both inflammatory and neurotrophic factors. These factors may play a role in neuronal damage, but also play protective and reparative roles by inducing neuroinflammation. However, definition as well as the mechanisms of neuroinflammation is not yet clear. We first define acute, chronic, and nonclassical neuroinflammation.

Glial cells are activated by a variety of stimuli via receptors on glial cells. Toll-like receptors (TLRs) are one of these receptors. In response to harmful stimuli, neurons produce factors as either eat-me or help-me signals. Glial cells, especially microglia and astrocytes, respond to these signals. The signals are initiated by factors from damaged neurons including cytokines, chemokines, and damage-associated molecular pattern (DAMP). Some of them reportedly activate glial cells via TLR, and function to protect neurons or further induce neuroinflammation. Thus, the interaction between neuron–glia and glia–glia is a main feature of neuroinflammation. Recent evidences suggest that glial cell communicates with other glial or neural cells via gap-junctions. The communication may also be important for the understanding of neuroinflammation. Oligodendrocytes also communicate with neurons. The communication may be critical in either myelination or demyelination. Damage of blood–brain barrier (BBB) is common feature of both inflammatory and degenerative neurological disorders. Thus, relation of BBB damage and functions of glial cell may also be important in the development of neuroinflammation.

In this book, we focused on neuron–glia interaction of various aspects for understanding of pathophysiology of neuroinflammation in development of inflammatory as well as degenerative neurological disorders.

Nagoya, Japan — Akio Suzumura
Okazaki, Aichi, Japan — Kazuhiro Ikenaka

Contents

Contributors

Joyce A. Benjamins Departments of Neurology and Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI, USA

Cinthia Farina Immunobiology of Neurological Disorders Lab, Division of Neuroscience and Institute of Experimental Neurology, San Raffaele Scientific Institute, Milan, Italy

Kazuhiro Ikenaka Division of Neurobiology and Bioinformatics, National Institute for Physiological Sciences, Okazaki, Aichi, Japan

Takashi Kanda Department of Neurology and Clinical Neuroscience, Yamaguchi University Graduate School of Medicine, Ube, Japan

Jun-ichi Kira Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Jasna Kriz Department of Psychiatry and Neuroscience, Faculty of Medicine Laval University, and Le Centre de recherche de l'Institut universitaire en santé mentale de Québec, Quebec, QC, Canada

Sung Joong Lee Department of Neuroscience and Physiology, Dental Research Institute, School of Dentistry, Seoul National University, Seoul, Republic of Korea

Robert P. Lisak Departments of Neurology and Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI, USA

Tetsuya Mizuno Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan

Fumitaka Shimizu Department of Neurology and Clinical Neuroscience, Yamaguchi University Graduate School of Medicine, Ube, Japan

Akio Suzumura Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan

Hideyuki Takeuchi Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Japan

Chapter 1
Acute, Chronic, and Nonclassical Neuroinflammation: Definitions in a Changing Scientific Environment

Robert P. Lisak and Joyce A. Benjamins

Abstract There is increased understanding of classical inflammation in the nervous system in both infection and autoimmune/immunopathologically mediated diseases as well as how persistence of inflammation and conversion to a more diffuse inflammation mediated predominately by endogenous cells of the nervous system lead to damage. More recently it has been appreciated that nonclassical inflammation, termed neuroinflammation, can start within the nervous system, often as an attempt at protection, when persistent, contributes to damage and furthering of the disease process itself, including in disorders that have been considered purely neurodegenerative in nature.

1.1 Introduction

There is a long standing interest in the pathogenesis of inflammatory diseases of the CNS and PNS with regard to the role of the inflammatory system in providing protection of tissue from effects of infectious agents as well as the role of inflammatory cells and mediators of inflammation. In the past several decades we have seen a dramatic increase in our knowledge about inflammation in the CNS and PNS that includes both in vitro and in vivo observations that cells of the inflammatory system, particularly lymphocytes and monocytes, contain subsets of cells which directly and by production of soluble mediators, can also downregulate inflammation, provide tissue protective and even regenerative stimuli (De Santi et al. 2011; Dhib-Jalbut et al. 2006; Hoke 2006; Karussis et al. 2006; Palace 2008; Re and Przedborski 2006; Schwartz 2001; Thippeswamy et al. 2005). In addition to cells and secretory factors

R.P. Lisak, M.D., F.R.C.P., F.A.A.N., F.A.N.A. (✉) • J.A. Benjamins, Ph.D.
Departments of Neurology and Immunology and Microbiology,
Wayne State University School of Medicine, Detroit, MI, USA
e-mail: rlisak@med.wayne.edu

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that are predominately downregulatory and protective, some of the same factors that are proinflammatory in disease states such as in multiple sclerosis (MS), acute disseminated encephalomyelitis (ADEM), acute inflammatory demyelinating polyneuropathy (AIDP), and chronic inflammatory demyelinating polyneuropathy (CIDP) and in animal models including experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune neuritis (EAN) can in later stages of classical inflammation become important in recovery and regeneration (Lisak 2007). Paradoxically, these same "proinflammatory" and "downregulatory" secretory factors, including chemokines and cytokines, also have important direct and indirect roles during normal development of the CNS and PNS as well as protective and reparative roles in the mature nervous system (Robinson et al. 1998; Bagri et al. 2002; Tsai et al. 2002; Mizuno et al. 2003; Chalasani et al. 2003; Lisak et al. 1997, 2006; Lisak and Benjamins 2007). We have seen that cells of the CNS and PNS, both glia and neurons, can respond to classic mediators of inflammation and not just serve as a target for inflammatory-mediated damage. In addition these same endogenous cells, especially glia, can produce some of these same factors which originally had been thought to be produced only by cells of the classic inflammatory system (Lisak and Benjamins 2007; Aloisi et al. 1992; Guo et al. 1998; Lieberman et al. 1989; Rus et al. 1992; Xiao et al. 1998a; Wagner and Myers 1996; Lisak et al. 2007). The reverse has also been demonstrated; cells of the immune system can produce growth factors, such as nerve growth factor (NGF), brain-derived nerve growth factor, and neurotrophin 3 (NT3) (Kerschensteiner et al. 1999; Hohlfeld et al. 2000; Stadelmann et al. 2002; Chen et al. 2003), originally thought to be produced only by endogenous cells of the nervous system. Even neurons and oligodendrocytes are now known to be active participants in inflammation within the CNS, rather than simply targets of inflammatory and other cytotoxic molecules (Lisak et al. 2007, 2011; Suzumura et al. 1986; Middleton et al. 2000; Rose et al. 2009).

Cytokines are able to induce regulation of genes for other cytokines as well as the levels of synthesis and secretion of cytokines (Lisak et al. 2006). In addition they are also able to regulate genes and the production of a wide array of inflammatory and growth factors by CNS cells and dramatically affect regulation of hundreds of genes and cellular phenotypes of endogenous cells of the CNS and PNS (Lisak et al. 1998, 2007, 2009; Ozaki et al. 2008). The discovery that microglia are members of the monocyte/macrophage lineage and are derived during development from the peripheral immune/inflammatory cells (Ting et al. 1983; Barron 1995; Simard et al. 2006), the yolk sac equivalent, has also opened up new areas of research including the role of cells of the CNS as part of the innate immune system. They therefore are similar yet different from peripheral bone marrow-derived circulating monocytes which can circulate and enter parenchyma, including the CNS parenchyma (Aguzzi et al. 2013). Microglia can upregulate major histocompatibility (MHC) class II antigens (Lisak et al. 2006, 2007; Suzumura et al. 1987), present antigen (Aloisi et al. 1998; Cash et al. 1993), express receptors that allow them to bind and react to microbial products and products of damaged and dead endogenous cells, and initiate inflammatory responses, including toll-like receptors (TLRs) (Aloisi et al. 1998; Albright and Gonzalez-Scarano 2004; Appel et al. 1995; Banati et al. 1993; Barron 2003;

Bedard et al. 2007; Benveniste 1997; Block and Hong 2005; Block et al. 2007; Carson et al. 1998; Chen et al. 2006; Constantinescu et al. 1996; Cuzner et al. 1994; Doi et al. 2009; Duke et al. 2004; Gehrmann et al. 1995; Hanisch 2002; Hanisch and Kettenmann 2007; Jack et al. 2005; Kettenmann et al. 2011; Kim and de Vellis 2005; Kreutzberg 1996; Magnus et al. 2005; Merson et al. 2010; Satoh et al. 1995; Ulvestad et al. 1994). It is also of interest that various stimuli, including cytokines and cells undergoing cell death, can also upregulate TLRs. Microglia can also be activated by other "response molecules" called pattern recognition receptors (PRRs) that recognize pathogenic reception-associated molecular patterns (PAMPs) classically associated with microbial molecules and damage-associated molecular patterns (DAMPs) seen with damaged cells (Kumar et al. 2011; Tabas and Glass 2013). TLRs are able to respond to both microbial products and molecules from damaged cells (Jack et al. 2005; Hertzog et al. 2003; Iliev et al. 2004; Kim et al. 2009). These responses can be protective but if prolonged can turn pathogenic (Aguzzi et al. 2013; Iliev et al. 2004; Takeuchi et al. 2005; Krasowska-Zoladek et al. 2007; Lee et al. 2006; Yoon et al. 2008). Microglia also have upregulated or can upregulate the necessary co-stimulatory molecules required for successful cell activation associated with antigen presentation (De Simone et al. 1995).

Astrocytes also participate in both positive and negative responses to microbial molecules as well as to damaged and dying cells (Sawada et al. 1995; Ma et al. 2013). This allows the CNS to both initiate inflammation and interact with the adaptive (cognate) immune system early in diseases characterized by classical inflammatory lesions as well as become the primary inflammatory/immune mechanism later in the course of the disease in certain diseases like multiple sclerosis (MS) (Gandhi et al. 2010). The role of Schwann cells and endogenous mononuclear cells within the PNS is not as certain, particularly in vivo (Lisak and Benjamins 2007; Scarpini et al. 1990; Skundric et al. 2001). Finally cell–cell interactions between endogenous cells of the CNS and PNS are involved in disease pathogenesis but also involved in normal development of the nervous system and likely in protection and repair as well (Lisak 2007).

1.2 Classic Inflammation in the Nervous System

Within the CNS, and perhaps to a lesser extent in the PNS, acute inflammation is usually viewed as precipitated by infectious processes or "autoimmune" or "immunopathogenic" diseases, but classical inflammation is also important in response to trauma and ischemia, albeit to a lesser degree (Zhang et al. 1994, 1995; Chen et al. 1994; Schwartz 2000; Schwartz et al. 1999; Yoles et al. 2001; Ziv et al. 2006). In different settings the cells and their secretory products (including cytokines, chemokines, and vasoactive substances), as well as humoral immune/inflammatory factors including immunoglobulins (antibodies), immunoglobulins bound to circulating antigen (immune complexes), and complement components are involved in the inflammatory response. These processes evolve into subacute and then chronic

inflammation before resolving in self-limited infectious disorders (although often leaving evidence of damage to the underlying tissue), but continuing in different patterns in other diseases, most striking in MS.

In the instance when direct infection of the CNS by many different microbial agents is the cause of inflammation, the innate immune system, including some of the endogenous cells of the nervous system that are part of the innate immune response, along with polymorphonuclear leukocytes (predominately neutrophils) brings about inflammation in the nervous system. This is clearly a protective function as long as it does not persist past the need, although unquestionably there can be some damage to the CNS during protective phases of acute inflammation, a type of innocent bystander damage. In parasitic infections as well as in some autoimmune/immunopathologically mediated disorders, eosinophils are involved, along with lymphocytes and exogenous cells of the monocyte/macrophage lineage. These types of classical inflammation are beyond the scope of this chapter.

Exogenously derived mononuclear cells predominate in classical inflammation, their presence and activation generally triggered by cells of the innate immune system. The cells of the innate immune system involved in inflammation in the CNS may be endogenous or part of the systemic immune system. Activation of the peripheral immune system cells results in entry of lymphocytes and mononuclear cells into the nervous system parenchyma during the acute phase of classic inflammation, through a complex process involving adhesion to vascular endothelium and then entry into the parenchyma itself (Agrawal et al. 2011; Alter et al. 2003; Brundula et al. 2002; Uhm et al. 1999; Yong et al. 2001). T cells activated in the peripheral immune system, through recognition of their specific antigen presented by antigen presenting cells (APCs; including monocytes, dendritic cells, and B cells) and the presence of co-stimulatory molecules, release cytokines and also upregulate adhesion molecules which are important in the adherence of inflammatory cells to the vascular endothelium and ultimately entrance into the parenchyma (see below). It has also been recently appreciated that B cells also enter the CNS and the process is also complex involving adhesion molecules as well.

Although the majority of these exogenous cells do not specifically recognize the presumed autoantigen (Cohen et al. 1987; Steinman 1996) or antigen of a microbial agent, this phase of classic inflammation is considered to be a function of the adaptive or cognate immune system. Proinflammatory cytokines produced by activated effector T cells in concert with cells of the CNS endogenous innate immune system or the APCs of the peripheral immune system lead to release of chemokines (small molecular weight cytokines) which attract cells, including the initial activated cells, which in turn results in activation and infiltration of additional hematogenous cells as well as activation of endogenous cells, in particular microglia and astrocytes. Cytokines released by Th1 and likely by Th17 cells stimulate the glial cells to upregulate genes specific for the so-called proinflammatory cytokines (Lisak et al. 2006). Production of cytokines and chemokines by the glial cells themselves has also been reported (reviewed in Lisak et al. 2006).

The process of entry of exogenous inflammatory cells involves the breaching of the normally intact blood–brain barrier (BBB) and changes in function of the

vascular endothelial cells, pericytes, astrocytes (foot processes of astrocytes are involved in maintaining the BBB), and microglia (Dore-Duffy 2008; Dore-Duffy et al. 1994). When antigen-specific T cells "see" their cognate antigen presented by an APC, likely the microglia in the CNS, in the presence of co-stimulatory molecules, further activation or reactivation occurs with release of cytokines and chemokines (Frohman et al. 2006). This leads to a complex series of interactions of different exogenous and endogenous cells as well as products of those cells that is still incompletely understood. It should also be noted that changes in oligodendrocytes and activation of microglia have been reported in parts of the brain in patients with RRMS, prior to pathologic evidence of classic perivenular mononuclear cell cuffs (Barnett and Prineas 2004). This has led some to speculate that the initial event that results in the classical acute inflammatory lesions is the result of a trigger by damaged oligodendrocytes and microglia activated by that damage (an oligodendroglial cytopathy). An alternative is that microglial activation is the initial abnormality, followed by damage in the white matter of the most sensitive cells, the oligodendroglia, and subsequent classical acute, subacute, and chronic inflammation.

In addition to the role of chemokines and cytokines in activating other inflammatory cells and endogenous cells of the nervous system, exogenous and endogenous inflammatory cells can cause direct damage to cells of the nervous system including oligodendrocytes and their myelin and neurons including axonal processes (Aguzzi et al. 2013; Banati et al. 1993; Block and Hong 2005; Block et al. 2007; Cardona et al. 2006; Chao et al. 1995; Sargsyan et al. 2005; Stefanova et al. 2007). Production of free oxygen radicals, nitric oxide and its metabolites, and excitatory amino acids and other transmitters contributes to damage as well. Cytokines are critical in upregulation of these toxic molecules (Aschner 1998; De Keyser et al. 2003; Farina et al. 2007; Guenard et al. 1994; John et al. 2005; Julien 2007; Williams et al. 2007a). In MS and in the animal model of MS, acute EAE, there is clearly synergy between the cellular immune response and complement-mediated damage likely elicited by the presence of immunoglobulins containing antibodies to constituents of the nervous system, in some experimental paradigms (Genain et al. 1995; Linington et al. 1988; Massacesi et al. 1995). In neuromyelitis optica (NMO), antibody to aquaporin 4, complement activation, Th17 cells, and Th17 typical cytokines are involved in the damage to astrocytes (NMO is initially an "astrocytopathy") with subsequent damage to other cells and their processes (Iorio et al. 2013; Lennon et al. 2004, 2005).

In some acute viral infections some damage is due to effects of the virus on cells of the nervous system, but it is also clear that the nervous system is damaged as an "innocent bystander" by the very cells involved in its defense (Cole et al. 1971; Gilden et al. 1971).

The mechanism of damage apparently can involve multiple molecular mechanisms including direct cytotoxicity, apoptosis, demyelination, and interference with metabolism and other functions of the cells of the target organ (Floden et al. 2005; Tolosa et al. 2011; Urushitani et al. 2001; Akassoglou et al. 1998; Andrews et al. 1998; Barres et al. 1992; Boullerne et al. 1999; Cammer 2001; Casaccia-Bonnefil et al. 1996; D'Souza et al. 1996; Gu et al. 1999; Jana and Pahan 2007; Jurewicz et al. 1998; Leuchtmann et al. 2003; Matute et al. 1997; Matysiak et al. 2002; Merrill

and Zimmerman 1991; Merrill et al. 1993; Selmaj et al. 1991a; Selmaj and Raine 1988; Zhang et al. 2005). In addition these "proinflammatory" cytokines inhibit the production of factors important for normal function of the nervous system as well as factors important for protection and regeneration (Lisak et al. 2007, 2009, 2011). Paradoxically some of the same cytokines can have protective effects on neurons and oligodendrocytes and their precursors (Merrill and Benveniste 1996; Benveniste and Merrill 1986; Benveniste 1998; Antel 2006; Arnett et al. 2001; Deierborg et al. 2010; Franklin et al. 1991; Kotter et al. 2001; Scurlock and Dawson 1999; Soane et al. 2001; Benjamins et al. 2011).

As lesions evolve there are changes in the subsets of mononuclear cells, which were not apparent with simple histological stains used in early studies. However, with newer molecular techniques that identify different phenotypic markers for these subsets of cells as well as identifying cell activation and upregulation of different products produced by different subsets of lymphocytes and monocytes/macrophages, it has become apparent that the situation is exceedingly more complex than originally thought. These cells predominately produce a different variety of cytokines and chemokines as well as other factors including what had been traditionally believed to only be produced by cells of the nervous system itself, i.e., growth factors (Chen et al. 2003; Hohlfeld et al. 2006; Ziemssen et al. 2002, 2005). The cytokines/chemokines and other factors produced by these so-called anti-inflammatory or downregulatory cells seem to inhibit the "proinflammatory" cells and the "proinflammatory" cytokines but also have direct protective and regenerative capacities. In addition to the direct protective and regenerative potential of the cytokines, these "anti-inflammatory" cells also have the capacity to induce production of growth factors and anti-inflammatory cytokines by endogenous cells of the nervous system (reviewed in Lisak et al. 2006, 2007, 2009).

It is critical to remember that the idea that there is a clear-cut dichotomy of cytokines into "proinflammatory/upregulatory" cytokines and "anti-inflammatory/downregulatory" cytokines is an oversimplification (Lisak 2007). It is also an oversimplification that cells cannot produce both "proinflammatory" and "anti-inflammatory" cytokines. As an example, while tumor necrosis factor-alpha is clearly important in pathogenesis of inflammatory disease of the nervous system, this cytokine may be important in downregulating autoimmune T cells, in remodeling CNS parenchyma for recovery and has other protective effects in the CNS and PNS (Selmaj and Raine 1988; Brosnan et al. 1988; Buntinx et al. 2004; Caux et al. 1993; Chandler et al. 1997; Chao and Hu 1994; Downen et al. 1999; Hofman et al. 1989; Larrick and Wright 1990; Laster et al. 1988; Neumann et al. 2002; Selmaj et al. 1991b; Chen et al. 1996). Much may depend on the balance of the two receptors for this cytokine (Heller and Kronke 1994; Skoff et al. 1998). IL-3 can be both pathogenic and protective (Caux et al. 1993; Chavany et al. 1998; Frei et al. 1986; Kannan et al. 2000; Wen et al. 1998). IL-1 contributes to the pathogenesis of inflammatory disorders of the CNS and PNS (Skundric et al. 2001; Leonard et al. 1995) but it may also have protective effects (Lisak et al. 1997, 2006; Bissonnette et al. 2004). The relative proportion of different cytokines, differences between

cell-bound and soluble cytokines, the relative proportion of different receptors, and the time of the evolution of the lesion are critical in whether a cytokine is contributing to disease or inhibiting disease (Lisak 2007). In the case of neurotrophic growth factors such as NGF and other members of the NGF family including brain-derived neurotrophic factor (BDNF) and NT3, the relative proportion of the pro-development/protection/repair receptors that signal through tyrosine kinases (trkA, trkB, and trkC) and the cell death-inducing p75NGFR, a member of the TNF receptor (TNFR) superfamily, as well as other interactions of the signaling pathways may determine whether these neurotrophic factors are protective or may contribute to cell damage (Bredesen and Rabizadeh 1997; Chao 2003).

In the instance of pathogens eliciting the classic inflammatory responses, acute with evolution to subacute and chronic phases, eventually there is clearing of the exogenous cells with evidence of changes in endogenous cells, often increases in the number and prominence of astrocytes and microglia along with loss of or evidence of damage to neurons/axons and oligodendrocytes/myelin. Changes in vessels including thrombosis and scarring are also seen. It has become clear that astrocytes and microglia are important in the formation and maintenance of synapses and have other protective effects on neurons, and thus changes in these cells induced by cytokines and chemokines can have consequences for normal nervous system function (Aguzzi et al. 2013; Albrecht et al. 2002, 2003; Horner and Palmer 2003; Mazzanti and Haydon 2003; Nedergaard et al. 2003; Slezak and Pfrieger 2003; Sofroniew 2005; Altman 1994). This is also true in nonclassical inflammation (see below). In the PNS the perisynaptic Schwann cells are important in formation and maintenance of the synapse, a situation similar to the role of the astrocyte, and perhaps the microglia, in the CNS.

1.3 Nonclassical Inflammation

In the case of diseases with presumed autoimmune etiology, many areas of the CNS continue to show changes of acute as well as subacute and chronic classical inflammation (Kutzelnigg et al. 2005; Trapp and Nave 2008; Lassmann 2010). In addition there is an evolution, not well understood, in the CNS, into what might best be described as a chronic nonclassical inflammatory response. In a disease like MS this includes a shift to activation of endogenous cells that are part of the innate immune response (Gandhi et al. 2010). In the recent emphasis on these endogenous cells which function as part of the innate immune system, the presence of lesser numbers of exogenous inflammatory cells, lymphocytes of different subsets, in a diffuse pattern in the CNS, seems to have been overlooked or discounted by many. This has led some to characterize this phase of MS, often in secondary progressive MS (SPMS) and primary progressive MS (PPMS), incorrectly, as noninflammatory/purely neurodegenerative. In PPMS we know very little about the early stages from the point of view of pathogenic mechanisms.

What is also important is that the cells of the innate immune system, particularly microglia and astrocytes, are activated and seem to be involved in the pathogenesis of, and perhaps an attempt to inhibit, diseases thought of as "purely" neurodegenerative, including Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD). This seems to be true whether one is dealing with the more common sporadic or less common inherited forms of these disorders (Aguzzi et al. 2013; Sargsyan et al. 2005; Henkel et al. 2004; Hickman et al. 2008; Naert and Rivest 2011; Borchelt 2006; Depino et al. 2003; Kim and Joh 2006). When such inflammation occurs in diseases in which there is no obvious preceding classical inflammation, as in diseases like AD, PD, and ALS, the term "neuroinflammation" has been used (Aguzzi et al. 2013). In stroke or trauma the situation would seem to be different than in these more classically degenerative diseases, although clearly differing from RRMS evolving into SPMS.

In the early stages of such a nonclassical inflammatory response triggered by cell damage and death, often of neurons, one can view this as a protective response including clearing misfolded proteins, dead cells, and extracellular material such as amyloid in Alzheimer's disease (Chen et al. 2006; Butovsky et al. 2005; Combs et al. 2001; El Khoury et al. 2003) and other proteins in other disorders (Tydlacka et al. 2008; Gow et al. 1998; Southwood et al. 2002; Anderson et al. 2008, 2009; Arai et al. 2006; Avila et al. 2004; Goris et al. 2007; Popescu et al. 2004). However, when this process goes on, that is, the nonclassical "proinflammatory" response does not switch off, perhaps because of the continued stimulus, the inflammatory response now contributes to CNS damage (Aguzzi et al. 2013). This has led to attempts to inhibit this inflammatory/immune response as a therapeutic strategy in these disorders (Krause and Muller 2010; Aisen 2002; Breitner 1996; Bachstetter et al. 2012). Unfortunately clinical trials of anti-inflammatory agents have yet to prove to be successful (Jaturapatporn et al. 2012). However, it must be remembered that nonclassical inflammation/immune response is just as likely to be as complex as the more classic inflammatory/immune response and simple suppression of all inflammation might well prove to be counterproductive since the initial activation presumably was an attempt to clear cells damaged by various degenerative stimuli. As an example, an increase in astrocytes could be detrimental to neuronal outgrowth as well as to entry of Schwann cells into the spinal cord, in an attempt to provide a source of myelination, either by presenting a physical barrier or by secreting toxic substances and/or factors that inhibit migration (Guenard et al. 1994; Julien 2007; Williams et al. 2007a, b; Sofroniew 2005; Ahlemeyer et al. 2002; Corley et al. 2001; Liuzzi and Lasek 1987; McKeon et al. 1991; Sidoryk-Wegrzynowicz et al. 2011; Shirvan et al. 1999, 2002). However, astrocytes, as previously noted, are important to formation and maintenance of the synapse, are involved in the integrity of the BBB, and by taking up excitatory amino acids and excess of electrolytes and via other activities, can protect the CNS and help with remyelination and repair (Williams et al. 2007a; Slezak and Pfrieger 2003; Sofroniew 2005; Xiao et al. 1998b; Ye and Sontheimer 1998). It is also of interest that proteins of the complement cascade may be involved in CNS degenerative diseases (reviewed in Aguzzi et al. 2013) in a different manner than as part of classical immunologically mediated demyelinating diseases as in RRMS (Lucchinetti et al. 2000; Storch et al. 1998),

and that proinflammatory cytokines induce upregulation of genes for some of these proteins in cultures of glial cells (Lisak et al. 2006). One could speculate, however, that complement could be important in pruning of synapses during development.

1.4 Chronic Meningeal Inflammation

Meningeal inflammation has always been recognized in the pathogenesis of microbial infections of the CNS without or with direct infection of the parenchymal cells themselves. The presence of inflammatory cells and inflammatory mediators as well as inflammation in blood vessels traversing the meninges can lead to parenchymal dysfunction and actual damage to the underlying parenchyma. These effects are important in disease symptomatology and outcome.

Recently the involvement of the meninges in MS has become clear, both in SPMS (Magliozzi et al. 2007; Serafini et al. 2004) and in some patients in the earliest stages of MS (Lucchinetti et al. 2011). While the meningeal lesions can be considered a type of classical acute, subacute, and chronic inflammation, the possible relationship of these meningeal lesions to neurodegeneration in the underlying cortical gray matter represents another type of nonclassical neuroimmunologic pathogenic mechanism. In SPMS and in early RRMS there are atypical germinal center-like structures which include B cells, and the underlying pathology of the cerebral cortex is proportionate to the extent of the cortical demyelination and damage to oligodendrocytes and neurons/axons (Lucchinetti et al. 2011; Howell et al. 2011). It should be emphasized that in types II and III cortical damage in MS is relatively acellular (Bo et al. 2003a, b). This has led to the hypothesis that in type III subpial damage (demyelination and axonal/neuronal damage), factors secreted by cells in the meninges, perhaps B cells, CD8 T cells, or both, diffuse into the cortex and produce the cortical damage. While B cells and their progeny, plasmablasts and plasma cells, could well be producing antibodies locally which diffuse into the cortex, there is no evidence for immunoglobulin or complement activation products in the cortex in MS. B cells produce cytokines which could produce this damage (Lisak et al. 2012). The presence of this meningeal inflammation in early stages of MS has also led some to believe that this is the primary lesion in the evolution of MS, with cells first entering the CNS via the meninges and/or choroid plexus, then subsequently leaving the CNS to reenter the systemic immune system. These cells that now recognize CNS antigens become activated in response to as yet unknown stimuli, perhaps during systemic inflammatory responses to infectious agents, and subsequently enter via the classical perivenular lesions of classical RRMS (Lucchinetti et al. 2011). It is of interest that in the development of lesions of EAE the earliest pathology is inflammation of the meninges (Alvord et al. 1959). Thus one could view this as still one more type of nontraditional inflammation, that is, one where there are degenerative changes accompanied by microglial activation as the result of a more classical inflammatory response adjacent to but not in the parenchyma of the CNS itself. Most recently it has been reported that in PPMS there is a more diffuse meningeal inflammatory response, without the focal atypical germinal follicle-like structures (Choi et al. 2012).

1.5 Summary

The chapters in this book detail with how glial cells, including oligodendrocytes, interact with neurons/axons and with one another in both classical and nonclassical inflammatory states including degenerative diseases of the nervous system. It is clear that the cells of the nervous system are active participants in these responses, particularly in the nonclassical inflammatory responses, both protective and destructive. An increase in our knowledge of these interactions is important for progress in treatment of diseases characterized by classic inflammation like RRMS and NMO, as well diseases where nonclassical inflammation seems to be most important like SPMS, PPMS, and some classical neurodegenerative diseases.

Conflict of Interest The authors have no conflicts of interest related to the contents of this chapter.

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Chapter 2
Neuroinflammation in Neurological Disorders

Jun-ichi Kira

Abstract Neuroglial inflammation is a pathological hallmark of neuroimmunological disorders, such as multiple sclerosis, as well as neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease. Activated microglia and reactive astroglia accompany the loss of neurons and myelin in these conditions. Both microglia and astroglia can exert neuroprotective and neurotoxic functions, which are stage-dependent. Both cell types can switch from an anti-inflammatory/neuroprotective to a proinflammatory/neurotoxic phenotype according to the surrounding environmental stimuli. Deciphering glial dual actions may provide insights for the management of neuroglial inflammation and the future development of new drugs targeting glia in neuroimmunological and neurodegenerative diseases.

2.1 Introduction

Demyelinating diseases, such as multiple sclerosis (MS) and neuromyelitis optica (NMO), are representative neuroimmunological diseases that affect the central nervous system (CNS). Demyelinating disorders are thought to be triggered by immune-mediated mechanisms although this has not been conclusively proven to date. MS and NMO subsequently develop neurodegeneration in addition to inflammatory demyelination. The accumulating disability and the resultant chronic disease progression in these conditions are likely determined by secondary neurodegeneration. In the chronic progressive phase of MS, T cell infiltration subsides while CNS-compartmentalized glial inflammation becomes dominant, which induces continuous tissue degeneration. Neurodegenerative disorders, such as

J. Kira, M.D., Ph.D. (✉)
Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
e-mail: kira@neuro.med.kyushu-u.ac.jp

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Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS), are triggered by neuronal abnormalities but are subsequently accompanied by neuroglial inflammation. Increasing evidence suggests that neuroglial inflammation determines disease progression, which is a clinical reflection of neurodegeneration. Therefore, neuroimmunological and neurodegenerative diseases share glial inflammation as an indispensable component of the disease processes, which underscores the importance of elucidating the mechanisms of glial inflammation. This review chapter describing neuroinflammation in neurological disease will focus on MS as a representative neuroimmunological disease afflicting the CNS, and ALS as a neurodegenerative disease accompanied with glial inflammation.

2.2 Neuroglial Inflammation in MS

2.2.1 Clinical Aspects Related to Neuroglial Inflammation and Neurodegeneration

Most MS patients initially develop a relapsing remitting disease course with a mean age of onset around 30 years of age (termed relapsing remitting MS, RRMS). After 10–20 years, approximately 50 % of RRMS patients enter a secondary progressive phase with or without superimposed relapses (termed secondary progressive MS, SPMS). Approximately 10–20 % of MS patients exhibit a relentlessly progressive disease course from the onset (termed primary progressive MS, PPMS). Clinical relapse is often accompanied or even preceded by appearance of contrast-enhanced magnetic resonance imaging (MRI) lesions in the CNS. Recent 7 T MRI studies clearly showed the presence of vessels in the center of MS lesions (Mistry et al. 2013), confirming that MS lesions develop around blood vessels. Pathologically, perivascular and diffuse lymphocytic infiltration is a common finding in active MS lesions. Thus, clinical relapse is likely to be caused by peripheral blood-borne inflammation around the blood vessels.

However, clinical relapses have only a weak effect on clinical progression (Confavreux et al. 2000). Irrespective of the initial disease course, a clinically progressive phase occurs in both SPMS and PPMS patients around 40 years of age and then proceeds at similar rate (Kremenchutzky et al. 2006). Large-scale epidemiological surveys revealed that MS patient disability, determined by Kurtzke's Expanded Disability Status Scale (EDSS) scores (Kurtzke 1983), progresses at approximately the same rate until the EDSS scores reach four, even though the progression rates varied until the development of an EDSS score of four (Confavreux and Vukusic 2006). These findings suggest that common pathogenic mechanisms may underlie clinical disability progression. At the progressive stage of MS, none of the recent disease-modifying therapies (DMT) acting on the peripheral immune system are effective, even though they have high efficacy for reducing annualized relapse rates. Thus, disease progression may have distinct mechanisms from relapse caused by peripheral immune-mediated inflammation.

Many MRI studies have reported that the T2 lesion burden in the white matter modestly correlates with disability (Fisniku et al. 2008) and rather the degree of gray matter and spinal cord atrophy correlates best with accumulating disability (Fisniku et al. 2008; Fisher et al. 2008; Bonati et al. 2011). These observations suggest that disease progression could be attributable to neuroglial inflammation compartmentalized in the CNS behind the blood–brain barrier (BBB) (Reynolds et al. 2011), which causes neurodegeneration regardless of the initial inflammatory relapses.

2.2.2 White Matter Pathology and Oligodendroglia in MS Lesions

MS predominantly affects CNS white matter that is rich in myelin. Actively demyelinating lesions are destructive lesions that are densely and diffusely infiltrated with macrophages/activated microglia that phagocytose myelin debris, as identified by Luxol fast blue staining and immunohistochemistry for myelin proteins (Lassmann et al. 1998; Lucchinetti 2007). Such lesions are associated with perivascular lymphocyte cuffing. Chronic active lesions display a rim of macrophages and activated microglia while chronic inactive lesions show no increase in macrophage/activated microglia numbers throughout the lesions. A mild global inflammation containing microglial activation and a diffuse low-level of T cells is seen even in normal-appearing white matter. Such diffuse inflammatory changes are more prominent in SPMS and PPMS than RRMS. In chronic MS, leakage from the BBB is absent, which corresponds to a paucity of gadolinium-enhanced lesions in PPMS and SPMS. Thus, compartmentalized inflammation behind the BBB is suggested based on these pathological findings.

It is widely accepted that MS pathology shows heterogeneity. Lucchinetti et al. (2000) classified four demyelinating patterns of MS lesions, and proposed that an individual only develops one pattern, suggesting a single mechanism is operative in individual patients. All lesions have inflammatory infiltrates composed of T cells and macrophages/activated microglia, while each pattern has its own specific features as follows.

Pattern I: *Active demyelination associated with the infiltration of T cells and macrophage/activated microglia in the absence of antibody and complement deposition*. These lesions are centered around veins and venules.

Pattern II: *Active demyelination associated with immunoglobulin and complement deposition*. Prominent deposition of immunoglobulins (mainly IgG) and complement C9neo antigen are found in association with degenerating myelin at the active plaque edge. This pattern is also centered around blood vessels.

Pattern III: *Distal oligodendrogliopathy characterized by selective myelin-associated glycoprotein* (*MAG*) *loss*. A profound loss of oligodendroglia at the active plaque border, DNA fragmentation, and oligodendroglial apoptosis are observed with T cell and macrophage/activated microglial infiltration but without the deposition of immunoglobulins and complement. Such lesions are not centered around blood vessels and the margin is ill-defined.

Pattern IV: *Oligodendroglial death with DNA fragmentation but without features of apoptosis in a small rim of periplaque white matter*. A near complete loss of oligodendroglia in active and inactive lesions is observed without remyelination. The border is sharply demarcated.

However, we and others have observed heterogeneous demyelinating patterns even within one autopsied individual, indicating such patterns may represent stage-dependent heterogeneity but not disease heterogeneity (Matsuoka et al. 2011). Immunoglobulin and complement deposits are found in lesions from about 50 % of autopsied MS patients (pattern II) (Stadelmann et al. 2011), suggesting that antibody and complement-mediated myelin phagocytosis might become the dominant mechanism in established MS lesions (Breij et al. 2008). Currently, the idea that an individual only develops one demyelinating pattern or can develop more than one pattern is still controversial.

Oligodendroglial cells are especially vulnerable to oxidative stress and glutamate toxicity associated with inflammation. Oligodendroglia express the AMPA/kainite receptor in the cell body and NMDA receptors in the processes. Oligodendroglia also express excitatory amino acid transporter (EAAT)-1 and -2 and are regarded as the principal cells for glutamate clearance in the white matter (Benarroch 2009). The accumulation of glutamate has been demonstrated in MS lesions by biopsy (Werner et al. 2001) and magnetic resonance spectroscopy (Srinivasan et al. 2005) while EAAT-1 and EAAT-2 are reduced in oligodendroglia (Pitt et al. 2003). In these circumstances, oligodendroglia may be vulnerable to toxicity from glutamate secreted by activated microglia. Oligodendroglia contain a large pool of iron but have a low capacity for anti-oxidative mechanisms, which render the cell especially sensitive to oxidative stress (Benarroch 2009).

2.2.3 *T Cells: A Key Player of the Effector Arm That Triggers CNS Inflammation*

Multiple sclerosis (MS) is thought to be an autoimmune disease that targets myelin antigens. This has been suggested from studies demonstrating an increased frequency of T cells showing inter- and intramolecular epitopes spreading against myelin proteins, increased levels of interferon (IFN)-γ, interleukin (IL)-17, and downstream proinflammatory cytokines in the cerebrospinal fluid (CSF), exacerbation of disease following the administration of IFNγ, and the increased frequency of T helper 1 (Th1) cells secreting IFNγ and Th17 cells secreting IL-17, which support the involvement of Th1 and Th17 cells in MS, at least for the inflammatory aspects of the disease (Ishizu et al. 2005; Tanaka et al. 2008; Matsushita et al. 2013). CD4+ T cells are present mainly in the perivascular areas while parenchyma infiltrates largely consist of CD8+ T cells (Babbe et al. 2000). Interestingly, CD8+ T cells outnumber CD4+ T cells in MS lesions (Booss et al. 1983); however, the roles of CD8+

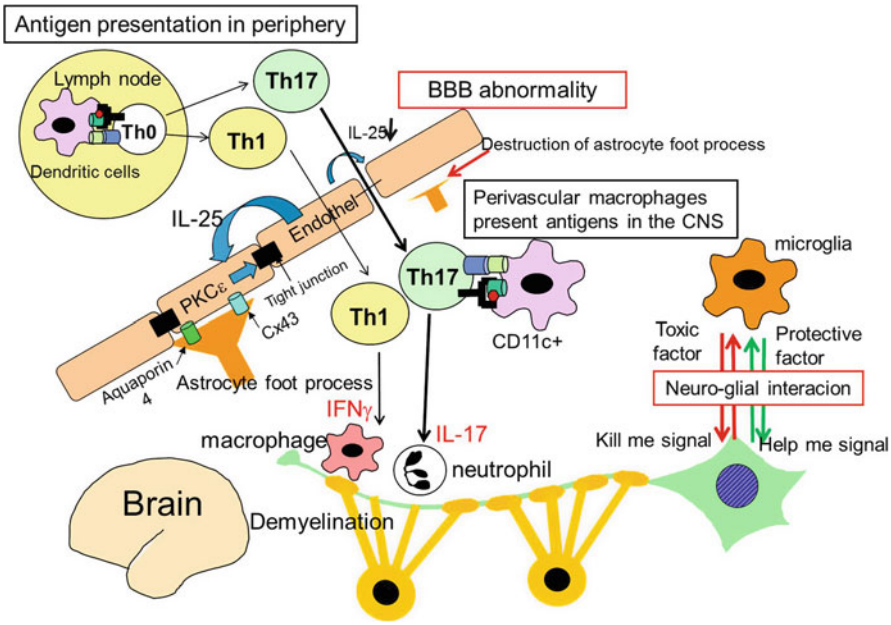

Fig. 2.1 Th17/Th1 cells are primed in the periphery and restimulated in the central nervous system (CNS). Naïve T cells (Th0) differentiate to Th1 or Th17 cells upon antigenic stimulation in the peripheral lymph nodes and enter the CNS via the blood–brain barrier (BBB). Perivascular macrophages present antigens to Th1 or Th17 cells in the perivascular space, and restimulated Th1 and Th17 cells traffic to the CNS parenchyma, secrete IFN**γ** and IL-17, and recruit macrophages and neutrophils, respectively. Microglia can produce either toxic or protective factors, sensing either "kill me" or "help me" signals from neurons. The destruction of astrocyte endfeet or decreased production of IL-25 from endothelial cells can cause the BBB to become "leaky"

T cells remain unclear. In an animal model of MS, experimental allergic encephalomyelitis (EAE), the early events in the formation of inflammatory lesions, involve a predominantly CD4+ T cell-mediated process. B cells and plasma cells also exist in the perivascular areas, but represent a minor component of inflammatory infiltrates in the CNS parenchyma (Friese and Fugger 2009; Frischer et al. 2009).

Studies from EAE demonstrated that myelin-specific CD4+ T cells could be transferred to naïve mice to induce a CNS demyelinating disease. Thus, it was hypothesized that in MS, naïve T cells are sensitized by myelin antigens in the peripheral lymph nodes, such as deep cervical lymph nodes, and differentiate to myelin antigen-specific Th1 or Th17 cells. These peripherally activated Th1 or Th17 cells express adhesion molecules that allow them to pass through the BBB and enter the CNS (Fig. 2.1). In EAE, adoptively transferred myelin antigen-specific T cells require several days to accumulate in the CNS. It was recently shown that such encephalitogenic T cells reside in bronchus-associated lymphoid tissue (Odoardi et al. 2012) and become eligible to enter the CNS.

T cells egress from postcapillary venules (high endothelial venules) and enter into the Virchow-Robins space (perivascular space) in the CNS. Here, activated T cells can firmly adhere to the surface of vascular endothelial cells via interactions between α4β1 integrin expressed on activated T cells and vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells lining the BBB. Anti-α4β1 integrin antibody, natalizumab, effectively blocks firm adhesion of T cells, thereby markedly suppressing MS relapses (Coisne et al. 2009). Thus, peripherally activated T cells can invade across endothelial cells and the endothelial basement membrane on the abluminal side and remain in the perivascular space delineated by the endothelial basement membrane and the parenchymal basement membrane, which is an extension of the subarachnoid space (Ransohoff and Engelhardt 2012). T cells require restimulation by perivascular macrophages to further traffic into the CNS parenchyma across the glia limitans perivascularis composed of parenchymal basement membrane and astrocyte endfeet (Ransohoff and Engelhardt 2012). Perivascular macrophages continuously repopulated from the peripheral blood can engulf CNS antigens in the perivascular space where myelin antigens are conveyed by the CSF flow pathway to the subarachnoid space, and present these antigens to T cells (Ransohoff and Engelhardt 2012). Subsequently, T cells restimulated by perivascular macrophages secrete matrix metalloproteinase (MMP)-2 and -9, which disrupt the basement membrane leading to destabilization of astrocyte endfeet anchored to the parenchymal basement membrane, and promote their entry into the CNS parenchyma (Bechmann et al. 2007; Tran et al. 1998). Once in the CNS parenchyma, T cells secrete proinflammatory cytokines and chemokines that further recruit macrophages, neutrophils, and activating resident microglia, which serve as effectors for tissue destruction, at least during the relapse phase.

The ability of natalizumab to markedly suppress relapses implies the critical importance of T cell involvement in CNS inflammation at relapse. However, according to MS pathology, there is considerable debate as to whether T cell infiltration is a primary event or secondary to oligodendroglial apoptosis and subsequent microglial activation. Barnett and Prineas (2004) observed oligodendroglial apoptosis without lymphocyte infiltration in autopsied cases with very early MS, and proposed that oligodendroglial apoptosis preceded the formation of all MS lesions and BBB "leakiness," and that microglial activation and T cell infiltration were secondary events. The source of the substantial debates regarding these issues is partly derived from the fact that factors causing initial oligodendroglial apoptosis remain unknown.

2.2.4 B Cells: Another Important Cell in the Effector Phase

Few plasma cells are observed in the CNS during the early stages of MS, but become increasingly prominent as disease progresses. In addition, there is an increased prevalence of oligoclonal IgG bands (OBs) in the CSF as the disease duration increases, which persist stably (Meinl et al. 2006). B cells exist in the perivascular areas and leptomeninges during all disease stages, but rarely in the CNS

parenchyma (Magliozzi et al. 2007). Autoantibody and complement-mediated myelin phagocytosis are assumed the dominant mechanism in established MS lesions, as mentioned in Sect. 2.2 (Breij et al. 2008). In the leptomeninges, ectopic lymphoid follicle-like structures have been observed in approximately 40 % of post-mortem SPMS cases (Magliozzi et al. 2007, 2010). These follicle-like structures consist mainly of CD20+ B cell aggregates interspersed with CD21+ CD35+ follicular dendritic cells (FDCs), CD4+ T cells, and CD8+ T cells. They are predominantly present in the deep cerebral sulci (Magliozzi et al. 2007, 2010). The majority of such meningeal lymphoid follicle-like structures are closely associated with large subpial demyelination (Magliozzi et al. 2007, 2010). MS cases with meningeal lymphoid-like structures showed a younger age at disease onset, a shorter time to wheelchair-bound disability, and a shorter time to progression than those without meningeal lymphoid-like structures (Magliozzi et al. 2007, 2010).

The importance of B cells in MS is clearly indicated by the fact that rituximab, that targets CD20 molecules expressed on B cells but not plasma cells, is highly efficacious in suppressing MS relapses (Hauser et al. 2008). In rituximab trials, B cell numbers decreased in parallel with the reduction of relapses, whereas total antibody levels did not decrease significantly. It is thus proposed that B–T cell interactions including antigen presentation or proinflammatory cytokine secretion by B cells is the critical step inhibited by rituximab, but not the inhibition of autoantibodies themselves. Interestingly, rituximab is also effective in NMO, where there is selective optic nerve and spinal cord demyelination in the presence of specific antibodies against astrocyte water channel protein, aquaporin-4 (AQP4) (Lennon et al. 2004, 2005), but without reducing anti-AQP4 antibody levels (Pellkofer et al. 2011). Therefore, B–T cell interactions and B cell cytokines are also thought to be critical in NMO. Highly specific autoantibodies in MS pathology remain to be identified. Autoantibodies against myelin oligodendrocyte glycoprotein (MOG) have been detected in children with atypical demyelinating disease (Brilot et al. 2009), but not in adult cases. The significance of anti-glycolipid antibodies and a recently described autoantibody against KIR4.1, an ATP-sensitive inward rectifying potassium channel expressed in astroglial endfeet and oligodendroglia (Srivastava et al. 2012), needs further confirmation in large-scale independent cohorts.

2.2.5 Gray Matter Lesions

Recently, gray matter lesions have gained much attention because they closely correlate with disability and disease progression. The introduction of double inversion recovery (DIR) MRI demonstrated that cortical lesions and cortical atrophy are present from the early stage of RRMS and become more prominent in SPMS (Fisniku et al. 2008; Fisher et al. 2008; Kutzelnigg et al. 2005; Vercellino et al. 2005). The absence of MRI evidence for noticeable inflammation suggests that neurodegeneration may take place in cortical lesions. Cortical lesion loads and atrophy are significantly associated with clinical progression (Geurts et al. 2005) whereas

white matter atrophy does not correlate with increasing disability (Fisher et al. 2008). Thus, cortical lesions may play a major role in the development of both physical and cognitive disability (Calabrese et al. 2010).

Pathologically, demyelination is present in the spinal cord and cerebral and cerebellar cortex but also in the deep gray matter, including the thalamus, basal ganglia, and hypothalamus, to varying degrees (Bö et al. 2003; Peterson et al. 2001). Cortical lesions are classified into three types: type I lesions are leukocortical lesions affecting both subcortical white matter and the lower layer of gray matter; type II lesions are entirely intracortical; and type III lesions involve the subpial gray matter regions (subpial demyelination) (Peterson et al. 2001). Frontal and temporal cortices, cingulate gyrus, and hippocampus are most frequently involved (Reynolds et al. 2011), and may explain the correlation between cognitive impairment and cortical pathology. In hippocampal demyelinated lesions, a reduction of synaptic density has also been reported (Dutta et al. 2011). However, cortical demyelination does not correlate with white matter pathologic changes (Bö et al. 2003), suggesting independent mechanisms may be operative.

Cortical lesions are accompanied with mild, if any, inflammatory infiltrates, but with increased numbers of activated microglia (Magliozzi et al. 2010; Bö et al. 2003; Peterson et al. 2001). Other differences between cortical and white matter lesions include the lack of significant leakage of plasma proteins, suggesting the BBB is preserved, and the absence of complement activation (Reynolds et al. 2011). In extensive subpial demyelination, increased numbers and activation status of microglia, increased axonal injury, and neuronal loss are greatest close to the pial surface (Magliozzi et al. 2007, 2010), implying that secretion of proinflammatory cytokines into the CSF from lymphocytes in the follicles may be responsible for the activation of microglia, cortical demyelination, and neuronal damage. In these cases, the loss of layer III and V pyramidal neurons exceeded 40 % and 50 %, respectively, and was accompanied by loss of interneurons in other cortical layers (Magliozzi et al. 2010). Cortical neuronal loss was also reported to occur diffusely even in normal-appearing gray matter (Magliozzi et al. 2010), suggesting that demyelination and neuronal loss may not be directly linked (Reynolds et al. 2011). Neuronal apoptosis and mitochondrial damage were thought to be responsible for the neuronal loss (Reynolds et al. 2011; Campbell et al. 2011; Dutta et al. 2006). However, other research groups did not confirm such a relation between meningeal lymphoid follicles and cortical demyelination (Kooi et al. 2009), and more studies are required to establish the roles of meningeal lymphoid follicles in MS.

2.2.6 Mechanisms of Axonal Injury

Acute axonal damage is accompanied by active focal inflammatory demyelination and is most prominent during the early stages of MS (Ferguson et al. 1997; Trapp et al. 1998) but decreases with disease progression (Frischer et al. 2009; Kornek and Lassmann 1999; Kuhlmann et al. 2002), suggesting that inflammation plays a

significant role in axonal loss. Cumulative axonal loss and resultant brain and spinal cord atrophy are significantly correlated with permanent disability (Frischer et al. 2009; Kuhlmann et al. 2002; Bjartmar et al. 2000). Acute damage can be detected by the presence of accumulated amyloid precursor protein (APP)-positive spheroids that reflect impaired axonal transport (Ferguson et al. 1997). APP-positive spheroids are more extensive during the first year of disease onset, and the number of acutely injured axons decrease with increasing disease duration (Kuhlmann et al. 2002). The extent of axonal loss correlates well with numbers of CD8+ T cells and macrophages/activated microglia that are present in close proximity (Kuhlmann et al. 2002) and numerous CD8+ T cells that infiltrate into CNS parenchyma transect axons possibly via major histocompatibility complex (MHC) class I-mediated self-antigen recognition (Trapp et al. 1998). Furthermore, reactive oxygen and nitrogen species and proinflammatory cytokines secreted by these cells may suppress axonal functions and cause mitochondrial damage (Dutta et al. 2006).

In limited numbers of MS cases, autoantibodies against nodal and paranodal antigens, such as neurofascin, contactin-2, and TAG-1, have been reported (Mathey et al. 2007; Derfuss et al. 2009). For example, anti-neurofascin antibody was found in one-third of MS patients, with higher prevalence in chronic progressive MS than in RRMS (Mathey et al. 2007). Neurofascin 186 expressed on the axolemma at the node of Ranvier concentrates voltage-gated sodium channels at the node while neurofascin 155 expressed on oligodendroglial membranes connects them to axons via binding to contactin-1 and Caspr1 (Ratcliffe et al. 2001; Sherman et al. 2005). Autoantibodies to nodal and paranodal antigens can induce axonal dysfunction in vivo and may be involved in axonal damage in MS (Desmazières et al. 2012).

2.2.7 Microglia and Monocyte/Macrophage in Demyelinating Diseases

The mononuclear phagocyte system in the CNS, including peripheral blood-borne monocytes/macrophages and resident microglia, plays major roles in the effector arm of demyelinating diseases by restimulating T cells within the CNS and by damaging and repairing CNS tissue.

2.2.7.1 Roles of Microglia and Monocytes/Macrophage in MS

The origin of microglia has long been a matter of debate, but recent studies indicated microglia are derived from extraembryonic yolk sac myeloid cells. Colony stimulating factor 1 receptor (CSFIR) is a cell-surface receptor for the cytokines CSF-1 and IL-34. CSF1R is usually expressed on monocytes and macrophages in the peripheral blood as well as on the surface of microglia in the CNS (Ransohoff and Cardona 2010). During fetal development, yolk sac myeloid cells colonize in the CNS due to IL-34 signaling through CSF1R. Once colonized, such cells lose

surface markers characteristic of mononuclear phagocytes, and are assumed to become microglia in adults (Ransohoff and Cardona 2010). CSF1 on CSF1R signaling is associated with survival, proliferation, regulation, and differentiation of microglia (Wang et al. 2012). In adult CNS, microglia consist of more than 10 % of all cells. In the resting state, microglia have a small body with extensively branched processes and are termed "ramified microglia." Microglia expressing CD11b, ionized calcium-binding adapter molecule 1 (Iba1), and CD68 constantly monitor the CNS environment (Ransohoff and Cardona 2010). Upon activation, the soma is enlarged while processes are retracted and these cells are termed "amoeboid microglia." Myeloid cell markers are enhanced on "amoeboid microglia."

Microglia are the only cells that express CX3CR1 in the CNS. CX3CR1-deficient mice develop severe EAE and increased neuron loss in a transgenic model of ALS (Cardona et al. 2006). Its ligand CX3CL1 is produced by neurons and downregulates microglial neurotoxicity. A lack of CX3CL1 input from neurons rapidly activates microglia (Ransohoff and Cardona 2010). Furthermore, plasma fibrinogen extravasated from disrupted BBB also can activate microglia (Ransohoff and Cardona 2010). Activated microglia produce numerous cytokines/chemokines, growth factors, reactive oxygen and nitrogen species via oxidative burst, and inducible nitric oxide synthase (iNOS). Activated microglia can express MHC class II molecules and costimulatory molecules. However, they never traffic to the draining lymph node, unlike dendritic cells in other tissues.

In the CNS, peripheral blood-borne perivascular and meningeal macrophages play a major role in antigen presentation to restimulate T cells. Without restimulation by relevant antigens, T cells do not survive in the CNS. The recruitment of monocytes/macrophages is mediated by CCL2–CCR2 signaling. Hypertrophic astrocytes in active MS lesions express CCL2 while its receptor, CCR2, is expressed on monocytes/macrophages (Mahad and Ransohoff 2003). CSF CCL2 levels are decreased in MS (Mahad et al. 2002), which is presumably because of its consumption by infiltrating cells (Mahad et al. 2002). CCR2-deficient mice develop mild EAE with neutrophil infiltration (Yamasaki et al. 2012). Thus, macrophages play major roles in antigen presentation and tissue destruction while microglia induce tissue damage. However, microglia may also have neuroprotective properties through phagocytizing tissue debris and producing neurotrophic substances.

2.2.7.2 Hereditary Microgliopathy Showing Widespread Myelin and Neuroaxonal Loss

Hereditary diffuse leukoencephalopathy with axonal spheroids (HDLS), a rare autosomal dominant disease characterized by cerebral white matter degeneration with axonal spheroids presenting cognitive decline, depression, and motor impairment, is caused by mutations in the *CSF1R* gene (Rademakers et al. 2012). As mentioned in Sect. 2.2.7.1, IL-34 signaling through CSF1R is related to microglial migration into the CNS during the embryonic period, while CSF1 signaling is associated with

survival, proliferation, and differentiation of microglia (Wang et al. 2012). We recently observed that microgliopathy caused by *CSF1R* mutation in HDLS causes myelin and axonal loss in the CNS, where CD4+ and CD8+ T cell infiltration occurs possibly through the actions of cytokines/chemokines produced by reactive microglia (Saitoh et al. 2013). In addition, a primary microglial disease known as Nasu–Hakola disease (NHD) is characterized by white matter degeneration and bone cysts. The recessive loss of function mutations in the gene encoding triggering receptor expressed on myeloid cells 2 (TREM2) and transmembrane adaptor signaling protein DAP12 that transduces TREM2 signals in NHD causes a lack of microglia and osteoclasts (Paloneva et al. 2000, 2002; Kondo et al. 2002). The TREM2–DAP12 protein complex is crucial for proliferation and survival of mononuclear phagocytes and is related to CSF1R signaling (Otero et al. 2009). That genetic mutation of indispensable molecules in microglial development and function causes diffuse myelin and axon degeneration underscores the critical roles of microglia in the maintenance of myelin and neurons. Thus, in HDLS and NHD, it has been suggested that microglial maintenance of myelin turnover is disrupted. Therefore, the disruption of normal microglial maintenance functions may exacerbate neurodegenerative process in neuroglial inflammation.

2.2.8 Roles of Astrocytes in Demyelinating Disease

Astrocytes normally have neuroprotective functions while in inflammatory circumstances they become neurotoxic. Such biphasic behavior of astrocytes makes neuroglial inflammation more complex.

2.2.8.1 Neuroprotective Aspects

Astroglia extend numerous processes, forming highly organized domains with little overlap between adjacent cells. Astroglia appose each other and interconnect via Cx43 gap junction channels to form functional networks. Highly ramified protoplasmic astrocytes in the gray matter ensheath synapses, forming tripartite synapses, while fibrous astrocytes in the white matter cover the nodes of Ranvier (Miller and Raff 1984). Astrocyte endfeet also have close contact with parenchymal basement membrane around vessels and contribute to maintenance of the BBB through the induction of tight junctions between endothelial cells (Janzer and Raff 1987). Astroglia also produce components of extracellular matrix, such as collagens, laminins, fibronectins, hyaluronan, chondroitin sulfate, and heparin sulfate (Zimmermann and Dours-Zimmermann 2008; van der Laan et al. 1997; van Horssen et al. 2007), which constitute the basal lamina around vessels. Astroglia constitutively express the membrane bound death ligand, CD95L, and can induce CD95L-mediated apoptosis of infiltrating T cells (Bechmann et al. 1999, 2002). Astroglia also secrete

tissue inhibitor of metalloproteinase (TIM), thereby limiting disruption of basement membrane and extracellular matrix by MMPs secreted by infiltrating T cells (Miljković et al. 2011). Ablation of proliferating astroglia exacerbates EAE and is associated with the massive infiltration of macrophages and T cells (Voskuhl et al. 2009), suggesting critical roles of astroglia in preventing the expansion of inflammation. We demonstrated that CSF levels of angiotensin and angiotensin-converting enzymes produced and secreted from astrocyte endfeet were significantly decreased in NMO (Matsushita et al. 2010) and MS (Kawajiri et al. 2008, 2009) patients, suggesting that injury of astrocyte endfeet and dampening of astrocytic barrier functions may occur in both MS and NMO.

Astroglia also produce a variety of growth factors that promote oligodendrocytes to form myelin (Moore et al. 2011) by influencing oligodendroglial progenitor cells (OPCs) (Gallo and Armstrong 2008). IL-6 and transforming growth factor (TGF)-β produced by activated astrocytes may promote neuroprotection (Allaman et al. 2011). A recent study showed that ablation of astroglia in glial fibrillary acidic protein (GFAP)-thymidine kinase transgenic mice with ganciclovir caused a failure of damaged myelin removal through decreased microglial activation during cuprizone-induced demyelination (Skripuletz et al. 2013). Thus, astroglia can deliver signals to microglia to clear myelin debris, thereby contributing to the regenerative process.

2.2.8.2 Neurotoxic Aspects

Activated astroglia morphologically demonstrate hypertrophy and increase expression of GFAP. Activated astroglia produce cytokines/chemoattractants as well as adhesion molecules for lymphocyte trafficking. For example, astroglia produce various proinflammatory cytokines, such as IL-1, IL-6, IL-12, IL-15, IL-23, IL-27, IL-33, CCL2 (MCP-1), CCL5 (RANTES), CXCL8 (IL-8), CXCL10 (IP-10), and CXCL12 (SDF-1). IL-12, IL-23, and IL-27 are essential for inducing Th1 and Th17 cells (Xu and Drew 2007; Kroenke et al. 2008; Markovic et al. 2009) while IL-15 is crucial for the activation of encephalitogenic CD8+ T cells (Saikali et al. 2010). CCL2 is a critical chemokine that attracts peripheral blood macrophages into the CNS (Yamasaki et al. 2012). Moreover, astroglia can express VCAM-1 and fibronectin CS-1, which are up-regulated in MS lesions (van Horssen et al. 2005; Engelhardt 2010). α4β1 integrin expressed on T cells interacts with its receptors, VCAM-1 and CS-1, thereby enabling T cells to traffic from the perivascular areas deep into the CNS parenchyma (Gimenez et al. 2004). Astroglia can express MHC class II and costimulatory molecules such as B7-1, B7-2, and CD40 (Chastain et al. 1812) dependent on the presence of IFNγ, TNF, and IL-1β (Dong and Benveniste 2001). Thus, astroglia may present autoantigens to T cells in the context of MHC class II molecules. Astroglia also produce iNOS via effects of endoplasmic reticulum stress chaperones (Saha and Pahan 2006), leading to the production of superoxide anion and peroxynitrite, which can damage oligodendrocytes harboring low antioxidant levels (Antony et al. 2004).

2.2.8.3 Hereditary Astrogliopathy

Alexander disease is caused by mutations in the *GFAP* gene (Brenner et al. 2001; Li et al. 2005), and thus is regarded as a primary astrocytic disease. Alexander disease shows leukodystrophy with macrocephaly. Its characteristic feature is the widespread presence of Rosenthal fibers containing mutant GFAP, heat shock protein 27, and αB-crystallin that are exclusively found in astrocyte foot processes and cell bodies (Quinlan et al. 2007). Rosenthal fibers are plentiful, especially in the astrocytic foot processes around blood vessels, and in subpial, subependymal, and periventricular zones (Liem and Messing 2009). Mutant GFAP may act as a toxic gain-of-function (Liem and Messing 2009). Alexander disease develops frontal dominant white matter degeneration while postnatal myelination progresses from the central sulcus to the occipital, frontal, and temporal poles. This suggests that Alexander disease is not dysmyelinogenic (Sawaishi 2009). Megalencephalic leukodystrophy with subcortical cysts (MLC) is another example of leukodystrophy caused by mutations in the *MCL1* gene that encodes a protein exclusively expressed in astrocytes (Leegwater et al. 2001). MLC1 is normally expressed in astrocyte endfeet around the blood vessels (Boor et al. 2005). These examples indicate astrocytes are important for the maintenance of myelin in the CNS and astrocytic endfoot dysfunction may induce widespread myelin loss.

2.2.9 Oligodendrocyte Precursor Cells and Remyelination in MS

Remission results from the resolution of inflammation, redistribution of ion channels along demyelinated axons, and remyelination. Many studies have demonstrated that demyelination in the MS brain and spinal cord can be followed by remyelination to a variable extent (Patrikios et al. 2006; Patani et al. 2007; Prineas et al. 1993). Remyelination is more prominent in the early stages of disease while chronic lesions have less or no remyelination. PPMS brains had a lower number of inflammatory active lesions and more complete remyelination than SPMS brains (Reynolds et al. 2011). Remyelination requires new oligodendroglia. OPCs expressing neuron glia 2, an integral membrane chondroitin sulfate proteoglycan, and platelet-derived growth factor receptor α, were shown to exist even in chronic MS lesions (Chang et al. 2000, 2002). Nonetheless, in chronic lesions demyelination tends to persist. These observations suggest that remyelination failure in MS is not attributable to the absence of OPCs, but rather the blockade of OPC differentiation to myelinating oligodendroglia. This blockade is explained by the existence of extracellular inhibitors or intrinsic intracellular blocking mechanisms. Examples of extracellular inhibitors include Jagged 1 expressed by astrocytes, which activates Notch 1 receptors on oligodendroglia to promote expression of Hes5 (John et al. 2002), a transcriptional inhibitor that blocks differentiation. LINGO-1 expressed on

astrocytes and macrophages (Satoh et al. 2007; Mi et al. 2005), PSA-NCAM abnormally expressed on demyelinated axons (Charles et al. 2002), and myelin debris (Kotter et al. 2006) can inhibit the differentiation of OPCs to myelinating oligodendroglia. A recent report showed that aggregates of fibronectin produced by astrocytes and leaked from damaged BBB inhibited oligodendroglial differentiation and remyelination (Stoffels et al. 2013). Increased expression of fibronectin in active MS lesions (Stoffels et al. 2013) may also inhibit remyelination. Furthermore, OPCs expressing Notch1 were stimulated by contactin expressed by demyelinated axons in chronic MS lesions while over-expression of TAT-interacting protein 30 kDa, a direct inhibitor of importin B, in OPCs blocked the translocation of Notch1-intracellular domains produced by cleavage of Notch1 receptor engagement (Nakahara et al. 2009). Thus, signals required for myelinogenesis are not operative in chronic MS lesions.

2.2.10 Connexins

Connexins (Cxs) form homotypic or heterotypic gap junctions between astrocytes, or between astrocytes and oligodendrocytes. Gap junctions appose two cells and form channels for direct intercellular communication through which intracellular second messengers such as calcium ions and other small molecules are exchanged. Astrocytic Cx43 and Cx30, oligodendrocytic Cx32 and Cx47, and astrocytic Cx43 and oligodendrocytic Cx32 double-knockout mice showed diffuse demyelination (Lutz et al. 2009; Magnotti et al. 2011; Menichella et al. 2003). Notably, Ezan et al. (2012) demonstrated that mice lacking Cx43/Cx30 in GFAP-positive astrocytes displayed astrocyte endfeet edema and a partial loss of AQP4. Thus, astrocytic and oligodendrocytic Cxs may play critical roles in maintaining CNS myelin.

Recently, we showed the extensive loss of Cxs43, 32, and 47 in demyelinated and myelin-preserved layers of acute lesions from patients with Baló's concentric sclerosis, a rare extremely severe variant of MS (Masaki et al. 2012). In the leading edge areas, where the expression of MAG was partly diminished with other myelin proteins well preserved, compatible with distal oligodendrogliopathy, astrocytic Cx43 was totally lost. Similar changes were also observed in MS and NMO cases culminating in death within 2 years after the disease onset (Masaki et al. 2013). It was reported that a significant reduction of Cx32 and Cx47 occurs in active lesions of MOG-induced EAE (Markoullis et al. 2012). In MOG- and myelin basic protein (MBP)-induced EAE (Brand-Schieber et al. 2005), astrocytic Cx43 was also diminished in active lesions, suggesting that myelin antigen-specific T cells may down-modulate Cx expression in oligodendrocytes and astrocytes. In the healthy state, Cx43 on astrocytes apposes Cx47 on oligodendrocytes, forming astrocyte–oligodendrocyte (A/O) gap junctions. A/O gap junctions are important for intercellular communication through this channel. Disruption of A/O gap junctions may cause the loss of glia syncytium, thereby inducing oligodendroglial damage and myelin loss (Fig. 2.2).

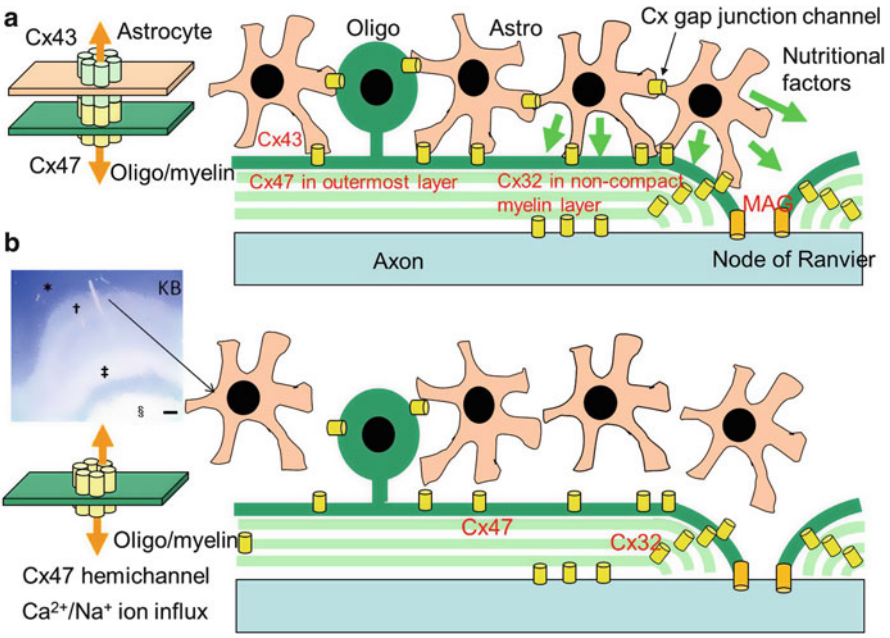

Fig. 2.2 Loss of astrocytic Cx43 and disruption of astroglia–oligodendroglia gap junction channels in the leading edge areas of Baló's disease lesions. (**a**) In the normal state, astrocyte–oligodendrocyte gap junction channels are formed by Cx43 and Cx43. (**b**) In the outer portion of the leading edge areas (*dagger*), astrocytic Cx43 is preferentially lost and therefore oligodendrocytic Cx47 forms a oligodendroglia

2.3 Neuroglial Inflammation in ALS

ALS is a progressive, fatal neurodegenerative disease where the loss of motor neurons in the spinal cord, brainstem, and motor cortex causes progressive motor paralysis. ALS usually develops sporadically; however, around 10 % of cases have a family history. A variety of susceptibility genes for familial ALS have been identified including mutations in $Cu^{2+}Zn^{2+}$ *superoxide dismutase* (*SOD1*) gene (20 % of cases). Mutations in *TADRBP*, *FUS*, *ANG*, *VCP*, *OPTN*, and *C9ORF72* genes are less frequent (Ince et al. 2011; Philips and Robberecht 2011). Although glutamate excitotoxicity, oxidative stress, neuroinflammation, and neurotrophic factor failure have been proposed to explain the pathogenesis of ALS, the mechanisms underlying ALS remain to be elucidated.

The appearance of reactive microglia and astroglia is a characteristic feature of ALS and other neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease. In this section, the review will focus on microglia and astroglia

that are thought to have key roles in neuroinflammation in neurodegenerative diseases. However, recent studies have revealed that oligodendroglia have close connections with neurons and support the metabolism of neuronal cells (Philips et al. 2013).

2.3.1 *Non-cell Autonomous Cell Death*

Neuroinflammation characterized by activated microglia and infiltrating T cells is one of the prominent pathological features of ALS (Philips and Robberecht 2011; Kawamata et al. 1992). Motor neuron-specific expression of mutant SOD1 (mSOD1) does not result in ALS-like disease (Pramatarova et al. 2001), and wild-type non-neuronal cells extend the survival of mSOD1-positive motor neurons in a mSOD1 transgenic ALS mouse model (Clement et al. 2003). The selective lowering of mSOD1 levels in microglia or astroglia slows disease progression, while reducing mSOD1 levels in neurons delays disease onset (Boillée et al. 2006; Yamanaka et al. 2008). These results suggest that mSOD1 in motor neurons determines disease onset while its expression in microglia and astroglia determines disease progression. Thus, motor neurons do not die by intrinsic mechanisms and motor neuron death is now regarded as non-cell autonomous cell death.

2.3.2 *Alterations of Cytokines/Chemokines and Growth Factors Influencing Neuroinflammation and Neurodegeneration in ALS*

We and others have demonstrated that proinflammatory cytokines/chemokines, such as CCL2 (MCP1), CCL4 (MIP1β), CXCL8 (IL-8), CXCL10 (IP10), IL-1β, IL-7, IL-9, IL-12 (p70), IL-17, IFNγ, and TNFα, were elevated in CSF from ALS patients (Tanaka et al. 2006; Mitchell et al. 2009; Tateishi et al. 2010). These proinflammatory cytokines were also elevated in spinal cord tissues (Weydt et al. 2004; Meissner et al. 2010). Interestingly, CCL2 and CXCL8 levels in CSF showed a significant positive correlation with disease severity while those of CCL4 and CXCL10 had a negative correlation with disease severity (Tateishi et al. 2010). Thus, the relationship of proinflammatory cytokines/chemokines with disease severity is not uniform, suggesting that CCL2 and CXCL8 may be neurotoxic while CCL4 and CXCL10 may be neuroprotective.

We also found increased granulocyte colony stimulating factor (G-CSF) levels in the CSF of ALS cases (Tanaka et al. 2006). G-CSF was expressed in reactive astroglia from ALS cases but not controls, while G-CSF receptor expression was significantly decreased in motor neurons in ALS cases (Tanaka et al. 2006). Thus, the neuroprotective effects of G-CSF secreted by astroglia do not occur because of the down-modulation of G-CSF receptors in motor neurons (Tanaka et al. 2006).

2.3.3 *Microglia in ALS*

Activated microglia produce a variety of proinflammatory cytokines/chemokines and oxidative molecules, such as NO and O_2. In contrast, they can also secrete neurotrophic factors and anti-inflammatory cytokines. Microglia producing proinflammatory cytokines are termed M1 microglia and exert deleterious effects and those that secrete anti-inflammatory cytokines are termed M2 microglia and exhibit neuroprotective activity. However, the clear-cut separation of M1 and M2 microglia may be difficult and the surrounding environment may modify the dual features of microglia. Another issue of note is that the classification of activated microglia residing in the CNS and infiltrating macrophages from the peripheral blood is often difficult when staining for cell-surface markers, and therefore immunohistochemical results should be interpreted cautiously.

Microglial activation is observed in the anterior horns, along the corticospinal tract, and in the motor cortex of postmortem ALS samples (Kawamata et al. 1992). However, some studies have reported a more widespread infiltration of activated microglia (Hayashi et al. 2001). Intriguingly, [11C]-PK11195 PET imaging observed the in vivo activation of microglia in motor cortices, dorsolateral prefrontal cortices, and thalami from ALS patients (Turner et al. 2004). In a G93A mSOD1 transgenic ALS mouse model, microglial activation occurred well before motor neuron loss and was present even at preclinical stages (Yamasaki et al. 2010; Kawamura et al. 2012; Nagara et al. 2013). In the sciatic nerves, macrophage infiltration occurs early, probably reflecting that the mSOD1 ALS model has characteristics of distal axonopathy (Fischer et al. 2004). Microglial activation is frequently associated with CD4+ and CD8+ T cell infiltration in ALS (Engelhardt et al. 1993), suggesting interactions between these two cell types. Activated microglia in the spinal cord from ALS patients up-regulate dendritic cell markers, such as CD11c and CD86 (Henkel et al. 2004). Similar up-regulation of antigen-presenting markers is also seen in the late stage of the mSOD1 transgenic ALS mouse model (Henkel et al. 2005). Therefore, microglia may present antigens to T cells (Henkel et al. 2004), similar to dendritic cells, during the late stages of ALS, while T cells may potentiate neuroinflammation (Chiu et al. 2008) or act neuroprotectively (Yamasaki et al. 2010; Beers et al. 2008). Although activated microglia may be toxic in the progressive stages, in mSOD1 ALS mice, motor neuron loss was not altered by ablation of proliferating microglia (Gowing et al. 2006). However, the same procedure worsened animal models of Alzheimer's disease (Simard et al. 2006), suggesting the roles of activated microglia are not similar among various neurodegenerative disorders.

In mSOD1-Tg mice, microglial proliferation and T cell infiltration occurred relatively early in the spinal cord (Alexianu et al. 2001), but not in the brainstem (Yamasaki et al. 2010). Furthermore, relatively preserved motor neurons were observed even in the disease progression stage (Chiu et al. 1995). Thus, we studied the actions of microglia following acute and chronic motor neuronal insults in mSOD1 ALS mice (Yamasaki et al. 2010; Kawamura et al. 2012) by unilateral hypoglossal nerve axotomy at young (8 weeks) and adult (17 weeks) ages. On day

21 following hypoglossal axotomy, the numbers of surviving neurons were markedly reduced in mSOD1 mice than non-transgenic littermates at 17 weeks of age but the same difference was not seen at 8 weeks, suggesting increased vulnerability of hypoglossal motor neurons to neuronal injury in adult mice (Yamasaki et al. 2010). On day 3 after axotomy, the number of microglia expressing glial cell-derived neurotrophic factor (GDNF) and insulin-like growth factor (IGF-1) that surrounded axotomized hypoglossal neurons was significantly lower in mSOD1-Tg mice than in non-transgenic mice at 17 weeks but not at 8 weeks, despite the good preservation of hypoglossal neurons. Infiltration of CD3+ T cells, mostly expressing CD4, occurred on day 7 (Kawamura et al. 2012). The migratory ability of cultured microglia from mSOD1 mice to MCP-1 was decreased compared with those from non-transgenic littermates (Yamasaki et al. 2010). G-CSF significantly restored the impaired migratory ability of mSOD1 microglia in vitro, while in vivo it increased the number of microglia surrounding axotomized neurons and improved neuronal survival following axotomy in mSOD1 ALS mice. Moreover, chronic administration of G-CSF significantly increased the numbers of GDNF-positive microglia surrounding spinal motor neurons in the anterior horns and increased the life span of mSOD1 ALS mice (Yamasaki et al. 2010). These findings suggest that microglia act protectively through the production of growth factors, and that decreased microglial protective ability of mSOD1 mice can be restored by appropriate growth factors, such as G-CSF. It is interesting to note that CNS microglia and peripheral blood monocytes/macrophages show decreased chemotactic activity in mSOD1 mice (Yamasaki et al. 2010). Decreased mobilization of microglia into the damaged CNS and macrophages into the peripheral nerves may explain the acceleration of motor neuron death, because these myeloid cells produce neuroprotective factors.

2.3.4 Astroglia in ALS

Astroglia usually have a neuroprotective function but can become neuroinflammatory upon interactions with their surrounding microenvironment. Reactive astroglia, characterized by increased expression of GFAP, exist in the anterior and posterior horns in the spinal cord from ALS patients and are present in the gray and subcortical white matter, not being limited to the motor cortex (Nagy et al. 1994; Kushner et al. 1991; Schiffer et al. 1996). Reactive astroglia can overexpress neurotoxic factors such as iNOS, which in turn produces reactive oxygen and nitrogen species (Sasaki et al. 2001).

Astroglia produce a variety of neurotrophic factors, such as GDNF, brain-derived neurotropic factor (BDNF), and IGF-1. Astroglia also produce VEGF that induces vascular proliferation and enhances neuronal survival (Jin et al. 2002; Tolosa et al. 2008). However, VEGF receptor 1 and 2 are decreased during disease progression, and, therefore, the over-expression of VEGF is unable to exert its protective effects in human ALS (Nagara et al. 2013). Astroglial expression of glutamate transporter EAAT2 and GLT1 is decreased in ALS patients and the mSOD1 ALS mice, respectively (Rothstein et al. 1992, 1995; Howland et al. 2002). Neurons can increase

astroglial glutamate transporter expression through the induction of kappa B-motif binding phosphoprotein factor to the GLT1 promoter (Yang et al. 2009), thereby reinforcing the clearance of glutamate. Decreased neuronal regulation may in turn accelerate excitotoxic neuronal death by overstimulation of AMPA and NMDA receptors.

2.3.5 T Cells in ALS

T cells are rarely seen in the normal CNS, which is an immunologically privileged site, while infiltrating T cells were observed in postmortem tissues of ALS patients (Engelhardt et al. 1993). Depletion of T cells in mSOD1 mice crossbred with RAG2–/– or CD4–/– mice resulted in accelerated motor neuron degeneration (Beers et al. 2008) where CD11b+ microglia were markedly reduced. In ALS models, depletion of T cells induces CNS neurotoxic substances, such as TNFα, and reduces neuroprotective factors, such as IGF-1, GDNF, and BDNF (Chiu et al. 2008; Beers et al. 2008). The adoptive transfer of regulatory or effector T cells from wild-type mice to mSOD1 Tg mice delayed disease progression (Banerjee et al. 2008) and the passive transfer of CD4+ CD25+ FoxP3+ regulatory T cells into mSOD1 mice ameliorated ALS (Beers et al. 2011). IL-4 produced by regulatory T cells suppressed the toxic properties of microglia (Beers et al. 2011). T cells from ALS CNS tissues may be neuroprotective; however, their protective function appears to be insufficient.

2.3.6 Experimental Therapy in ALS

The presence of glial inflammation in ALS encouraged the use of anti-inflammatory and immunomodulatory drugs, and several case reports have described beneficial effects of anti-inflammatory therapies, even in familial ALS cases (Saiga et al. 2012). This suggested that some inflammatory components are involved in human ALS pathogenesis. However, clinical trials investigating these drugs demonstrated no efficacy. Trials of nonspecific immunosuppression with cyclophosphamide or cyclosporin, whole body irradiation, or bone-marrow stem cell transplantation have all been unsuccessful (Appel et al. 2008). Expression of cyclooxygenase 2 (COX2), which is critical in producing proinflammatory prostaglandins, is increased in ALS patients and mSOD1 mice (Almer et al. 2001). However, although the COX2 inhibitor celecoxib is effective at delaying the disease course in ALS model mice, it has no effect in human ALS (Drachman et al. 2002; Pompl et al. 2003). Minocycline down-modulated microglial activation and improved the survival rate of mSOD1 ALS mice (Zhu et al. 2002), but it was not effective in humans with ALS (Scott et al. 2008). To date, no single anti-inflammatory drug has been successful in human ALS even though some are effective in animal models. Not all inflammatory components are toxic and some might act protectively. This may explain in part why general anti-inflammatory procedures are not beneficial in human ALS.

Either *TNFα* (Gowing et al. 2008) or *IL-1β* gene knockout (Nguyen et al. 2001) did not ameliorate disease in mSOD1 ALS mice. Thus, it is unlikely that a single cytokine is responsible for the exacerbation of motor neuron degeneration in ALS, but rather the combined effects of such proinflammatory cytokines may accelerate motor neuron death.

Although the administration of neurotrophic factors, such as GDNF, BDNF, IGF-1, and VEGF, increased the survival of mSOD1 Tg mice (Kaspar et al. 2003; Azzouz et al. 2004; Storkebaum et al. 2005), these factors could not sufficiently delay clinical progression in human ALS patients. This may be explained by the differences in bioavailability and dosage between mice and humans. In our study, mSOD1-harboring microglia increased GDNF expression upon stimulation with G-CSF while concurrently such GDNF-positive microglia also expressed iNOS, a potential neurotoxic factor (Yamasaki et al. 2010; Kawamura et al. 2012). These findings support the idea that M1 and M2 microglia are not clearly separable and that microglia may simultaneously produce both protective and toxic substances. Thus, the nonselective targeting of microglia may not be successful in future clinical trials for neurodegenerative diseases.

2.4 Conclusion and Future Perspectives

Neuroglial inflammation has dual actions; protective and toxic. Neuroglial inflammatory reactions are distinct between neuroimmunological and neurodegenerative diseases and even among neurodegenerative disorders. Detailed analyses of glial activation states may increase our understanding and influence how we can decipher and control glial dual actions, thereby enabling new drug development targeting glia in humans.

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Chapter 3
Factors from Intact and Damaged Neurons

Tetsuya Mizuno

Abstract Neuron–glial interactions play important roles in the maturation and maintenance of the central nervous system (CNS). Glial cells including astrocytes and microglia support and survey neuronal activity, whereas neurons provide trophic support for glial cells through the production of various molecules such as cytokines, chemokines, and neurotrophins. CNS diseases, including neurodegenerative diseases, demyelinating disease, epilepsy, and brain ischemia, often involve the disruption of neuron–glial interactions. Furthermore, neuroinflammation induced by glial activation contributes to the pathogenesis of these diseases. Damaged neurons can trigger glial activation, resulting in the production of inflammatory molecules and phagocytosis of injured neurons by glial cells. On the other hand, these neurons can also suppress glial activation through the induction of anti-inflammatory cytokines and chemokines. Here we review the roles of various molecules induced by intact or damaged neurons with respect to neuron–glial interactions.

3.1 Introduction

Neuron–glial interactions are important to the regulation of the development, maturation, and maintenance of the central nervous system (CNS). Astrocytes and microglia, which are representative glial cells, support and survey neuronal activity through the production of various molecules such as cytokines, chemokines, and neurotrophins (NTs).

T. Mizuno, M.D., Ph.D. (✉)
Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, 464-8601, Japan
e-mail: tmizuno@riem.nagoya-u.ac.jp

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Microglia play pivotal roles in synaptic remodeling and plasticity in the healthy brain. Synaptic pruning by microglia is necessary for brain development. Indeed, deficient synaptic pruning results in an excess of dendritic spines and immature synapses (Paolicelli et al. 2011). Furthermore, phagocytosis of neuronal debris by microglia helps maintain neuronal networks (Neumann et al. 2009). Microglial phagocytosis is dependent upon neural activity and the microglia-specific phagocytic signaling pathway including complement receptor 3/C3 (Schafer et al. 2012). On the other hand, neurons regulate microglia via signals such as fractalkine, transforming growth factor-β (TGF-β), cluster of differentiation (CD) 22, CD200, and neurotrophins under physiological conditions (Biber et al. 2007). Neurons damaged by CNS diseases (including neurodegenerative diseases, demyelinating disease, epilepsy, and brain ischemia) produce signals such as glutamate, high-mobility group protein 1 (HMGB1), chemokine (C–C motif) ligand 21 (CCL21), and nucleotides that can trigger microglial activation (Biber et al. 2007; Koizumi et al. 2007). Activated microglia then produce proinflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor-α (TNF-α); glutamate; chemokines; complement components; and reactive oxygen species (ROS) including superoxide anions and hydroxy radicals (Barger and Basile 2001; McGeer and McGeer 2001; Takeuchi et al. 2005, 2006). These molecules further accelerate neuronal damage. Damaged neurons, however, are not merely passive targets of microglia, but rather suppress microglial activity through the production of fractalkine, C–C chemokine receptor type (CCR)-5, IL-34, macrophage colony-stimulating factor (M-CSF), and TGF-β.

Astrocytes provide nutritional support for neurons, maintain the extracellular ion balance, and increase the uptake of glutamate via glutamate transporters (Magistretti and Pellerin 1999; Bouvier et al. 1992). They also produce antioxidants such as glutathione and superoxide dismutases, and exert antioxidant effects on neurons (Dringen et al. 1998; Mokuno et al. 1994). Neuronal activity regulates the function of astrocytes; for instance, the expression of glutamate transporter GLT-1 is increased in astrocytes when astrocytes are cultured with neurons, and, conversely, the expression is decreased in astrocytes cultured without neurons (Swanson et al. 1997). Neurons also regulate both the organization of GLT1 in developing astrocytes and their position relative to synapses (Benediktsson et al. 2012). Neurons are thought to provide trophic support for astrocytes; however, the precise mechanism is unknown. In the present review, we focus on the molecules derived from intact or damaged neurons, and clarify their effects on glial function in physiological or pathological conditions (Fig. 3.1).

3.2 Molecules Derived from Intact Neurons

Neurons maintain their own synaptic transmission by regulating the function of microglia and astrocytes. Various molecules secreted from intact neurons are involved in neuron–glial interactions. In this report, we provide more information on fractalkine, TGF-β, CD22, CD200, and neurotrophins.

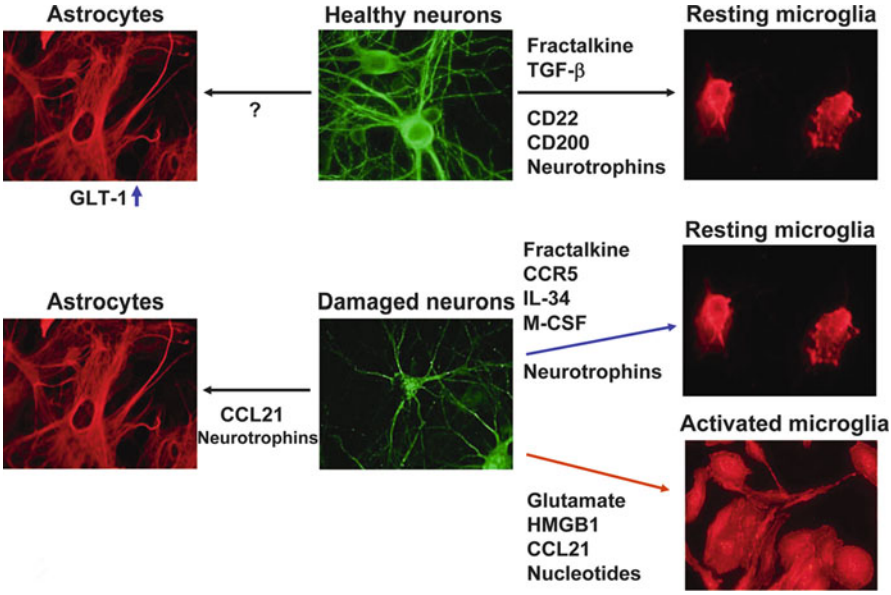

Fig. 3.1 Glial cells are affected by factors secreted from healthy and damaged neurons. Fractalkine, TGF-**β**, CD22, CD200, and neurotrophins secreted from healthy neurons keep microglia in a quiescent state. Fractalkine, CCR5, IL-34, M-CSF, TGF-**β**, and neurotrophins secreted from damaged neurons suppress microglial activation. On the other hand, glutamate, HMGB1, CCL21, and nucleotides activate microglia. Healthy neurons up-regulate GLT-1 in astrocytes. Details on the trophic support for astrocytes by neurons remain unknown

3.2.1 Fractalkine

The CX3C chemokine fractalkine is a 373-amino acid protein that has a chemokine domain on top of an extended mucin-like stalk (Bazan et al. 1997). Fractalkine exists in the membrane-anchored or soluble form. Neurons secrete fractalkine, and its receptor CX3CR1 is exclusively expressed in microglia (Harrison et al. 1998). Fractalkine contributes to maintain microglia in a resting ramified phenotype. It also plays a pivotal role in memory formation and synaptic plasticity. The hippocampus contains IL-1β binding sites (Takao et al. 1990). Under physiological conditions, mice lacking CX3CR1 show cognitive dysfunction in contextual fear conditioning and Morris water maze tests; deficits in motor learning; and significant impairment in long-term potentiation (LTP) via increases in IL-1β produced by microglia (Rogers et al. 2011).

3.2.2 TGF-β

TGF-β is a neurotrophic cytokine responsible for the initiation and maintenance of neuronal differentiation and synaptic plasticity (Zhang et al. 1997). TGF-β is mainly

produced by astrocytes and microglia; however, neurons also produce TGF-β and regulate glial cells. For instance, cortical neurons induce astrocyte differentiation from radial glial cells through the TGF-β1 pathway (Stipursky and Gomes 2007). TGF-β also regulates the proliferation of postnatal progenitors and Müller glia in the rat retina (Close et al. 2005). Furthermore, TGF-β enhances the expression of CX3CR1 in microglia (Chen et al. 2002).

3.2.3 CD22

CD22 is known as a B-cell transmembrane protein that functions by mediating cell–cell interactions with T cells, and is an endogenous ligand for the CD45 receptor (Stamenkovic et al. 1991). Neurons express CD22 and secrete a soluble form of CD22, whereas microglia express the CD45 receptor. Reports suggest that CD22 acts on microglia to inhibit the production of proinflammatory cytokine such as TNF-α (Tan et al. 2000; Mott et al. 2004).

3.2.4 CD200

CD200 is a membrane glycoprotein expressed in neurons that suppresses immune activity via the microglial CD200 receptor (CD200R) (Wright et al. 2000). It has been reported that a lack of CD200 results in the rapid onset of experimental autoimmune encephalomyelitis (EAE) via nitric oxide (NO) production (Hoek et al. 2000), and in the impairment of LTP via toll-like receptor (TLR) activation in microglia (Costello et al. 2011). The soluble form of CD200 induces IL-10 in microglia, and IL-10 protects neurons from microglia-induced neurotoxicity. The up-regulation of CD200R by endocannabinoid anandamide also exerts a neuroprotective effect on Theiler's virus-induced demyelinating disease (Hernangómez et al. 2012).

3.2.5 Neurotrophins

Neurotrophins including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3, and NT-4 are normally secreted by neurons and glial cells, and have pivotal roles in cell survival, neurite growth, synaptic plasticity, and neurotransmitter release in the CNS (Barde 1989; Davies 1994). Neurotrophins also affect the function of glial cells; for example, NGF and BDNF promote microglial proliferation, whereas NT-4 enhances microglial viability (Zhang et al. 2003).

3.3 Molecules Derived from Degenerated Neurons

Degenerated neurons induced by glutamate or oligomeric amyloid β (Aβ) release soluble factors known as help-me, find-me, or eat-me signals. These factors can induce or suppress glial activation. The difference in glial response may depend on the degree of neurodegeneration; however, the precise mechanism is unknown.

3.3.1 Glutamate

Glutamate is an excitatory neurotransmitter, and excessive amounts are released as a result of neurodegenerative processes in Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). Microglia express the AMPA and kainate subtypes of ionotropic glutamate receptors, which mediate TNF-α production (Noda et al. 2000). Metabotropic glutamate receptor (mGluR)-2, mGluR3, and mGluR5 are also expressed in microglia (Taylor et al. 2005; Piers et al. 2011). Excessive glutamate activates microglia through mGluR2 and promotes microglial neurotoxicity. Microglial mGluR5, on the other hand, exerts a neuroprotective effect by inhibiting the production of NO and TNF-α (Piers et al. 2011).

3.3.2 HMGB1

HMGB1 is a ubiquitously expressed nonhistone DNA-binding protein, and is included in the class of molecules termed "damage-associated molecular patterns" (DAMPs). DAMPs are released from injured or infectious cells and activate immune cells to induce inflammation. HMGB1 is secreted from damaged neurons; for instance, it is upregulated in the postischemic brain (Kim et al. 2006). Secreted HMGB1 accumulates in the media of NMDA-treated primary cortical neuron cultures, and induces neuronal cell death via RAGE–ERK and RAGE–p38 MAPK interactions (Kim et al. 2011). Moreover, HMGB1 activates microglia and acts as an endogenous toxic molecule. HMGB1 released from dying neurons is reported to inhibit microglial Aβ clearance and enhance the neurotoxicity of Aβ (Takata et al. 2004).

3.3.3 CCL21

The chemokine CCL21 is released from damaged neurons by glutamate toxicity. CCL21 activates microglia via two different receptors, CXCR3 and CCR7, and triggers chemotaxis of microglia through CXCR3 (de Jong et al. 2005). CCR7 is upregulated in reactive astrocytes upon intracerebral lipopolysaccharide (LPS) treatment

(Gomez-Nicola et al. 2010). CCL21 thus affects both types of glial cells. In spinal cord injury, CCL21 does not change the mRNA expression of these receptors, but upregulates P2X4 receptors in spinal cord microglia, resulting in an untreatable pathological pain reaction (Biber et al. 2011).

3.3.4 Nucleotides

Nucleotides adenosine triphosphate (ATP) and uridine diphosphate (UDP) function as find-me and eat-me signals for recognition and phagocytosis, respectively, which are released from degenerated neurons. Microglia, on the other hand, express ionotropic (P2X4 and P2X7) and metabotropic (P2Y1, P2Y2, P2Y6, and P2Y12) purinergic receptors, and migrate to damaged or dead neurons and phagocytose them. ATP regulates microglial branch dynamics in the intact brain, and mediates a rapid microglial response towards injury (Davalos et al. 2005). Microglial chemotaxis by ATP is induced via P2Y12 receptors (Haynes et al. 2006). UDP triggers microglial phagocytosis via P2Y6 receptors that are upregulated when neurons are damaged (Koizumi et al. 2007).

3.3.5 Fractalkine

Secreted from glutamate-damaged neurons, fractalkine promotes microglial phagocytosis of neuronal debris through the release of milk fat globule-EGF factor 8 (MFG-E8) and induces the expression of the antioxidant enzyme heme oxygenase (HO)-1 in microglia. These actions by microglia are neuroprotective against glutamate toxicity (Noda et al. 2011). Fractalkine upregulates MFG-E8 (Leonardi-Essmann et al. 2005) and suppresses the production of NO, IL-6, and TNF-α induced by activated microglia (Mizuno et al. 2003). MFG-E8 significantly attenuates oAβ-induced neuronal cell death through microglial phagocytosis of oAβ (Li et al. 2012). In addition, its expression is reduced in AD (Boddaert et al. 2007). In regard to HO-1, its end-products, such as biliverdin, carbon monoxide, and iron, provide cellular and tissue protection through anti-inflammatory, anti-apoptotic, or anti-oxidative effects (Morse et al. 2009). Indeed, the up-regulation of HO-1 in the CNS is beneficial to counteract neuroinflammation and in turn neurodegenerative diseases (Syapin 2008). The roles of fractalkine and MFG-E8 in neuron–microglia interactions are shown in Fig. 3.2.

In addition to inducing MFG-E8 and HO-1, fractalkine has other effects in the CNS. For instance, exogenous fractalkine reduces ischemia-induced cerebral infarct size, neurological deficits, and caspase-3 activation in mouse model of permanent middle cerebral artery occlusion (Cipriani et al. 2011). Furthermore, fractalkine signaling is deficient in AD, and CX3CR1 deficiency worsens AD-related neuronal

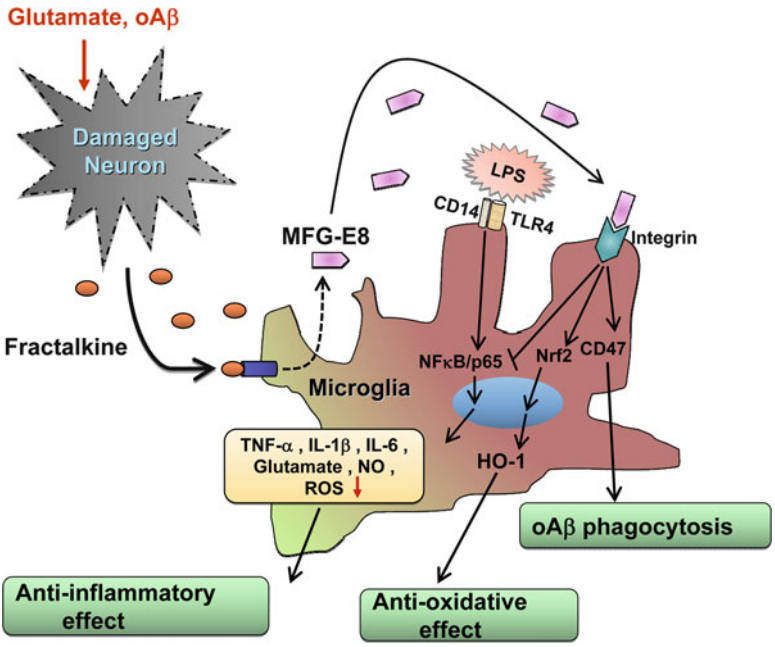

Fig. 3.2 The roles of fractalkine and MFG-E8 in damaged neuron–microglia interactions. Fractalkine, which is secreted from damaged neurons by glutamate or oA**β**, induces MFG-E8 in microglia. Fractalkine–MFG-E8 system exerts neuroprotection by phagocytosis, anti-oxidation, and anti-inflammation

and behavioral deficits. These effects were associated with cytokine production but not with plaque deposition (Cho et al. 2011). In contrast, CX3CR1 deficiency is reported to reduce Aβ deposition in AD mouse models including APP/PS1 and R1.40 transgenic mice (Lee et al. 2010). Thus, fractalkine–CX3CR1 signaling in AD warrants further exploration.

3.3.6 CCR5

CCR5 is the chemokine receptor of three β-chemokines, CCL3, CCL4, and CCL5, and is expressed in astrocytes and neurons, but not in microglia. CCR5 is upregulated in brain ischemia (Spleiss et al. 1998) and HIV-associated dementia (Kaul et al. 2007). It has been reported that a lack of CCR5 increases the severity of brain injury following occlusion of the middle cerebral artery (Sorce et al. 2010). The neuroprotective effect of CCR5 is thought to suppress microglial activation. Moreover, the cytokines CCL3 and CCL4 prevent excitotoxicity induced by NMDA (Bruno et al. 2000).

3.3.7 IL-34

Mainly expressed in neurons, the cytokine IL-34 regulates microglial function through the colony-stimulating factor (CSF)-1 receptor. IL-34 stimulates the differentiation and proliferation of monocytes and macrophages (Lin et al. 2008). We have shown that microglia treated with IL-34 play neuroprotective roles against oAβ toxicity. IL-34 induces microglial proliferation and the production of antioxidant enzyme HO-1 and insulin-degrading enzyme (IDE), which is known as Aβ degrading enzyme. Moreover, single intracerebroventricular injection of IL-34 ameliorates the impairment of associative learning in an APP/PS1 transgenic mouse model of AD. In the contextual learning test, IL-34 treatment significantly reverses the contextual freezing response as compared to vehicle-injected APP/PS1 transgenic mice (Mizuno et al. 2011). IDE activity affects the level of Aβ. It has been reported that the levels of membrane-bound IDE protein and activity are significantly decreased in the hippocampal formation of subjects affected by mild cognitive impairment who are at high risk to develop AD (Zhao et al. 2007). In IDE/APP double-transgenic mice, the transgenic overexpression of IDE decreases Aβ levels and prevents amyloid plaque formation (Leissring et al. 2003). Recently, we found a novel mechanism of IL-34 in microglial neuroprotection: IL-34 dose-dependently induces TGF-β in microglia, and the TGF-β attenuates oAβ neurotoxicity in neuron–microglial cocultures (Ma et al. 2012).

3.3.8 M-CSF

In addition to IL-34, M-CSF is also a ligand of the CSF-1 receptor; however, IL-34 and M-CSF differ in structure and CSF-1 receptor binding domains. They also have different bioactivities and signal activation kinetics (Chihara et al. 2010). M-CSF enhances the acidification of lysosomes in macrophage and microglia, and leads to the degradation of internalized Aβ (Majumdar et al. 2007). For instance, intraperitoneal injection of M-CSF increases the number of microglia, induces microglial phagocytosis of Aβ, and prevents memory disturbance in APP/PS1 mice (Boissonneault et al. 2009). Thus, M-CSF also exerts microglial neuroprotective properties.

3.3.9 Neurotrophins

Neurotrophins, including NGF and BDNF, are another group of soluble factors used by neurons to control immune cell functions. NGF inhibits the expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules CD86 and CD40 in microglia (Neumann et al. 1998; Wei and Jonakait 1999). BDNF suppresses the release of NO from activated microglia (Mizoguchi et al. 2009). In contrast, it has been reported that the expression of BDNF receptor TrkB is

induced on astrocytes in white matter lesions in multiple sclerosis and EAE mice, and, furthermore, that astrocytes stimulated with BDNF amplify EAE-induced neurodegenerative processes via NO production (Colombo et al. 2012). Thus, the effects of BDNF on glial cells are unresolved.

3.4 MicroRNAs

MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate the expression of multiple target genes and are involved in many fundamental biological processes, such as embryonic development, cell proliferation, differentiation, and apoptosis. Recent reports have shown that some miRNAs are produced by neurons and regulate glial cells.

miR-124 is known to promote neuronal differentiation by targeting the mRNA of the antineural function protein SCP1, which represses neuronal gene expression in non-neuronal cell types (Makeyev et al. 2007). miR-124 also promotes microglia quiescence: it has been reported that during EAE, miR-124 is down-regulated in activated microglia (Ponomarev et al. 2011). Furthermore, peripheral administration of miR-124 causes systemic deactivation of macrophages, reduced activation of myelin-specific T cells, and marked suppression of EAE. miR-124 produced by neuronal cells is thought to be transferred into macrophages and microglia through exosomal shuttle vesicles (Ponomarev et al. 2013).

Hypoxic microglia produce Fas ligand (FasL) and induce neuronal apoptosis. miR-21 suppresses FasL production in microglia (Zhang et al. 2012). Thus, miR-21 inhibits microglial toxic effect.

On the other hand, miR-155 is induced by inflammatory stimuli such as interferon-gamma (IFN-γ) and TLR agonists (Ruffell et al. 2009). miR-155 promotes microglial activation by down-regulating the suppressor of cytokine signaling 1 (SOCS-1) and induces NO and inflammatory cytokines in microglia (Cardoso et al. 2012). IFN regulatory factor 3 is reported to suppress miR-155 and to inhibit inflammatory gene expression in astrocytes (Tarassishin et al. 2011). miR-206 is increased in human AD brains and Tg2576 AD transgenic mice. It decreases BDNF expression via translational repression. A neutralizing inhibitor of miR-206 increases BDNF and improves the memory function (Lee et al. 2012). This suggests that miR-206 may activate microglia. However, the production of miR-21, miR-155, and miR-206 by neurons remains unknown.

3.5 Conclusion

It is clear that molecules derived from healthy or damaged neurons regulate glial activation. Healthy neurons produce various cytokines, chemokines, membrane CD proteins, and neurotrophins under physiological conditions. Fractalkine maintains

microglia in a resting ramified form, and TGF-β, CD22, and CD200 suppress the microglial production of inflammatory molecules. Neurotrophins also enhance microglial proliferation. Neurons regulate glutamate uptake of astrocytes; however, it remains unclear whether neuron-derived soluble factors affect astrocytes. Damaged neurons, on the other hand, produce molecules that exert microglial neurotoxicity or neuroprotection. Glutamate, HMGB1, CCL21, and nucleotides induce microglial activation. Fractalkine, CCR-5, IL-34, M-CSF, and neurotrophins suppress microglial activation or exert antioxidant effects. miRNAs also play pivotal roles in neuron–glial interactions as outlined above. The conditions that determine microglial toxic or protective effects remain to be elucidated. Clarifying these issues may contribute to the understanding of neurodegenerative disorders.

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Conflict of interest Tetsuya Mizuno declares that he has no conflict of interest.

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Chapter 4
Interactions Between Neurons and Microglia During Neuroinflammation

Akio Suzumura

Abstract Microglia often accumulate around degenerating neurons. These macrophage-like immune cells produce a variety of neurotoxic and neuroprotective factors. Thus, the accumulation of glia in various neurologic disorders does not reflect only gliosis, but likely results in an active contribution to neuroinflammation, neural degeneration, and cell regeneration. We previously showed that glutamate is the most neurotoxic factor released by activated microglia, and suppressing glutamate release from microglia can inhibit disease progression in various animal models of neurodegenerative disorders. Interferon-γ (IFNγ) is also neurotoxic after binding to IFNγ receptor alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptor complexes. On the other hand, when exposed to harmful stimuli, neurons also produce and release various factors that serve as "help-me" signals. For example, the CX3C chemokine fractalkine, interleukin-34, and fibroblast growth factor-2 are secreted from damaged neurons; these help-me signals induce various microglial activities to rescue neurons, including upregulated phagocytosis of toxicants and damaged debris, and production of antioxidant enzymes and other neurotrophic factors. Elucidating the interactions between neurons and microglia will help uncover the mechanisms underlying chronic neuroinflammatory conditions, and may provide insights into new therapeutic strategies for neurodegenerative disorders.

A. Suzumura, M.D., Ph.D. (✉)
Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University, Furo-cho, Chikusa, Nagoya, Aichi, Japan
e-mail: suzumura@riem.nagoya-u.ac.jp

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4.1 Introduction

Microglia are macrophage-like immune cells in the central nervous system (CNS). Microglia accumulate in the lesions that form in neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and ischemic and infectious diseases. Microglia are thought to play both toxic and protective functions for neuronal survival (Farfara et al. 2008). When activated, microglia undergo morphologic changes to an ameboid state, proliferate, migrate toward lesions, release various soluble factors, and phagocytose foreign substances and cellular debris. The migration of microglia to injured areas is controlled by chemokines and nucleotides (Honda et al. 2001; El Khoury and Luster 2008). Phagocytosis may prevent senile plaque expansion in AD by removing amyloid β (Aβ) deposits (Bard et al. 2000). Microglia not only engulf Aβ protein but also phagocytose apoptotic cells and neuronal debris.

Degenerating neurons also release signaling molecules, including nucleotides, cytokines, and chemokines, to recruit microglia and enhance their activities (Hoarau et al. 2011; Fuller and Van Eldik 2008). These factors act as "find-me," "eat-me," and "help-me" signals. The best characterized eat-me signal is phosphatidylserine, which is present on the cellular membranes of apoptotic cells (Sambrano and Steinberg 1995; McArthur et al. 2010). Nucleotides are also thought to act as eat-me signals. Microglia express various P2X and P2Y nucleotide receptors, which regulate chemotaxis and phagocytosis (Fuller and Van Eldik 2008; Horvath and DeLeo 2009; Hoarau et al. 2011). Microglia also express many other surface receptors that interact with targets and initiate phagocytosis (Fuller and Van Eldik 2008). These receptors include phosphatidylserine receptor, the lipopolysaccharide (LPS) receptor CD14 (Liu et al. 2005), the scavenger receptor CD36 (Stolzing and Grune 2004), the purine receptor P2Y6 (Hoarau et al. 2011), and toll-like receptors (TLRs) (Landreth and Reed-Geaghan 2009). The CX3C chemokine fractalkine (FKN) receptor CX3CR1 is almost exclusively expressed on microglia in the CNS. CX3CR1 reportedly contributes to the progression of neurodegenerative diseases by altering microglial activities (Cardona et al. 2006). Removing CX3CR1 from microglia was shown to cause progressive neuronal cell death in an animal model of neurodegenerative disease. Neurons themselves produce cytokines and chemokines, such as FKN. We previously reported that neurons produce interleukin-34 (IL-34), whereas its receptor, colony-stimulating factor 1 receptor, is primarily expressed on microglia (Mizuno et al. 2011). Fibroblast growth factor-2 (FGF-2) is also produced by damaged neurons. These factors may contribute to the induction and maintenance of neuroinflammation. Clarifying signaling pathways that mediate interactions between neurons and microglia may uncover therapeutic targets in neurodegenerative diseases. In the following sections, we discuss mechanisms underlying communication between microglia and neurons.

4.2 Effects of Microglia-Derived Factors on Neurons

4.2.1 Neurotoxic and Neuroprotective Effects

Microglia produce a variety of neurotoxic and neuroprotective factors. Thus, some authors have referred to this cell population as a double-edged sword (Fig. 4.1). Microglia produce inflammatory cytokines, such as IL-1β, tumor necrosis factor (TNF)-α, IL-6, IL-12, and interferon-γ (IFNγ). They also produce superoxide compounds, nitric oxide, and excitatory amino acids. These factors may disrupt neuronal functions. Microglia also produce various neuroprotective factors, such as brain-derived neurotrophic factor, glia-derived neurotrophic factor, and nerve growth factor. When stimulated, microglia express the antioxidant enzyme heme oxygenase-1 and such amyloid-degrading enzymes as insulin-degrading enzyme and matrix metalloproteinase-9.

4.2.2 Inflammatory Cytokines

In vitro and in vivo studies indicate that inflammatory cytokines derived from microglia may be involved in neurodegeneration, either directly or indirectly.

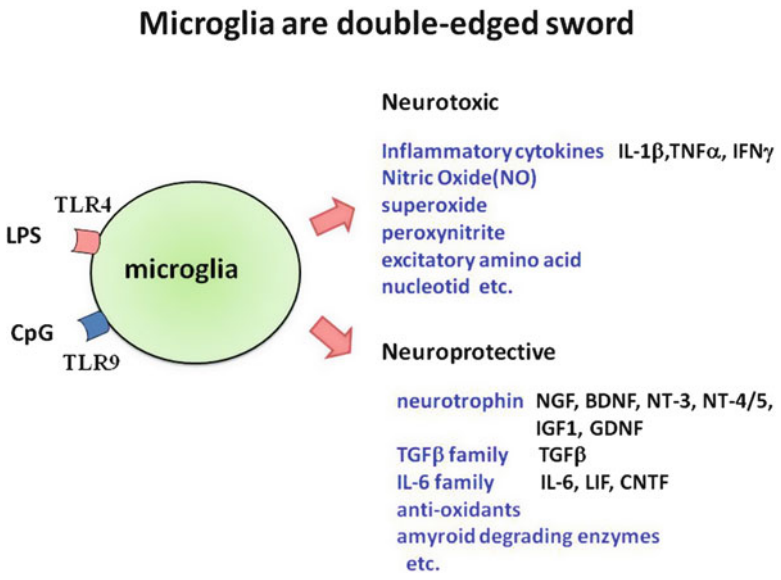

Fig. 4.1 Factors produced by activated microglia. LPS binds TLR4 on microglia, resulting in the production of neurotoxic and neuroprotective factors. Therefore, these cells are often described as a double-edged sword. *NGF* nerve growth factor, *BDNF* brain-derived neurotrophic factor, *TGF* transforming growth factor, *GDNF* glia-derived neurotrophic factor, *LIF* leukemia inhibitory factor, *CNTF* ciliary neurotrophic factor

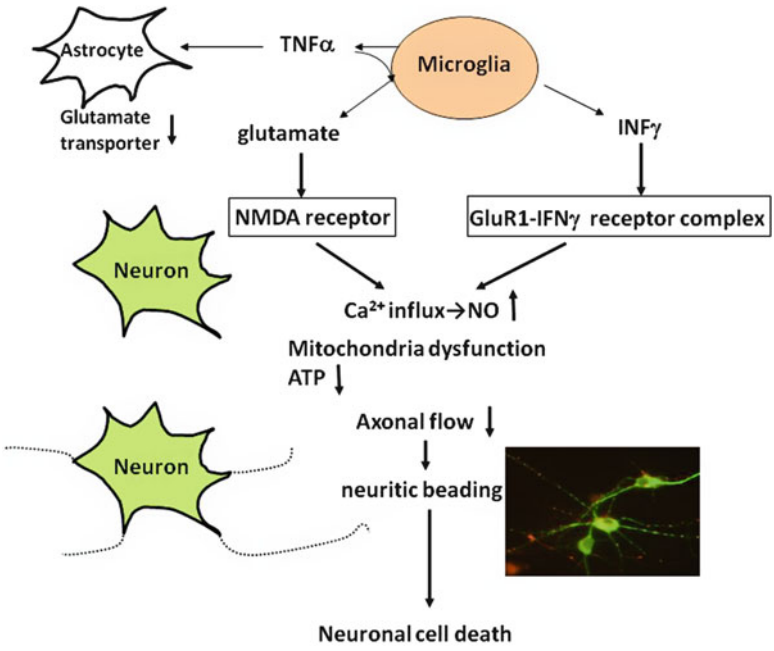

Fig. 4.2 Neuronal damage induced by activated microglia. Glutamate is neurotoxic via NMDA receptors, whereas IFN**γ** is neurotoxic via AMPA–GluR1–IFN**γ** receptor complexes. Both signaling pathways induce calcium ion influx and mitochondrial dysfunction, which results in neuritic beading and neuronal cell death

Although a growing pool of evidence suggests that inflammatory cytokines—for example, IL-1β and TNF-α—are neurotoxic, this hypothesis remains controversial. Both of these cytokines do not cause neuronal death in healthy brain tissue or normal neurons (Rothwell et al. 1977) and a few studies have suggested neuroprotective roles for TNF-α and IL-1β (Strijbos and Rothwell 1995; Bruce et al. 1996). We examined the effects of inflammatory cytokines on cultured neurons. Alone, each of the microglia-derived cytokines did not induce neuronal cell death. IFNγ, however, directly induced neuronal dysfunction, which manifested as dendritic bead formation in mouse cortical neurons and enhanced glutamate neurotoxicity via α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA) receptors but not *N*-methyl-D-aspartate (NMDA) receptors. In the CNS, IFNγ receptors form neuron-specific, calcium-permeable receptor complexes with the AMPA receptor subunit GluR1. Through this receptor complex, IFNγ phosphorylates GluR1 at serine 845 via the JAK1.2/STAT1 pathway, increases calcium ion influx and nitric oxide production, and decreases ATP production, leading to dendritic bead formation (Mizuno et al. 2008). These mechanisms of neuronal excitotoxicity may occur in both inflammatory and neurodegenerative diseases of the CNS (Fig. 4.2).

4.2.3 Excitatory Amino Acids

Glutamate released by activated microglia induces excitotoxic neuronal death, which likely contributes to non-cell autonomous neuronal death in certain neurodegenerative diseases, including ALS and AD. We showed that glutamate is the most neurotoxic factor released from activated microglia (Takeuchi et al. 2006, 2011). Upon activation, microglia use glutaminase to produce large amounts of glutamate from extracellular glutamine, and release the glutamate through gap junction hemichannels, but not through glutamate transporters. Although blocking glutamate receptors and inhibiting microglial activation are potential therapeutic approaches in various neurodegenerative diseases, glutamate receptor blockers also perturb physiologic glutamate signaling and inhibitors of microglial activation suppress the neurotoxic and neuroprotective roles of microglia and have not been shown to markedly affect disease progression. Instead, gap junction hemichannels could be developed for the treatment of neurodegenerative diseases. However, the well-known gap junction inhibitor carbenoxolone failed to ameliorate disease progression in animal models of AD and ALS, although it suppressed glutamate release from activated microglia in vitro. Based on glycyrrhetinic acid, we synthesized INI0602, a novel gap junction hemichannel blocker that permeates the blood–brain barrier. INI0602 inhibited excessive glutamate release from activated microglia in vitro and in vivo and no notable toxicity was observed. Blocking gap junction hemichannels significantly suppressed neuronal loss in the spinal cord and extended survival in a transgenic ALS mouse model bearing a mutation in human superoxide dismutase 1. Moreover, INI0602 significantly reduced memory impairments in a transgenic mouse model of AD bearing both mutated human amyloid precursor protein and presenilin 1 (Takeuchi et al. 2011). Our results suggest that gap junction hemichannel blockers may provide a new therapeutic strategy to target neurotoxic microglia and prevent microglia-mediated neuronal death in various neurodegenerative diseases.

4.2.4 Miscellaneous

Microglia also produce various factors that are toxic to neurons and involved in neurodegenerative disorders like AD (Zhong et al. 2002). Activated microglia can damage or kill neurons in vitro by generating nitric oxide (Boje and Arora 1992; Meda et al. 1995), various toxic oxygen species (Tanaka et al. 1994), L-cysteine (Yeh et al. 2000), and tissue plasminogen activator (Flavin et al. 2000). Nitric oxide and superoxide react to form neurotoxic peroxynitrite (Estevez et al. 1998), which may have a role in AD; the levels of nitrotyrosine—a product of the reaction between peroxynitrite and tyrosine—increase in AD (Smith et al. 1997).

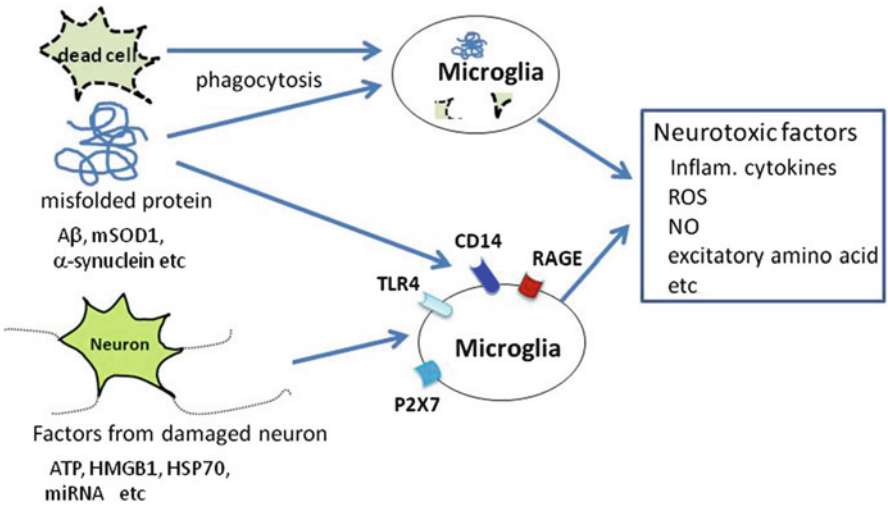

Fig. 4.3 Mechanisms of microglial activation during neuroinflammation

4.2.5 Factors that Activate Microglia

Although microglia activated via TLR4 or CD14 with LPS are highly neurotoxic, how microglia are activated in neurodegenerative disorders is unclear. Recent studies suggest that misfolded protein (e.g., Aβ, α-synuclein), and factors from damaged neurons called damage-associated molecular patterns, including ATP, high morbidity group box 1, heat shock protein 70, and microRNAs, can bind surface receptors on microglia (TLR4, CD14, P2X, and RAGE, among others) to initiate microglial activation (see Chap. 3) (Fig. 4.3).

4.3 Neural Factors That Affect Microglia

Recently, several lines of evidence have suggested that damaged neurons are not merely passive targets of microglia, but rather regulate microglial activity through cytokines, nucleotides, and chemokines. Degenerating neurons produce signaling molecules that regulate microglia-mediated phagocytosis and neuroprotection. Some of this signaling may be controlled by chemokines and chemokine receptors, which are widely expressed throughout the CNS (Tran and Miller 2003). These factors may function as help-me signals (see Chap. 3), whereas others may activate microglia to further damage neurons.

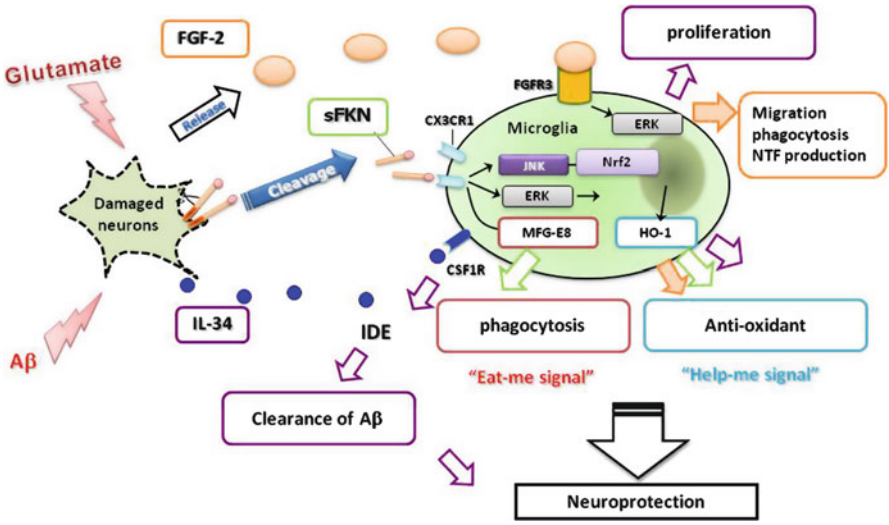

Fig. 4.4 Factors from injured neurons that activate microglia as help-me signals. Damaged neurons produce and secrete FKN (CX3CL1), IL-34, and FGF-2. Microglia express receptors for these factors, including CX3CR1, colony-stimulating factor 1 receptor, and FGF receptors. FKN, IL-34, and FGF2 activate microglia as eat-me or help-me signals

4.3.1 FKN

The CX3C chemokine FKN (CX3CL1), which occurs as soluble and membrane-anchored forms, may play a pivotal role in signaling between degenerating neurons and microglia. FKN and its receptor CX3CR1 are highly expressed in brain tissue (Pan et al. 1997; Nishiyori et al. 1998; Harrison et al. 1998; Hatori et al. 2002), particularly in neurons and microglia. We previously demonstrated that FKN functions as an intrinsic inhibitor of microglial neurotoxicity (Mizuno et al. 2003). CX3CL1 directly induces various microglial activities, including migration, proliferation (Pan et al. 1997), and inhibition of Fas-ligand-induced cell death (Boehme et al. 2000), glutamate-induced neurotoxicity (Noda et al. 2011), and proinflammatory cytokine production (Zujovic et al. 2000). Recently, we showed that soluble CX3CL1 directly enhances microglial clearance of neuronal debris, an effect that is mediated through phosphatidylserine receptors and production of milk fat globule-EGF factor 8 protein (Fig. 4.4) (Leonardi-Essmann et al. 2005; Noda et al. 2011). Neurons produce soluble CX3CL1. Membrane-anchored CX3CL1 is cleaved by several proteases, including members of the disintegrin and metalloprotease (ADAM) family (ADAM-10 and ADAM-17) and cathepsin S (Garton et al. 2001; Tsou et al. 2001; Hundhausen et al. 2003; Clark et al. 2007). When neurons are injured or exposed to glutamate, CX3CL1 is immediately shed. Little, however, is known about potential connections between Aβ-induced neuronal toxicity and CX3CL1 shedding.

Microglia respond to CX3CL1 through CX3CR1. CX3CL1 is protective against activated microglia-induced neurotoxicity (Noda et al. 2011). CCL2 appears to activate CX3CR1 expression via CCR2 and p38 MAPK activation (Green et al. 2006). CX3CR1 deficiency increases susceptibility to neurotoxicity in mice administered LPS and mouse models of Parkinson's disease and ALS (Cardona et al. 2006). A recent report also detailed CX3CL1-induced neuroprotection in a rat model of Parkinson's disease (Pabon et al. 2011). In addition, some studies have shown the worsening of pathologic features in an AD mouse model after knocking out CX3CR1 (Fuhrmann et al. 2010). Therefore, FKN from damaged neurons may be a help-me signal for microglia.

4.3.2 IL-34

The cytokine IL-34 is broadly expressed in various organs, including the heart, brain, lung, liver, kidney, spleen, and colon (Lin et al. 2008). We examined the effects of IL-34 on microglia because it has been shown to induce monocyte and macrophage proliferation through the colony-stimulating factor 1 (CSF-1) receptor. The function and production of IL-34 in the CNS, however, was unclear. We found that IL-34 is primarily produced by neuronal cells and microglia express CSF-1 receptor. IL-34 promoted microglial proliferation and the clearance of soluble oligomeric Aβ (oAβ), which plays a critical role in AD-associated synaptic dysfunction and neuronal damage. IL-34 increased the expression of insulin-degrading enzyme, enhancing oAβ clearance, and induced the expression of the antioxidant enzyme heme oxygenase-1 in microglia, reducing oxidative stress without producing neurotoxic molecules. Consequently, microglia treated with IL-34 attenuated oAβ-related neurotoxicity in primary cocultures of neurons and microglia. Intracerebroventricular administration of IL-34 in an APP/PS1 transgenic mouse model of AD ameliorated the impaired associative learning phenotype and reduced oAβ levels by upregulating levels of insulin-degrading enzyme and heme oxygenase-1. These findings suggest that enhancing the neuroprotective roles of microglia using IL-34 may be an effective approach against oAβ-mediated neurotoxicity in AD (Mizuno et al. 2011).

4.3.3 FGF-2

FGF-2 (also called basic FGF) plays a role in communication between degenerating neurons and microglia. We found that neurons damaged by oAβ 1–42 or glutamate secreted FGF-2, but these neurotoxic agents did not affect FGF-2 release from astrocytes or microglia (Noda et al. 2011). FGF-2 promoted microglial migration and the phagocytosis of neuronal debris through FGF receptor 3 (FGFR3). FGF-2 was neuroprotective and induced microglial migration via FGFR3 and the

extracellular signal-regulated kinase (ERK) signaling pathway. Moreover, Wnt signaling directly controlled FGFR3–ERK signaling in microglia. Taken together, these results show that FGF-2 secreted from degenerating neurons may act as a neuroprotective help-me signal by activating microglial migration and the phagocytosis of debris.

4.4 Concluding Remarks

Microglia produce factors that can be toxic or protective for neurons. Neurons send a variety of signals to microglia, including factors that signal "find me," "eat me," or "help me." Bidirectional communication between microglia and neurons is likely critical to maintaining a healthy CNS and also may contribute to the development of chronic neuroinflammation.

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Conflicts of interest The author has no conflicts of interest to declare.

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Chapter 5
Neuron–Astrocyte Interactions in Neuroinflammation

Jasna Kriz

Abstract Reactive astrogliosis is a prominent feature of the brain inflammatory response and it represents a hallmark of many CNS pathologies including Alzheimer's and Parkinson's diseases and amyotrophic lateral sclerosis. While in physiological conditions astrocytes serve as multifunctional housekeeping cells, once activated they may affect neuronal survival in many different ways. Depending on the type of the stimuli and/or pathological conditions reactive astrogliosis may lead to either neuroprotective or neurotoxic inflammatory responses. Here we summarize the current knowledge of the origins and neuropathological features of reactive astrogliosis. Furthermore, we discuss the role and the potential of astrocytes as resident brain immune cells with particular emphasis on how astrocyte immune profiles may determine the cross talk between activated astrocytes and neurons in acute brain injuries such as stroke versus nonresolving, chronic inflammation associated with neurodegenerative disorders. Finally, because of the complex nature of the brain inflammatory response and its relevance in drug discovery programs, we highlight the value and importance of live imaging models in which different elements of neuroinflammation, including astrocytes activation and glia/neuron cross talk, can be visualized and studied in real time.

Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
BBB	Blood–brain barrier

J. Kriz, M.D., Ph.D.(✉)
Department of Psychiatry and Neuroscience, Faculty of Medicine Laval University, and Le Centre de recherche de l'Institut universitaire en santé mentale de Québec, F3000 (F3467) 2601, de la Canardière, Québec (Quebec), Canada G1J 2G3
e-mail: jasna.kriz@fmed.ulaval.ca

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CCD	Coupled charged device
CNS	Central nervous system
ERE	Estrogen responsive element
Fluc	Firefly luciferase
FTD	Fronto-temporal dementia
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
ICAM-1	Intercellular adhesion molecule 1
IFN-γ	Interferon gamma
IL-1β	Interleukin-1beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
MCAO	Middle cerebral artery occlusion
MHC	Major histocompatibility complex
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NPCs	Neuronal progenitor cells
PD	Parkinson's disease
ROS	Reactive oxygen species
SOD1	Cu/Zn Superoxide Dismutase 1
TDP-43	Tar binding protein 43
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor alpha

5.1 Astrocytes: Housekeepers of the Brain

Astrocytes are the major resident glial cell population within the central nervous system (CNS) comprising nearly 35 % of the total brain cell population. Previously considered as simple cellular layer that fills interneuronal space, it is now recognized that astrocytes are multifunctional housekeeping cells involved in a permanent cross talk with neurons and other neighboring glial cells (for review, see Parpura et al. 2012). In physiological conditions and in healthy brain the astrocytes perform numerous functions that are essential for neuronal survival (Pekny and Nilsson 2005; Parpura et al. 2012). For example, astrocyte–neuron cross talk through the release of several neurotrophic factors is instrumental in maintenance of the brain homeostasis and the brain energy metabolism (Pellerin and Magistretti 1994; Ransom et al. 2003). Moreover, astrocytes play a pivotal role in modulating extracellular glutamate level contributing both to the functional synapse and to the prevention of glutamate-induced excitotoxic neuronal injury (Pellerin and Magistretti 1994; Parpura and Zorec 2010). Astrocytes are also central to the formation of the neurovascular unit and regulation of the blood–brain barrier (BBB). Namely the close interactions between astrocytes, neurons, and blood vessels make astrocytes a key element involved in coupling of neuronal activity and cerebral blood flow

(Gordon et al. 2008; Choi et al. 2012) inducing either constriction or dilation of the neighboring blood vessels (for review, see Attwell et al. 2010). Altogether, understanding these multiple functions should be taken into consideration when questioning the role of astrocytes in neuroinflammation. Inflammation is a defense reaction against diverse insults designed to remove noxious agents and to limit their detrimental effects. Although CNS has been long considered as an immune privileged site, inflammation does occur in the brain and neuroinflammation has been increasingly considered as a prominent feature of many chronic neurodegenerative disorders including Alzheimer's disease (AD) and Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), etc. (for review, see Maragakis and Rothstein 2006). Inflammatory response is also a key element and integral part of the complex pathophysiological cascade triggered by ischemic and/or other types of brain injuries. Experimentally and clinically, brain ischemia is followed by acute and prolonged inflammatory response characterized by the activation of resident glial cells (microglia and astrocytes), production of inflammatory cytokines, and leukocyte infiltration in the brain, events that may contribute to brain pathology (Stoll et al. 1998; Lo et al. 2003; Kriz and Lalancette-Hébert 2009; Iadecola and Anrather 2011). However, whether inflammatory processes and associated glial phenotypes are deleterious or beneficial to recovery is presently a matter of debate and controversies.

5.2 Reactive Astrogliosis as a Hallmark of Brain Inflammatory Response

Astrocyte activation is one of the key components of the cellular responses to brain injuries and chronic neurodegeneration (Ridet et al. 1997; Hall et al. 1998; McGeer et al. 1991; Nagele et al. 2004; Pekny and Nilsson 2005). The passage from the quiescent to reactive astrocytes observed in many neuroinflammatory conditions is associated with the strong up-regulation of the intermediate filament, glial fibrillary acidic protein (GFAP), and under certain conditions the reactive astrocytes may also up-regulate their progenitor markers such as vimentin and nestin (Ridet et al. 1997; Eng et al. 2000; Pekny and Nilsson 2005). While to date it has been widely established that strong transcriptional activation of GFAP is hallmark of many neuropathological conditions the precise molecular mechanisms underlying the GFAP induction remain less clear. Interestingly, however, recent work by Bae et al. (2006) reveals that one of the important molecules involved in transcriptional regulation of GFAP is nuclear factor kappa B (NF-κB) thus suggesting that GFAP up-regulation is indeed strongly associated with neuroinflammation. Reactive astrogliosis is the universal reaction to brain injuries; however, the reason for this response and how it may effect neuronal survival have been somewhat controversial. It has been suggested that reactive astrogliosis comprises two major events; mobilization (and hypertrophy) and proliferation of mature astrocytes around the site of injury (Fawcett and Asher 1999; Silver and Miller 2004). However, the most comprehensive analysis of the morphological changes associated with the reactive astrogliosis

has been presented by Wilhelmsson et al. (2006). In their efforts to visualize the morphological changes linked to reactive astrogliosis and to identify their respective occupied domain they actually redefined the concept of reactive astrocyte Wilhelmsson et al. (2006). Namely, by using novel dye filling method the authors described and assessed the full three-dimensional shape of quiescent and reactive astrocytes in two different models of neurotrauma including denervated hippocampus and lesioned cerebral cortex. Interestingly in both experimental paradigms, the reactive astrocytes increased the numbers and the thickness of their cellular processes but did not extend to occupy a greater volume of tissue than nonreactive astrocytes. Importantly, the domain of an individual astrocyte did not change between activated and the resting cells. Actually in both experimental paradigms the most striking difference between quiescent and reactive astrocytes was in the numbers of visible processes leaving the soma Wilhelmsson et al. (2006).

Another important question raised here is to what extent and/or whether reactive astrocytes have a potential to proliferate? The evidence to date has been rather controversial. While mature astrocytes normally do not divide, a subpopulation of reactive GFAP positive cells does proliferate in response to injury. Because increase in GFAP immunoreactivity is commonly used as a marker of reactive astrogliosis this raises the question of whether the proliferating GFAP+ cells arise from endogenous glial progenitors or from mature astrocytes that start to proliferate in response to injury. By targeting quiescent astrocytes by either genetic or viral manipulations and following these cells in their reactive response to injury allowed the fate mapping analysis and establishment of the lineage relation between reactive and quiescent astroglia (Buffo et al. 2008). Using this powerful molecular technique the authors observed that considerable proportion of quiescent astrocytes indeed resume proliferation in response to injury suggesting that astrocytes are not permanently postmitotic cells and in certain conditions may retain the capacity to resume proliferation (Buffo et al. 2008). Interestingly, however, in the experimental model of chronic neurodegenerative disease, in the mutant $SOD1^{G93A}$ mice (mouse model of ALS) the origin of the GFAP+ cells' contribution to "reactive astrogliosis" seems to be rather different (Lepore et al. 2008; Magnus et al. 2008). In the studies employing transgenic mouse model in which the herpes simplex virus-thymidine kinase (TK) is expressed in GFAP+ cells the authors investigated the role of proliferating GFAP cell in ALS (Lepore et al. 2008). Here it is important to mention that in this mouse model astrocyte proliferation can be completely blocked by the ganciclovir treatment (Bush et al. 1999). In the context of ALS, first the GFAP-TK mouse was crossed with $SOD1^{G93A}$ mouse model and once double transgenic animals were obtained the GFAP+ astrocyte/cells were ablated by ganciclovir treatment (Lepore et al. 2008). Intriguingly in this experimental model ablation of dividing astrocytes did not affect overall astrogliosis nor the clinical course of disease suggesting that astrocyte proliferation does not play an important role in ALS pathogenesis (Lepore et al. 2008). However, an additional interesting observation came from this study. Namely the data analysis revealed that the probable source of reactive astrogliosis in ALS models was NG2+ glial progenitors. This was also confirmed by Magnus et al. (2008). They examined in the mouse model of ALS (in the adult spinal cord of Cu/

Zn Superoxide Dismutase 1 (SOD1) mutant mice) whether glial progenitors became activated and contribute to the astroglial response in this model. Interestingly, the progenitor marker NG2 was increased in parallel with GFAP during the symptomatic phase of disease. Furthermore, in in vitro conditions the treatment of SOD1 mutant expressing glial progenitors with pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) (unlike the wild-type glial progenitors) induced marked proliferation and translocation of the transcription factor Olig2 from the nucleus to the cytoplasm consequently resulting in astrocyte differentiation. Taken together the results from this study suggest that glial progenitor cells from SOD1G93A mutant mice differentially respond to pro-inflammatory cytokines and contribute to reactive astrogliosis (Magnus et al. 2008). Thus it may be concluded that both mature astrocytes and multipotent progenitors may proliferate; however, their contribution to reactive astrogliosis and associated increase in the GFAP immunoreactivity may be context- and injury-dependent. What should not be forgotten when comparing differential inflammatory profiles and origin of reactive astrocytes is that in $SOD1^{G93A}$ mice (and in some other genetic disease models) mature astrocytes as well as glial progenitors express high levels of the mutated gene which may considerably affect functional properties of astrocytes and other cell types implicated in the inflammatory response and disease pathology.

5.3 Astrocytes as Resident Brain Immune Response

Neuroinflammation is a complex tissue response to different types of pathological stimuli and it comprises reaction of all cell types within the CNS. Microglia are the principal immune cells of the brain and their activation is one of the earliest features of almost any changes in microenvironment often preceding astrogliosis (Carson et al. 2006). While numerous studies have confirmed the role of microglia as antigen presenting cells within the CNS the role of astrocyte remains controversial. However, some evidence suggests that astrocytes have also potential to act as immunocompetent cells (for review, see Yuanshu and Benveniste 2001). Namely, it has been reported that astrocytes activation in certain conditions can be associated with the expression of the major histocompatibility complex (MHC) class II molecules which play a critical role in induction of immune response through the presentation of the antigen to CD4 T helper cells. Furthermore, previous studies revealed that reactive astrocytes again in certain conditions may express molecules such as intercellular adhesion molecule 1 (ICAM-1) (Aloisi et al. 1998), B7, and occasionally CD40 (Nguyen and Benveniste 2000; Aloisi et al. 1998; Tan et al. 1998; Abdel-Haq et al. 1999). While there have been discrepancies in the literature regarding the ability of astrocytes to act as the antigen presenting cell, on the other side it has been widely accepted that these cells can produce several pro- and anti-inflammatory molecules as well as a variety of trophic factors. For example, following pro-inflammatory stimuli, brain injury and/or chronic brain pathology-activated astrocytes can produce interleukin-1beta (IL-1β), TNF-α, IL-6, as well as nitric oxide

(NO) and reactive oxygen species (ROS). Furthermore, astrocytes are in particular strong producers of IL-6 in diseased brain and the toxicity of the astrocytic IL-6 has been demonstrated in the transgenic mouse models where IL-6 is expressed under GFAP promoter. In this mouse model high levels of IL-6 have been associated with neurodegeneration, breakdown of the BBB, and increased expression of the complement proteins (Campbell et al. 1993). Moreover, destructive potential of astrocytic IL-6 has been also demonstrated in the transgenic mouse model (GFAP-IL6) whereas IL-6 production has been restricted to cerebellum (Quintana et al. 2009). Following immunization protocols these mice develop tissue damage characterized by exaggerated inflammatory response restricted to cerebellum which further was associated with severe ataxia (Quintana et al. 2009). On the other hand, the results of the recent study indicate that the astrocyte IL-6 has a major pro-survival role at early ages of intrauterine life (Quintana et al. 2012). Some additional evidence further supports the dual role of astrocyte in brain inflammatory response. Astrocyte are the cells involved in the brain innate immune response and recent evidence suggests that many of the toll-like receptor (TLR) ligands may induce pro-inflammatory and neurotoxic profiles in astrocyte (Ma et al. 2012). Interestingly, however, in previous studies Bsisbi and colleagues reported that contrary to TLR3 response in macrophages, activation of the TLR3 pathway in astrocytes was associated with expression of anti-inflammatory cytokines including IL-9, IL-10, and IL-11 promoting neuroprotection and increasing neuronal survival in organotypic slice assays (Bsibsi et al. 2006). This may suggest that TLRs as key molecules of innate immune system may exert context-specific and rather differential effects. In our previous work using the TLR2 reporter mouse models and analyzing induction of the TLR2 following pathogen- and/or danger-associated molecular patterns, contrary to a robust induction of TLR2 observed on microglial cells (mRNA as well as on protein levels), we did not observe any up-regulation of the TLR2 on astrocytes (Lalancette-Hébert et al. 2009).

5.4 Functional Role of Activated Astrocytes: Astrocyte–Neuron Cross Talk in Acute Injuries

In animal models as well as in human disease astrogliosis is prominent feature and it is thought to be associated with inflammation-induced neurotoxicity. However, growing evidence suggests that astrocytes as microglia act as dual edge swords. Depending on the injury and disease context, including here spatial and temporal components, astrocyte activation may be viewed as beneficial event characterized by production of growth factors and neurotrophins thus promoting neuronal survival and supporting neuronal growth. On the other hand reactive astrogliosis may be detrimental for neuronal function by production of potentially neurotoxic molecules and by causing a major impediment to CNS repair processes due to a glial scar formation (for review, see Trendelenburg and Dirnagl 2005; Pekny and Nilsson 2005; Hamby and Sofroniew 2010).

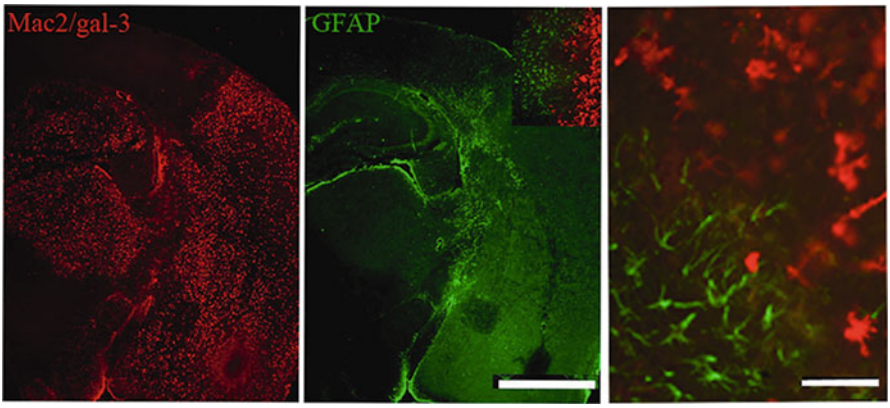

Fig. 5.1 Differential spatial distribution of activated microglia and astrocytes 48 h following experimental stroke (60 min transient left middle cerebral artery occlusion (MCAO)). Activated microglial cells were detected by fluorescent immunolabeling of Mac-2/gal-3 (*red*) while activated astrocytes were labeled by glial fibrillary acidic protein (GFAP) (*green*). Note there is no spatial overlap between activated microglia and astrocytes. Scale bars: 500 and 50 μm

In the context of cerebral ischemia, reactive astrogliosis is strongly associated with increase in GFAP immunoreactivity and the GFAP levels have been widely used as an alternative marker of neuronal damage (Herrmann and Ehrenreich 2003; Vissers et al. 2006; Petzold et al. 2006). The process of astrocytes activation following brain ischemia is initiated approximately 4–6 h after ischemic attack and it develops during initial few days and may persist up to 7 days and longer after stroke (Stoll et al. 1998; Cordeau et al. 2008). Astrocytes become hypertrophic while microglial cells retract their processes and assume an amoeboid morphology that is typical for activated microglia. Previous studies have demonstrated that following middle cerebral artery occlusion (MCAO) from 2 days reperfusion onward, there was a marked increase in intensively stained GFAP positive astrocytes in the areas surrounding ischemic lesions (Stoll et al. 1998; Cordeau et al. 2008). As shown in Fig. 5.1, contrary to activated microglial cells stained with the activation marker Mac-2/Gal-3 and situated within the core of infarction, 48 h after experimental stroke the GFAP positive astrocytes are barely detectable in the lesion core but they markedly increase in numbers and in the intensity of the GFAP staining in the peri-infarct area. The distinct spatial distribution of the astrocytes is also confirmed by using spectral imaging and 3D reconstruction of the GFAP signals obtained from the GFAP-luc reporter mouse after stroke (Cordeau et al. 2008) (see Fig. 5.2). In addition to distinct spatial distribution between activated microglia/macrophages and astrocytes we also observed marked difference in the temporal activation profiles. As described by Cordeau et al. (2008) the GFAP signal peaked 48–72 h after stroke and it was barely detectable 7 days after stroke, while microglial activation may last several months after initial stroke (Lalancette-Hébert et al. 2009). Although the functional significance of reactive astrogliosis in acute injuries is still debated, evidence suggests that it is involved in the formation of the astroglial scar and

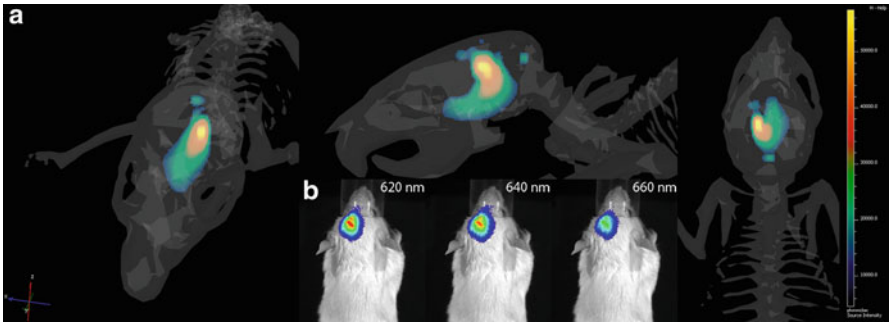

Fig. 5.2 In vivo 2D and 3D imaging of astrogliosis after ischemic injury. 3D reconstruction of in vivo biophotonic/bioluminescent GFAP signals emitted from the brain of the living GFAP-luc mice 48 h after MCAO. (**a**) Using diffuse light imaging tomography DLIT-algorithms and structural images the data was transformed to 3D images. *Red* areas represent the regions of the brain with highest intensity of the photon emission. The localization of the highest intensity area was measured and presented in three axes (*x*, *y*, and *z*) in mm from the scull surface (*upper*). (**b**) Representative images show collection of imaging samples at three different wavelengths across the emission spectrum of the bioluminescent source (firefly luciferase) with the substantial fraction of the light 620 nm. The scales on the right are the color maps for photon density and source intensity

considered as major impediment to axonal regeneration. However, recent studies employing GFAP knockout mouse suggested that the role of this intermediate filament protein in brain injuries and the glial scar formation may be more complex than initially thought (Pekny 2001). Contrary to expectations, the absence of the GFAP protein has been associated with increased susceptibility for ischemic brain damage following MCAO (Pekny et al. 1999) and by marked alterations in post-traumatic glial scarring and tissue healing (Nawashiro et al. 1998). Additional evidence of the protective role of functional astrocyte and scar tissue formation in the brain response to injury came from the studies performed on the GFAP-TK mice (Bush et al. 1999). The results have shown that selective ablation of the proliferating astrocytes following traumatic (Bush et al. 1999) or spinal cord injury (Faulkner et al. 2004) worsens the outcome. Namely, in both experimental paradigms defective astrocyte response was associated with marked increase in neuronal and oligodendrocytic death, increase in lesion size, and diminished functional recovery (Bush et al. 1999; Faulkner et al. 2004). Taken together, these findings suggest that reactive gliosis may play protective role in the brain response to acute injuries.

One of the rather intriguing results we obtained in our "stroke" experiments was marked gender difference in neuroinflammatory astrocyte response to ischemic injury. While previous studies on non-injured astrocytes demonstrated cyclic, estrus-dependent variations of GFAP expression in certain nuclei of the rat brain (Garcia-Segura et al. 1994; Stone et al. 1998), a putative estrogen responsive element (ERE) binding site has been detected in the 5′-upstream region of the human and rat GFAP promoter (Laping et al. 1994) thus suggesting that the level of circulating gonadal hormones would predict and/or modulate glial response to brain injury. Importance

of gonadal hormones in modulation of astrocyte response to injuries was further confirmed in our hormone-deprivation experiments. Namely, ischemic lesions were in general smaller in female mice when compared to GFAP-luc males. In addition, the infarcts were significantly smaller in females during estrus and in the females on estrogen replacement therapy (pharmacological doses), thus confirming a direct neuroprotective effect of estrogen in ischemia (Cordeau et al. 2008). However, contrary to the findings in male mice where positive correlation was observed between bioluminescent signal intensities/GFAP up-regulation and the size of infarctions, there was no correlation between GFAP up-regulation/astrocyte responses and infarct size in any of the experimental groups employing female GFAP-luc mice, thus suggesting that GFAP up-regulation/astrocyte response to ischemic injury may not have the same functional significance in male and female mice.

5.5 Astroglia–Neuron Cross Talk in Chronic Neurological Disorders

Inflammatory response and associated reactive astrogliosis are hallmark of many neurological disorders including AD and PD, ALS/fronto-temporal dementia (FTD), and many other similar conditions (for review, see Maragakis and Rothstein 2006). While inflammation may represent a natural and beneficial mechanism that helps the nervous system to recover from acute injury, such as cerebral ischemia, spinal cord and brain injuries, or nerve degeneration, the tissue response is however different in chronic neurological disorders, with an issue of nonresolving inflammation (Nagele et al. 2004; Maragakis and Rothstein 2006; Carson et al. 2006; Nathan and Ding 2010; Parpura et al. 2012). In chronic neurodegenerative disorders inflammation may persist for several years or even decades. In these conditions, astrocytes, as well as other glial cells, are exposed to an ongoing inflammatory stimulation that eventually reduces the glial capacity to release neurotrophic factors and provide support to neurons. Results obtained from the studies on triple transgenic AD mouse model (comprising both amyloid and tau pathology) revealed the age-dependent astroglia atrophy as well as astrogliosis (Olabarria et al. 2010). However, to what extent and/or how these morphological changes affected astrocyte function in this model is less clear (Olabarria et al. 2010). Growing line of evidence suggests that astrocytes are involved in the pathogenesis of AD at multiple levels. As resident immune cells they act as an important source of cytokines and the major site of expression of the inducible nitric oxide synthase (iNOS), in human disease as well as in related mouse models (Heneka et al. 2005). On the other hand Koistinaho et al. (2004) (Pihlaja et al. 2011) have shown that astrocytes are actively involved in the degradation and clearance of Aβ from the brain involving direct and indirect actions, as modifiers of microglial phagocytosis. Hence, in chronic conditions such as AD astrocytes may be driving neurodegeneration by release of various neurotoxic pro-inflammatory mediators, but also trying to resolve the pathological stimuli by modifying uptake and degradation of Aβ.

The neuron/astroglia cross talk is equally complex in ALS especially in genetically inherited disease where function of non-neuronal cells is additionally affected by the presence of mutated proteins such as SOD1 and more recently Tar binding protein-43 (TDP-43) (for review, see Rowland and Shneider 2001; Lagier-Tourenne and Cleveland 2009). Although restricted over-expression of the mutated SOD1 in astrocytes (using GFAP promoter) resulted in reactive astrogliosis but surprisingly did not cause motor neuron degeneration in this transgenic model (Gong et al. 2000) more recent evidence suggest that expression of mutated SOD1 protein in astrocytes may indeed be neurotoxic. Namely, in the parallel papers by Di Giorgio et al. and Nagai et al. the expression of the mutated SOD1 in astrocytes, but not other cell types, induced selective death of motoneurons in cultures, thus suggesting that astrocytes may play a role in the specific degeneration of motor neurons in ALS (Di Giorgio et al. 2007; Nagai et al. 2007). Further supporting the role of astrocytes in ALS is the converse set of experiments where mouse models with deletable transgenes ($SOD1^{G37R}$ flanked by LoxP sequences to allow excision by the Cre recombinase in specific cell types) have been generated (Boillee et al. 2006; Yamanaka et al. 2006). The deletion of mutant SOD1 transgene from Cd11b positive cells increased the life span of Lox $SOD1^{G37R}$ mice by approximately 100 days, whereas deletion of the SOD1-mutated gene from GFAP expressing cells also resulted in significant increase in survival of ALS mice (Boillee et al. 2006; Yamanaka et al. 2006). However, the strongest evidence yet for direct involvement of astrocytes in ALS pathology came from the studies on astrocytes generated from human postmortem tissue from familial and sporadic ALS patients (Haidet-Phillips et al. 2011). In this study the astrocytes were derived form the adult postmortem neuronal progenitor cells (NPCs) proved to be tripotent thus capable of differentiating into neurons, oligodendrocytes, and astrocytes. Importantly the NPCs-derived astrocytes markedly up-regulate inflammatory gene expression while cocultured with motor neurons derived from familiar and sporadic ALS patients. Interestingly the network-based pathway analysis of different networks ranked by statistical scoring analysis identified the NF-κB signaling complex as a highest ranked network, followed by IFN-α and stress kinases network, all involved with numerous interactions with inflammatory genes (Haidet-Phillips et al. 2011). That inflammatory pathways and in particular NF-κB signaling pathway may play a role in TDP-43-related ALS/FTD pathogenesis has been recently reported by Swarup et al. (2011a). In this work, the real-time RT-PCR data analysis from the spinal cord samples from sporadic ALS cases and control individuals revealed approximately a threefold increase in mRNA coding for TDP-43 and p65 NF-κB (Swarup et al. 2011a). Moreover, it has been demonstrated that TDP-43 interacts and co-localizes with NF-κB p65 in glial and neuronal cells of ALS patients as well as in a mouse model. Further analysis revealed that TDP-43 acts as a co-activator of p65 resulting in exaggerated glial inflammatory response and increased neurotoxicity while treatment with NF-κB inhibitor withaferin A reduced ALS disease symptoms (Swarup et al. 2011a). Here, it is important to mention that in mouse models expressing genomic fragments of human wild-type and mutant TDP-43 one of the first signs of pathological changes in presymptomatic period (as well as in SOD1 mutant mice) was early increase in markers of glial activation, in particular GFAP, suggesting a

complex dialog between stressed neurons and astrocytes before clinical onset of disease (Keller et al. 2009; Swarup et al. 2011b).

5.6 Live Imaging of Neuroinflammation and Astrogliosis: What We Can Learn from the Studies Using GFAP-Reporter Mouse Model

To date, most of the basic knowledge about functional changes associated with glial cells' activation profiles, including astrogliosis, is based on detailed immunohistological/morphological evaluation of the cell-specific markers. Due to a static nature of this approach, it is possible that some early events may have been overlooked due to a lack of sensitive markers for detection of subtle and/or transient changes in early cellular phenotypes. To address this issue over the past several years in the laboratory we developed and validated series of mouse models of bioluminescence and fluorescence allowing the noninvasive and time-lapse imaging of processes associated with brain injuries and repair including astrogliosis, microgliosis, and neuronal damage and regeneration (Cordeau et al. 2008; Lalancette-Hébert et al. 2009; Gravel et al. 2011). The strategy was to generate transgenic mice expressing dual reporters, the firefly luciferase (Fluc) and the green fluorescence reporter GFP, whose transcription is dependent upon the selected gene promoter (Lalancette-Hébert et al. 2009; Gravel et al. 2011).

In order to visualize neuroinflammatory changes and astrocyte activation from the brain of live animals in our studies we took advantage of the mouse model expressing Fluc under transcriptional control of the murine GFAP promoter, initially described by Zhu et al. (2004). Although GFAP-luc mouse has proved to be a very useful tool in in vivo analysis of neuroinflammatory response in stroke (Cordeau et al. 2008) (see Fig. 5.2) and in the murine experimental autoimmune encephalomyelitis (Luo et al. 2008), the most intriguing and interesting results using this model have been obtained in the studies focused on early disease pathogenesis in ALS and ALS/FTD models. Here, it is important to mention that ALS, AD, and other neurodegenerative disorders are chronic diseases that may have an asymptomatic phase spanning over several decades; therefore, using sensitive imaging approaches may help discover early biomarkers of disease. We generated double transgenic SOD1G93A/GFAP-luc mutant (Keller et al. 2009) as well as the TDP-43/GFAP-luc mice (Swarup et al. 2011b) and by using sensitive coupled charged device (CCD) camera, an in vivo biophotonic/bioluminescence imaging, in this models we were able to visualize early and disease-specific changes in astrocyte phenotypes. Importantly in both experimental models changes in astrocyte profiles/increase in the GFAP signals preceded clinical onset of disease. Our initial analysis revealed that similar tendency has been also observed in the mouse model of AD (unpublished observations), thus suggesting that increase in GFAP signals and associated increase in the expression of inflammatory molecules may be considered as an early biomarker in chronic neurodegeneration. Moreover, using the GFAP-luc

reporter mouse in preclinical in vivo pharmacological studies revealed that the GFAP biophotonic signals can be used as a valid readout in preclinical studies investigating therapeutic efficacy of immunomodulatory molecules such as minocycline and withaferin A in ALS and ALS/FTD models (Keller et al. 2011; Swarup et al. 2011a). Finally, our studies suggest that biophotonic signals imaged from the live animals can be used as valid biomarkers to screen for novel drugs/biocompatible molecules (Maysinger et al. 2007; Hutter et al. 2010; Lalancette-Hébert et al. 2010) and/or to visualize distinct pathological events and therapeutic efficacy in ALS and in other neurological disorders (Keller et al. 2009, 2011; Swarup et al. 2011a, b).

Over the last years, studies targeting glial cells, including astrocytes, in neurodegenerative diseases taught us that inflammation is a double-edged sword (Wyss-Coray and Mucke 2002; Hamby and Sofroniew 2010) leaving the question of adequacy of this approach open, especially when considering that the border between beneficial and detrimental effects of glial cell activation seemed to be very narrow. Growing line of recent evidence suggests that inflammation is promising but rather complex therapeutic target and in order to find novel and efficient neuroinflammation-based therapies there is need for better understanding (decoding) of glia neuron cross talk in diseased as well as in healthy brain.

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Chapter 6
Neuron–Oligodendrocyte Interactions in Neuroinflammation

Kazuhiro Ikenaka

Abstract Axon–oligodendrocyte progenitor cell (OPC) interactions are required for proper development of axons and OPCs. (1) Axons direct the OPC to differentiate and initiate myelination. (2) Signals from the myelinating oligodendrocyte (OL) trigger reorganization of axonal proteins. (3) The conduction velocity increases further because of reciprocal signaling between the OL and the myelinated axon. (4) Myelin is crucial for maintenance of the axon. Neuroinflammation affects neuron–OL interactions during each of these stages, and results in demyelination, which causes redistribution of axonal proteins and inefficient impulse conduction. However, in the early phases, OPCs present at the lesion remyelinate the naked axon and promote axon recovery. When cyclical demyelination and remyelination occur, remyelination is hampered by several inhibitors. Thus, a new type of axon–OL interaction is established, resulting in chronic demyelination.

6.1 Introduction

The most representative of the neuron–oligodendrocyte (OL) interactions is myelination. Myelin is a multilamellar structure that ensheathes axons and enhances conduction velocity by inducing saltatory conduction. In the central nervous system (CNS), myelination is accomplished by a series of orchestrated interactions between axons and oligodendrocytes (OLs). During early stages of CNS development, axons also interact with OPCs. OPCs are generated in several loci in the ventricular zone and migrate to the future white matter, where many axons are waiting for their arrival. Axons and OPCs communicate with each other vigorously, by releasing

K. Ikenaka, Ph.D. (✉)
Division of Neurobiology and Bioinformatics, National Institute for Physiological Sciences, 5-1-Higashiyama, Myodaiji, Okazaki, Aichi 444-8787, Japan
e-mail: ikenaka@nips.ac.jp

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humoral factors and making direct contact. At sites of axon–OPC contact, unmyelinated axons assemble a vesicular release apparatus that supports the activity-dependent release of neurotransmitters such as glutamate, and even form synapses (or synaptic-like structures) with OPCs (Kukley et al. 2007; Ziskin et al. 2007). These interactions are required for proper development of several morphological and functional features of OPCs and axons. First, the signaling from the axon directs the OPC to differentiate and initiate myelination. In mammals, the myelin membrane inhibits axonal extension; therefore, myelination can only be initiated after neural circuits have been established. Thus, myelination must be strictly regulated by the axon. Second, the myelinating OL signals to the axon, triggering reorganization and localization of membrane proteins that had been evenly distributed along the unmyelinated axon. The localization of distinct sets of membrane proteins along the length of a myelinated axon creates four unique regions: the node of Ranvier, the paranode, the juxtaparanode, and the internode. Creation of these regions is essential for the induction of saltatory conduction. Myelin also increases the diameter of axons. Third, after saltatory conduction has been induced, axonal activity in the axon leads to increases in the intracellular calcium concentration in the OL. The activated OL in turn signals to the myelinated axon to further increase the conduction velocity (Yamazaki et al. 2007). Fourth, myelin plays an important role in the maintenance and survival of the axon (Lee et al. 2012).

Neuroinflammation affects neuron–OL interactions during each of these stages. Multiple sclerosis (MS) is the most common disabling CNS disease in young adults. It is characterized by recurrent periods of relapse and progression that result from multifocal brain and spinal cord inflammation. Thus, in this chapter, MS will be mainly considered as a cause of neuroinflammation. Herein, I will describe neuron–OL interactions in normal conditions for each of the stages described above, and then describe how these interactions are affected by neuroinflammation.

6.2 Neurons Stimulate Differentiation of Oligodendrocyte Progenitor Cells

Cross talk between OPCs and axons begins when OPCs encounter naked axons in the future white matter. First, the axons direct the OPCs to delay further differentiation. Nerve growth factor (NGF) is a potent regulator of the axonal signals that control myelination, and reduces OL myelination (Chan et al. 2004). NGF and its cognate receptor, TrkA, induce the expression of leucine-rich repeat (LRR) and Ig domain-containing, Nogo receptor-interacting protein (LINGO-1) (Lee et al. 2007) that inhibits OL differentiation (Mi et al. 2005). OL differentiation is also regulated by Notch signaling. OPCs/OLs in the developing rat optic nerve express Notch1 receptors and retinal ganglion cells express Jagged1, a ligand of the Notch1 receptor, along their axons. Jagged1 expression decreases with a time course that parallels myelination in the optic nerve (Wang et al. 1998). Conversely, Notch1 interactions with contactin 1 (also known as neural cell surface protein F3), which is clustered at axonal/paranodal junctions, promote OL differentiation by initiating Deltex1

signaling (Hu et al. 2003). Thus, the timing of both OL differentiation and myelination is controlled by the Notch pathway. Another inhibitor of OPC differentiation, PSA-NCAM, is first expressed on all growing fibers and negatively regulates myelin formation. Then axonal expression is down-regulated and myelin deposition occurs only on PSA-NCAM-negative axons (Charles et al. 2000). Thus, OPC differentiation is inhibited by unmyelinated axons through several distinct pathways.

Once the neural circuitry of the CNS has been established, the axons now direct OPCs to differentiate and initiate myelination. The electrical activity coursing through the axon could easily be considered as one of the candidates for this signal, because electrical activity increases after circuit formation. In mice reared in complete darkness for 20 and 30 days, delay in myelination of the optic nerve fibers caused a reduction in the number of myelinated axons by some 12–13 % (Gyllensten and Malmfors 1963). Artificial opening of the eyes of young rabbits on the fifth postnatal day led to accelerated myelination: the myelin basic proteins and proteolipid proteins nearly doubled between the seventh and the tenth postnatal days when compared to controls; 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) activity also increased by about 60 % (Tauber et al. 1980). These results clearly demonstrate that electrical activity controls OPC differentiation.

Axonal electrical activity has been shown to control the production and release of growth factors that regulate OPC proliferation. Therefore, the level of activity determines the number of OLs that develop in a given region (Barres and Raff 1993). OPCs express adenosine receptors, which are activated in response to action potential firing. Adenosine acts as a potent neuron–glial transmitter to inhibit OPC proliferation, stimulate differentiation, and promote the formation of myelin. This neuron–glial signal provides a molecular mechanism for promoting OL development and myelination in response to impulse activity (Stevens et al. 2002). A mechanism for nonsynaptic, nonvesicular release of adenosine triphosphate (ATP) from axons through volume-activated anion channels (VAACs) activated by microscopic axon swelling during action potential firing was identified. ATP release from cultured embryonic dorsal root ganglion axons persisted when bafilomycin or botulinum toxin was used to block vesicular release, whereas pharmacological inhibition of VAACs or prevention of action potential-induced axon swelling inhibited ATP release and disrupted activity-dependent signaling between axons and astrocytes (Fields and Ni 2010). The cytokine leukemia inhibitory factor (LIF) is released by astrocytes in response to ATP liberated from axons firing action potentials, and LIF promotes myelination by mature OLs. This activity-dependent mechanism promoting myelination could regulate myelination according to functional activity or environmental experience and may offer new approaches to treating demyelinating diseases (Ishibashi et al. 2006).

An important finding to understand communication between axon and OPCs is that OPCs form synaptic contacts with axons in cerebral white matter in neonatal rodents (Kukley et al. 2007; Ziskin et al. 2007). Glutamate is released from synaptic vesicles along axons of mouse dorsal root ganglion neurons in culture and promotes myelin induction by stimulating formation of cholesterol-rich signaling domains between OLs and axons, and increasing local synthesis of the major protein in the myelin sheath, myelin basic protein, through Fyn kinase-dependent signaling. This axon–OL

signaling would promote myelination of electrically active axons to regulate neural development and function according to environmental experience (Wake et al. 2011).

During early stages of development, white matter injury due to hypoxia, ischemia, and the resultant neuroinflammation often results in periventricular leukomalacia (PVL), which is the predominant form of brain injury and the most common cause of cerebral palsy in premature infants. In a mouse model of PVL, synapses between axons and OPCs are profoundly damaged (Shen et al. 2012). Synaptic damage could disrupt the communication between axons and OPCs, and may cause PVL. Ischemia is also damaging to OLs, because the reduced energy supplies and the increased intracellular Ca^{2+} levels can injure OLs and damage myelin. Although mature OLs are sensitive to hypoxia and ischemia (Matute et al. 2006), immature OLs in the perinatal brain are even more sensitive, most likely because they express a variety of glutamate receptors. Influx of extracellular Ca^{2+} through AMPA and kinate receptors is probably the most important mechanism of Ca^{2+} overload in OLs (Matute 2011).

6.3 Oligodendrocytes Induce Clustering of Axonal Channels and Increase Axon Diameter

While axon-derived signal induces the OL to initiate myelination, myelin-derived signals in turn induce the axonal ion channels to redistribute. For example, the voltage-gated sodium channel Nav1.6 clusters at the nodes of Ranvier (Caldwell et al. 2000) and the voltage-gated potassium channels Kv1.1 and Kv1.2 cluster at the juxtaparanodal regions (Wang et al. 1993). Correct clustering of these channels is essential for the induction of saltatory conduction. As shown in Fig. 6.1, intracellular complexes regulate the formation of ion channel clusters and paranodal junctions, which are induced by interactions between neurofascin 155 (NF155), contactin, and contactin-associated protein 1 (Caspr/CNTNAP1).

When demyelination occurs, sodium and potassium channels redistribute and delocalize, but they do so with different kinetics. Potassium channels delocalize much faster than sodium channels do (Ishibashi et al. 2003). In the absence of a myelin sheath, clustered sodium channels and unclustered potassium channels could contribute to the conduction block observed in patients with MS.

Myelin and OLs also affect axonal caliber. Axon caliber may be influenced by intrinsic neuronal factors and extrinsic factors related to myelination. Caliber expands and neurofilaments accumulate only along regions of the axon with OL in mouse optic nerve during development. Very proximal portions of axons within a region of the optic nerve from which OLs are excluded remain unchanged. More distally, these axons rapidly expand an average of fourfold as soon as they were recruited to become myelinated between postnatal days 9 and 120 (Fig. 6.2). Axons ensheathed by OL processes, but not yet myelinated, were intermediate in caliber and neurofilament number. Thus, signals from oligodendrocytes, independent of myelin formation, are sufficient to induce axonal caliber expansion (Sánchez et al. 1996).

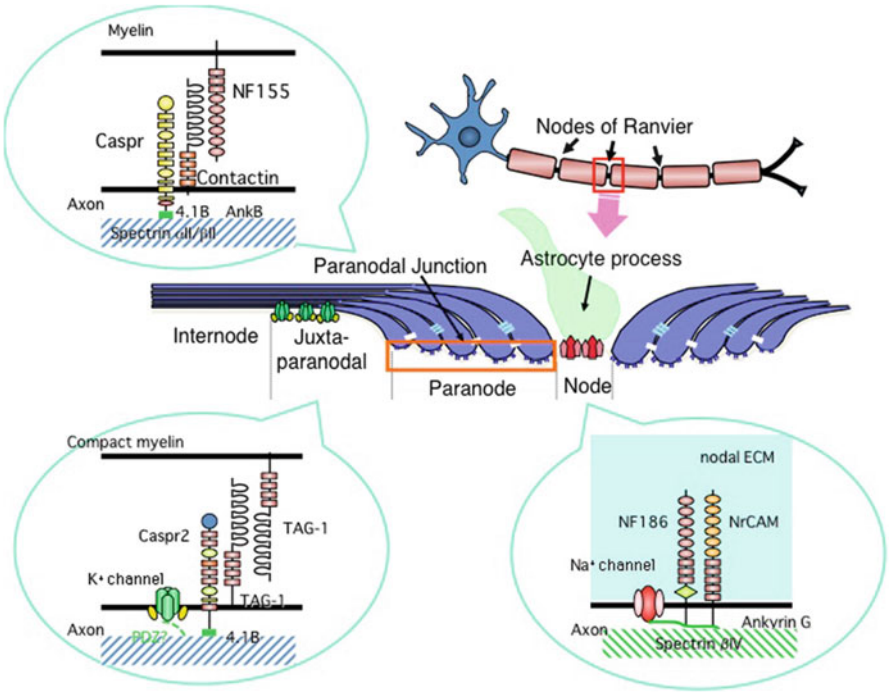

Fig. 6.1 Molecular composition of the node of Ranvier, paranode, and juxtaparanode. *AnkB* ankyrin B, *Caspr* contactin-associated protein, *ECM* extracellular matrix, *NF* neurofascin, *NrCAM* neuronal cell adhesion molecule, *TAG* transient axonal glycoprotein

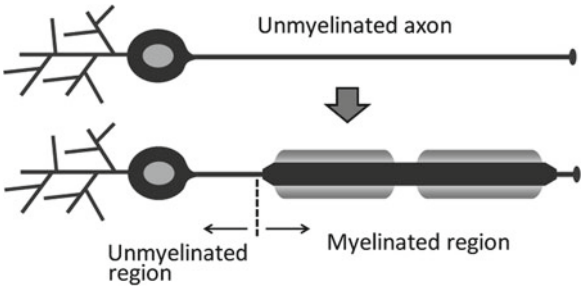

Fig. 6.2 Myelination induces local expansion of axonal caliber

6.4 Axon–Myelin Interactions After Myelin Formation

Many people are aware that myelination increases the axonal conduction velocity. However, less consideration is given to the nature of the communication between axons and OLs after myelination is complete. Lev-Ram and Ellisman (1995) and

Micu et al. (2007) demonstrated that Schwann cells and OLs respond to axonal activity by increasing the intramyelin Ca^{2+} concentration. Additionally, a groundbreaking study by Yamazaki et al. (2007) revealed that activated OLs-derived signals could further increase the conduction velocity of axons in which saltatory conduction had been induced. Therefore, axonal activity can be detected by myelinating OLs, and this activity can activate OLs and lead to regulation of the conduction velocity. One particularly interesting aspect of this relationship that is not known is whether OL signaling affects the conduction velocity of all the axons it ensheathes or only the axon in which it detected electrical activity.

OLs also support axon survival and function through mechanisms independent of myelination, and their dysfunction leads to axon degeneration in several diseases. A possible cause of this degeneration has recently been determined (Lee et al. 2012). Lee et al. showed that the most abundant lactate transporter in the CNS, monocarboxylate transporter 1 (MCT1, also known as SLC16A1), is highly enriched within OLs and that disruption of this transporter produces axon damage and neuron loss in animal and cell culture models. Thus, OLs support neurons and their axons by providing them with lactate. In cases of prolonged demyelination, a diminished lactate supply could be one of the reasons for axonal degeneration.

6.5 Axon–Myelin Interactions During Demyelination

In patients with MS, neuroinflammation causes degeneration of OLs and the myelin sheath; thus, all neuron–OL interactions could be affected. However, OPCs are present abundantly throughout the CNS, and are capable of generating OLs and remyelinating the naked axons. Although it is true that many thin myelin-containing structures have been observed in the shadow plaques that surround the MS lesions, indicating that remyelination is ongoing, the central regions of the lesions contain naked axons. Signs of remyelination are usually not found in these lesions, and they become chronically demyelinated. Although it has been believed that OPCs are depleted in chronic lesions because of extensive regeneration of OLs, Chang et al. (2002) demonstrated that, in rodents, that may not always be true. Thirty-four of the 48 chronic lesions of MS contained OLs with multiple extended processes that associated with demyelinated axons but failed to myelinate them. In some regions, the densities of premyelinating oligodendrocytes (25 mm^{-2} of tissue) were similar to those in the developing rodent brain (23 mm^{-2}) (Chang et al. 2002). These results show premyelinating OLs are present in MS chronic lesions; thus, remyelination is not limited by an absence of OPCs or their failure to generate OLs, but instead the axons are not receptive for remyelination.

Various factors that inhibit OL differentiation and maturation have been identified in chronically demyelinated lesions (Fig. 6.3). Many of these factors are expressed during normal development and are required to delay OPC differentiation until neural circuits have been established (see Sect. 6.3).

During development PSA-NCAM is expressed at the axonal surface and acts as a negative regulator of myelination. PSA-NCAM, normally absent from adult brain,

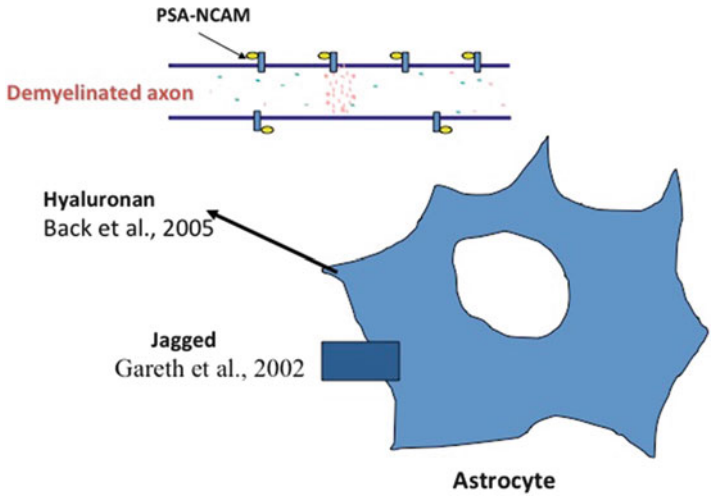

Fig. 6.3 Inhibitors of remyelination found in chronically demyelinated lesions. *Green dots* show voltage-gated potassium channels, and *red dots* sodium channels. *PSA-NCAM* polysialylated-neural cell adhesion molecule

was reexpressed on demyelinated axons in the chronic lesions (Charles et al. 2000). Within shadow plaques, remyelinated axons did not express PSA-NCAM. Re-expression of PSA-NCAM could act as an inhibitor of remyelination and participate in disease progression in MS.

The Notch pathway is also reexpressed in the adult CNS in cases of MS. John et al. (2002) found that within and around active MS plaques lacking remyelination, Jagged1 was expressed at high levels by hypertrophic astrocytes, whereas Notch1 and Hes5 localized to cells with an immature OL. In contrast, there was negligible Jagged1 expression in remyelinated lesions. These data implicate the Notch pathway regulates remyelination in MS. In contrast Stidworthy et al. (2004) found that OPC-targeted Notch1 ablation in cuprizone-treated Plp-creER Notch1(lox/lox) transgenic mice yielded no significant differences in remyelination parameters between knock-out and control mice. Thus, in contrast to developmental myelination, adult expression of Notch1 and Jagged1 neither prevented nor played a major rate-determining role in remyelination. Therefore, the role of Notch signaling during demyelination and remyelination is still controversial.

Nakahara et al. (2009) found that one Notch ligand, contactin, was saturated on demyelinated axons, Notch1-positive OPCs accumulated in contactin-positive lesions, and the receptor was activated to generate Notch1-intracellular domain (NICD). However, nuclear translocalization of NICD, required for myelinogenesis, was virtually absent in these cells. NICD and related proteins carrying nuclear localization signals were associated with the nuclear transporter importin but were trapped in the cytoplasm. Abnormal expression of TIP30, a direct inhibitor of importin, was observed in these OPCs. Therefore, an intrinsic nucleocytoplasmic transport blockade within OPCs may be involved in the pathogenesis of remyelination failure in MS.

The glycosaminoglycan hyaluronan, and in particular its high molecular weight (HMW) form, is synthesized by astrocytes and accumulates in demyelinated lesions from individuals with MS and in mice with experimental autoimmune encephalomyelitis (Back et al. 2005). OPCs did not mature into myelin-forming cells in demyelinating lesions where HMW hyaluronan was present. Furthermore, the addition of HMW hyaluronan to OPC cultures reversibly inhibited progenitor cell maturation, whereas degrading hyaluronan in astrocyte–OPC cocultures promoted OL maturation. HMW hyaluronan may therefore contribute substantially to remyelination failure by preventing the maturation of OPCs that are recruited to demyelinating lesions.

6.6 Conclusions

OLs are sensitive to inflammation; thus, interactions between axons and OLs are severely affected by neuroinflammation. This is especially true when inflammation induces demyelination. OLs not only contribute to the induction of saltatory conduction but they also regulate conduction velocity, affect axonal caliber, and support axonal survival. It is important to consider all of these factors when treating demyelinating diseases. Therefore, the outcomes of artificial remyelination (such as those induced by Schwann cell transplantation) should be carefully analyzed to determine if the remyelinated axons function normally.

Conflict of Interest Kazuhiro Ikenaka declares that he has no conflict of interest.

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Chapter 7
Neuron–Glia Interaction via Neurotrophins

Cinthia Farina

Abstract Initially described as target-derived survival factors for neurons, neurotrophins are now known to exert pleiotropic actions as they also regulate neuronal development, function, and plasticity. These processes however are not cell autonomous but derive from the complex interplay between neurons and glia cells.

In this chapter we offer an overview of the current knowledge on the functions supported by neurotrophins in neurons, and focus the attention on the role of neurotrophins in the cross talk between neurons and myelinating cells, microglia, and astrocytes.

Abbreviations

BDNF	Brain-derived neurotrophic factor
DRG	Dorsal root ganglion
LINGO-1	LRR and Ig domain containing nogo receptor interacting protein
LRR	Leucine-rich repeats
LTD	Long-term depression
LTP	Long-term potentiation
MAG	Myelin-associated glycoprotein
NGF	Nerve growth factor
NgR	Nogo receptor
NO	Nitric oxide
NT	Neurotrophin(s)

C. Farina, Ph.D. (✉)
Immunobiology of Neurological Disorders Lab, Division of Neuroscience and Institute of Experimental Neurology, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy
e-mail: farina.cinthia@hsr.it

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OL	Oligodendrocyte(s)
OMgp	Oligodendrocyte myelin glycoprotein
SC	Schwann cell(s)
TK	Tyrosine kinase

7.1 The Neurotrophin System: Ligands and Receptors

In mammals the family of neurotrophins (NT) has four members which share high homology in sequence and structure: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4 (NT4). All NT are translated from single coding exons and synthesized as larger precursors (proneurotrophins) of 30–34 kDa that associate non-covalently to form homodimers. Proneurotrophins are then cleaved intracellularly by furin and proconvertases to produce mature NT of circa 13 kDa (Reichardt 2006) (Fig. 7.1).

NT exert their actions by two structurally unrelated classes of receptors: the p75NTR receptor and the Trk receptors (Fig. 7.1). The p75NTR receptor is a member of the tumor necrosis factor (TNF)-receptor superfamily and has an extracellular domain with four cysteine-rich motifs, a single transmembrane domain, and a cytoplasmic tail containing a death domain similar to those present in other members of this family. Although this receptor does not have catalytic properties, it interacts with several adapter proteins, as NRIF and NRAGE, that further transmit the information (see Reichardt 2006 for details). p75NTR binds to all four neurotrophins equally well (Reichardt 2006), with a 2:2 stoichiometry (Aurikko et al. 2005; Gong et al. 2008).

The Trk receptors are tyrosine-kinase (TK) membrane receptors and include three receptors (TrkA, B, and C). Their extracellular domain exhibits three

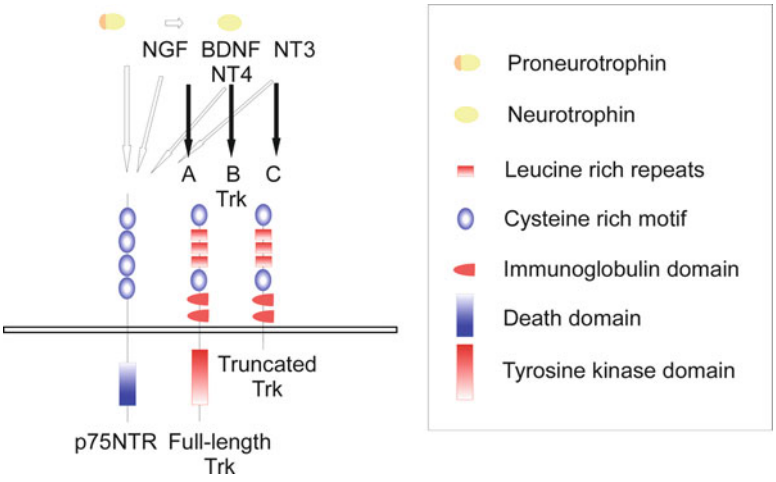

Fig. 7.1 The neurotrophin system

leucine-rich repeats (LRR) flanked by cysteine clusters and two immunoglobulin-like domains next to the second cysteine domain. The Trk receptors bind specifically distinct members of the NGF family of NT, with TrkA, TrkB, and TrkC interacting preferentially with NGF, BDNF or NT4/5, and NT3, respectively. In addition, NT3 can activate the other Trk receptors with less efficiency. The intracellular portion contains the catalytic tyrosine-kinase (TK) domain surrounded by several tyrosines that serve as phosphorylation-dependent docking sites for cytoplasmic adaptors and enzymes (Reichardt 2006). Binding of NT leads to dimerization of Trk receptors, resulting in activation through transphosphorylation of the kinases present in their cytoplasmic domains. Truncated Trk isoforms lacking the TK domain occur in nature and may form dimers with, and thereby inhibit the activation of, the full-length isoforms (Eide et al. 1996). The observation that, for example, truncated TrkB allows binding, internalization, and subsequently release of BDNF (Biffo et al. 1995) led to the hypothesis that truncated receptors function as reservoirs of BDNF. More recent work demonstrated that truncated Trk receptors are also important signal transducing molecules (Rose et al. 2003; Ohira et al. 2005; Colombo et al. 2012).

Neurotrophins were first identified as promoters of neuronal survival (Huang and Reichardt 2001) (Fig. 7.2). These effects are clearly mediated by Trk receptors, as specific neuronal cell populations are lost in Trk gene knockout animals (Huang and Reichardt 2003). Moreover, NT play a key role in synaptic plasticity, as they take part to structural (e.g., growth vs. shrinkage/retraction of dendritic spines) and functional (e.g., long-term potentiation (LTP) vs. long-term depression (LTD)) events in synaptic regulation (Lu et al. 2005). Here, distinct tasks are performed by Trk and p75NTR receptors, with TrkB signalling supporting synapse formation and LTP, and p75NTR signalling contributing to synapse retraction and LTD (Lu et al. 2005). Further, tuning of Trk actions is exerted by p75NTR receptor, which either interferes with Trk signalling or directly forms heterodimers with Trk receptors, obtaining agonistic or antagonistic effects on Trk-mediated signal transduction. In fact, it was demonstrated that p75NTR can enhance ligand binding (Davies et al. 1993; Esposito et al. 2001) and retrograde transport (Curtis et al. 1995), and promote Trk signalling (Makkerh et al. 2005), axon growth, and target innervation (Bentley and Lee 2000; Harrison et al. 2000). By contrast, p75NTR leads to axon pruning in postnatal life by attenuating Trk signalling (Singh and Miller 2005; Singh et al. 2008).

Interestingly, proneurotrophins, which may escape intracellular processing and be released by neurons, constitute the main form of NT in brain (Fahnestock et al. 2001) and are biologically active, as they bind selectively to p75NTR but not Trk receptors. In this case, interactions between proNT and p75NTR in complex with another protein called sortilin lead to neuronal apoptosis (Lee et al. 2001; Nykjaer et al. 2004; Teng et al. 2005) (Fig. 7.2).

p75NTR acts as an inhibitory coreceptor also for other receptors (Fig. 7.2). Eph receptors and ephrins are two families of proteins involved in nervous system patterning (Murai and Pasquale 2011). ephrinA ligands are GPI-anchored to the membrane and regulate growth cone mobility during establishment of neuronal connections (Winslow et al. 1995; Janis et al. 1999; Bundesen et al. 2003). Interestingly, formation of the complex ephrinA–p75NTR mediates retinal axon repulsion during development after proNT binding to p75NTR (Lim et al. 2008; Marler et al. 2010).

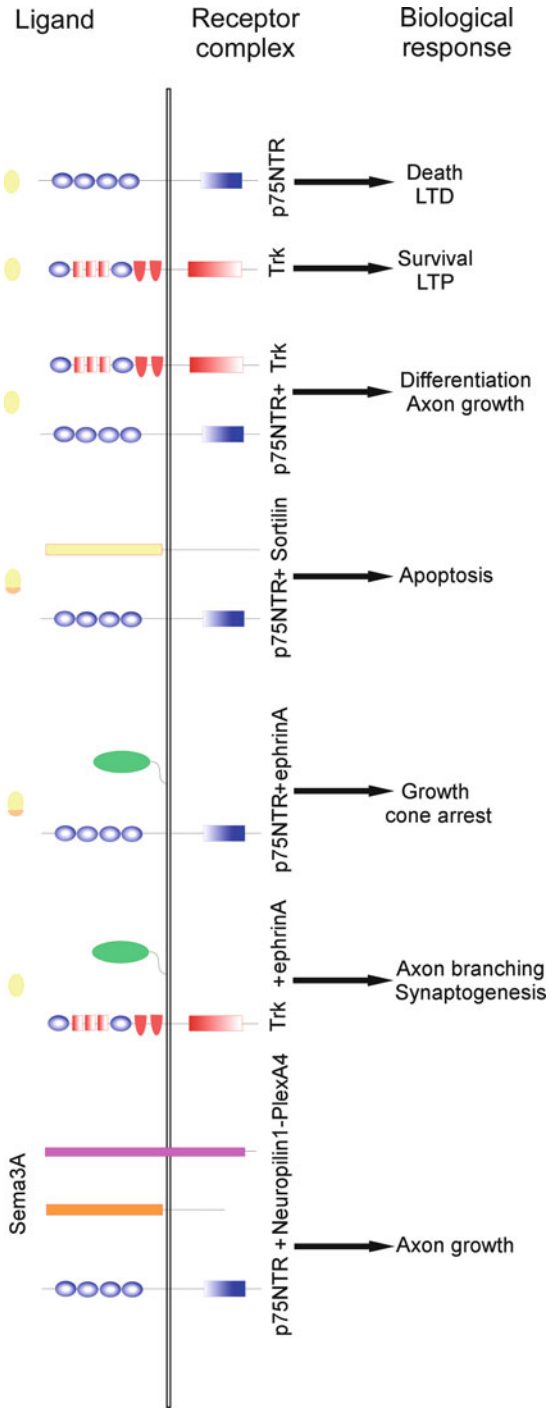

Fig. 7.2 NT system and biological effects in neurons

In parallel, ephrinA enhances BDNF-induced TrkB signalling leading to enhanced axon branching and synaptogenesis (Marler et al. 2008). Thus, the coordinated interaction of ephrins with distinct neurotrophin receptors results in growth cone arrest, branching, and specialization of the synaptic membrane. Finally, another group of axonal growth inhibitory cues regulated by p75NTR is that of class 3 semaphorins (Sema3) (Roth et al. 2009). Sema3A binding to the receptors neuropilin 1 and Plexin A4 modulates axonal pathfinding and pruning. There, p75NTR interaction with Sema3A receptor complex reduces receptor activation (Ben-Zvi et al. 2007).

Several neuronal features (e.g., survival and synaptic activity) rely on the interaction between neurons and glia cells via neurotrophins. The next paragraphs highlight the current knowledge on the role of NT as mediators in neuron–glia cross talk.

7.2 Neurotrophins in Neuron–Myelin Forming Cell Interaction

A superb example of neurotrophin-mediated cell–cell interaction is the formation of myelin, which consists of the unidirectional wrapping of multiple layers of membrane around an axon initiated at the side of the axon–glial junction. It is probably no accident that the evolutionary expansion of the neurotrophin family early in vertebrates coincides with the evolution of myelin (Hallbook et al. 2006). It is well established that both peripheral and central neurons as well as Schwann cells (SC) and oligodendrocytes (OL) express NT and their receptors. Thus, NT system integrates neurite growth with myelination processes either by mediating axonal signals or by acting directly on myelinating glia (Figs. 7.3, 7.4, and 7.5).

During development growth inhibitory signals regulate axon guidance and mediate synapse selection (Flanagan and Vanderhaeghen 1998; Yu and Bargmann 2001). In the postnatal period the regulation of neurite growth is a fundamental issue especially when considering the impact on axonal regeneration after injury. It is known that injured central nervous system (CNS) offers a physical barrier to regeneration due to the formation of a gliotic scar. In addition, a series of molecular signals may dampen axonal outgrowth. Among them oligodendrocyte myelin glycoprotein (OMgp), myelin-associated glycoprotein (MAG), and Nogo-A are myelin-derived factors which inhibit axonal regeneration upon binding to nogo receptors (NgRs) (Chen et al. 2000; GrandPre et al. 2000; Fournier et al. 2001; Venkatesh et al. 2005) or PIR-B (Fujita et al. 2011a). Being a GPI-linked membrane receptor, NgR needs to associate with additional membrane proteins to deliver the signal into the cell. Importantly, p75NTR neurotrophin receptor is part of this complex together with LRR and Ig domain containing nogo receptor interacting protein LINGO-1 (Wang et al. 2002). Likewise, PIR-B association with Trk receptors mediates myelin suppression of axon growth (Fujita et al. 2011a) and this effect depends on p75NTR which interacts with PIR-B/Trk complex (Fujita et al. 2011b). Finally, in addition to growth inhibition, myelin delivers signals for axon degeneration which depend on p75NTR but can be overcome by robust Trk receptor activation (Park et al. 2010).

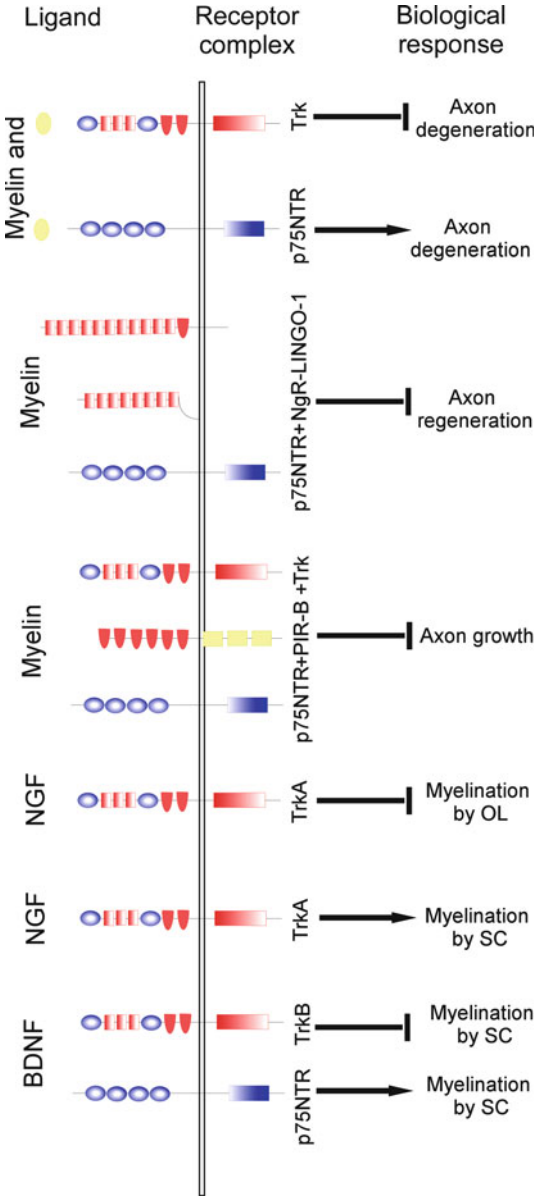

Fig. 7.3 NT system in neurons regulating myelination and responses to myelin-derived factors

Neurotrophins are essential regulators of myelination during development and of remyelination after injury. The identification of the precise cellular and molecular basis of myelination has been complicated by the severe neuronal phenotype experienced by NT and NT receptor knockout mice, so that changes in myelination there most probably result from neuronal alterations. In vitro myelination assays, encompassing the coculture of defined populations of neurons (normally dorsal root

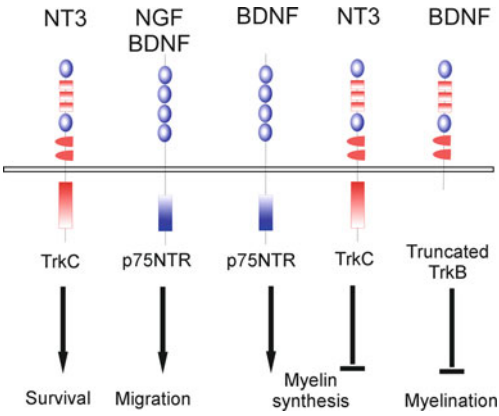

Fig. 7.4 NT system and biological effects in Schwann cells

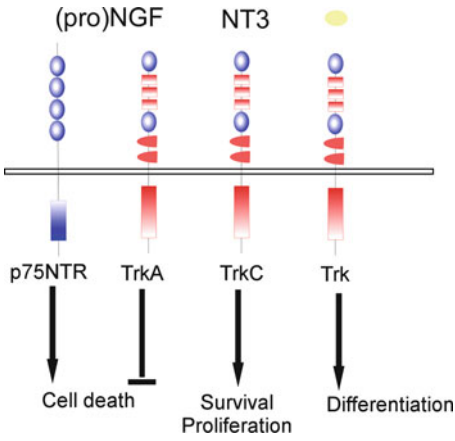

Fig. 7.5 NT system and biological effects in oligodendrocytes

ganglion (DRG) neurons) with SC or, more recently, oligodendrocytes have been used to recapitulate fundamental aspects of in vivo myelination. In this experimental set-up NGF promotes myelination of TrkA-positive DRG neurons by SC, while it inhibits OL myelination by inducing expression of axonal LINGO-1 (Chan et al. 2004; Lee et al. 2007). Interestingly, the neurotrophin BDNF enhances myelination of NGF-dependent peripheral neurons, an effect dependent on neuronal expression of the p75NTR receptor, whereas it inhibits myelination of BDNF-dependent neurons via the full-length TrkB receptor (Xiao et al. 2009) (Fig. 7.3). These evidences highlight how distinct axonal signals may control myelination.

Furthermore, NT have direct effects on myelin forming cells, beginning with glial development to the migration of myelin forming cells along the axons until the ensheathment of the axon and the active synthesis of myelin proteins (Figs. 7.4 and 7.5). Regarding peripheral myelination, NT3 regulates survival of SC which express TrkC (Meier et al. 1999; Woolley et al. 2008), and NGF and BDNF direct SC migration through p75NTR (Bentley and Lee 2000; Anton et al. 1994; Yamauchi et al. 2004) (Fig. 7.4). However, the expression of the myelin proteins in SC and the

formation of internodes are inhibited by NT3 and TrkC signalling, while supported by BDNF binding to p75NTR (Chan et al. 2001; Pruginin-Bluger et al. 1997; Cosgaya et al. 2002). Additionally, the truncated TrkB receptor is induced during active myelin formation and negatively regulates myelination by SC (Cosgaya et al. 2002) (Fig. 7.4). Thus, peripheral myelination is the result of time-controlled expression of NT and their receptors: NT3 supports survival of SC via TrkC, while p75NTR signalling regulates SC migration; downregulation of TrkC signalling at the beginning of myelination removes an inhibitory signal, while expression of the BDNF–p75NTR axis supports myelination; and, finally, induction of the truncated TrkB, which scavenges extracellular BDNF, switches off the process.

Regarding central myelination (Fig. 7.5), TrkC activation upon binding to NT3 regulates survival and proliferation of OL (Barres et al. 1993, 1994; Cohen et al. 1996). BDNF, NGF, and NT3 were found to promote differentiation of basal forebrain OL, while NGF and NT3 but not BDNF induce differentiation of cortical OL (Du et al. 2003). The regional effects of NT are due to the distinct expression of NT receptors on OL, as cortical OL lack TrkB expression (Du et al. 2003, 2006).

A few evidences indicate a role for NT also in regulating cell death of oligodendrocytes (Fig. 7.5). An in vitro study shows that NGF can induce cell death of mature cortical oligodendrocytes but not of OL precursor cells via p75NTR (Casaccia-Bonnefil et al. 1996) and that forced expression of TrkA may rescue cells from apoptosis (Yoon et al. 1998). Indeed, OL express p75NTR after spinal cord injury and are apoptotic. Consistently, proNGF levels increase in injured spinal cord and are effective in inducing p75NTR-mediated OL death in culture (Beattie et al. 2002).

Remyelination is thought to be a process which recapitulates myelination. Transplantation of fibroblasts engineered to release BDNF and NT3 into the injured spinal cord results in enhanced axonal growth, OL precursor proliferation, and improved myelination (McTigue et al. 1998). Additional studies support the view that administration of NT in vivo may be effective in sustaining remyelination (Tuszynski et al. 1998; Cao et al. 2005; Girard et al. 2005).

Altogether, the role of the NT in myelination is complex and may be characterized by opposite effects depending on the cell type and on the members of the NT system present at distinct stages of the neuron–glia interaction, suggesting that careful and balanced timing of expression of these molecules is responsible for process control.

7.3 Neurotrophins in Neuron–Microglia Interaction

Microglia are myeloid cells entering the nervous tissue at early stages during development and reaching a maximum at the end of the second postnatal week when intense synaptogenesis is occurring. Though exerting immune cell functions (Aloisi 2001; Farina et al. 2007), microglia secrete a variety of growth factors, including neurotrophins, implicated in all aspects of neuronal functions (Hanisch 2002).

Several evidences indicate that microglia have the ability to kill neurons by secreting glutamate, TNF-alpha, Fas ligand, interleukin 1b, nitric oxide (NO), and

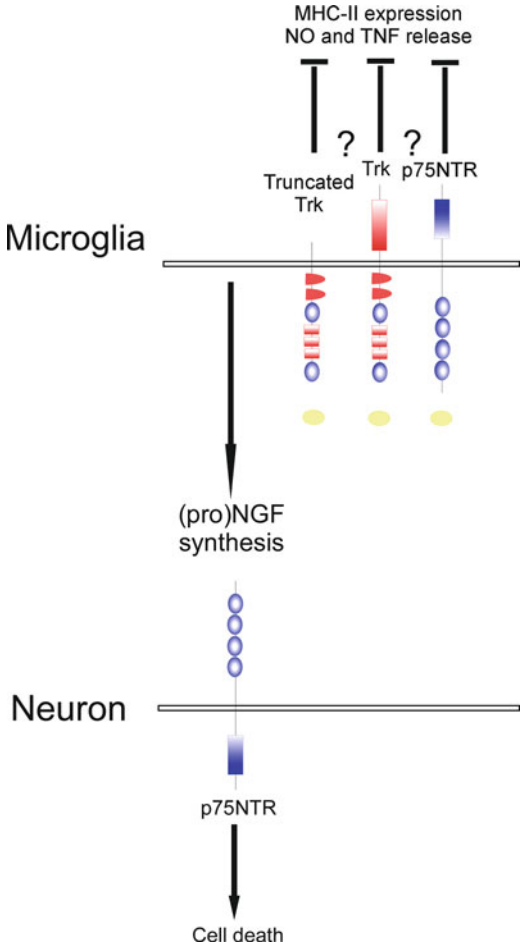

Fig. 7.6 NT system in the interaction between neurons and microglia

reactive oxygen species (ROS). At the same time microglia may increase neuronal survival through the release of trophic factors and anti-inflammatory factors. Interestingly, neurotrophins may support the detrimental side in microglia–neuron interaction (Fig. 7.6). In embryonic chick retina about half of the neurons die in a restricted time window that follows invasion of neural tissue by microglia. However, neuronal death is reduced when the embryonic chick retina is dissected before colonization by microglial cells, and is restored by the addition of microglia (Frade and Barde 1998). Importantly, this effect is blocked by antibodies to NGF (Frade and Barde 1998), indicating that microglia NGF induces developmental neuronal death. In vitro experiments demonstrated that retinal microglia release proNGF, which in turn drives photoreceptor cell death via binding to p75NTR (Srinivasan et al. 2004). Similarly, in a model of light-induced retinal degeneration p75NTR upregulation on Muller glia cells leads to decreased production of basic fibroblast growth factor (bFGF), a survival factor for photoreceptor cells (Hayashi et al. 1997). Blockade of

p75NTR prevents bFGF reduction, thereby promoting photoreceptor survival (Harada et al. 2000). Importantly, following retinal degeneration microglia release neurotrophins, which block bFGF synthesis in Mueller glia (Harada et al. 2002), highlighting how neuronal fate can be determined by microglia–Mueller glia interaction via neurotrophins.

Microglia regulate also synaptic function. Mice with loss of function of DAP12, a transmembrane protein specifically expressed in developing microglia, display altered synaptic function and plasticity. Neurotrophins are known to modulate synaptic transmission and LTP (Klintsova and Greenough 1999). In particular, BDNF affects function and trafficking to synaptic sites of glutamate receptors (Levine et al. 1998; Levine and Kolb 2000). Consistently, the observed synaptic phenotype in DAP12-deficient mice is characterized by a dramatic decrease in the postsynaptic density of the full-length TrkB isoform. Thus a microglial defect may lead to synaptic impairment by targeting the BDNF–TrkB pathway in neurons. Importantly, in the spinal cord microglia respond to ATP stimulation with release of BDNF, which causes allodynia by activating microglia themselves (Zhou et al. 2011) and by inducing a depolarizing shift in the anion reversal potential in neurons (Coull et al. 2005). This shift inverts the polarity of currents activated by GABA (gamma-amino butyric acid) (Coull et al. 2005), as has been shown to occur after peripheral nerve injury.

By contrast, the observation that dopaminergic sprouting following striatal injury is accompanied by the accumulation of BDNF-expressing microglia at the wound site (Batchelor et al. 1999) suggests that microglia BDNF supports tissue repair. Indeed, NT release from microglia may be enhanced by stimuli derived from neurons themselves. In vitro experiments show that, when exposed to neuronal factors, microglia promote the survival and maturation of catecholaminergic, GABAergic, and cholinergic neurons via neurotrophin synthesis (Nakajima et al. 2007).

Microglia are also target of neurotrophic action as they express neurotrophin receptors (Nakajima et al. 1998). Neurotrophins suppress the LPS-induced release of nitric oxide (Nakajima et al. 1998; Tzeng and Huang 2003; Mizoguchi et al. 2009) and of TNF-alpha (Tzeng and Huang 2003) by microglia, and contribute to the low expression of major histocompatibility class II molecules on microglia (Neumann et al. 1998) (Fig. 7.6), indicating that NT may dampen the inflammatory side of microglia.

7.4 Neurotrophins in Neuron–Astrocyte Interaction

Astrocytes are the most abundant glia cells in the CNS and are of neuroectodermal origin. They are fundamental for brain structure and function. In fact they regulate ion homeostasis in the extracellular space, keep balance between clearance and release of the neurotransmitter glutamate, produce metabolic substrates for neurons, and take care of the structural maintenance of neuronal synapses (Nedergaard et al. 2003). Clearly, astrocyte dysfunction can be detrimental for neurons. In fact, on the one hand astrocytes may protect neurons from ROS- and NO-induced cell death by

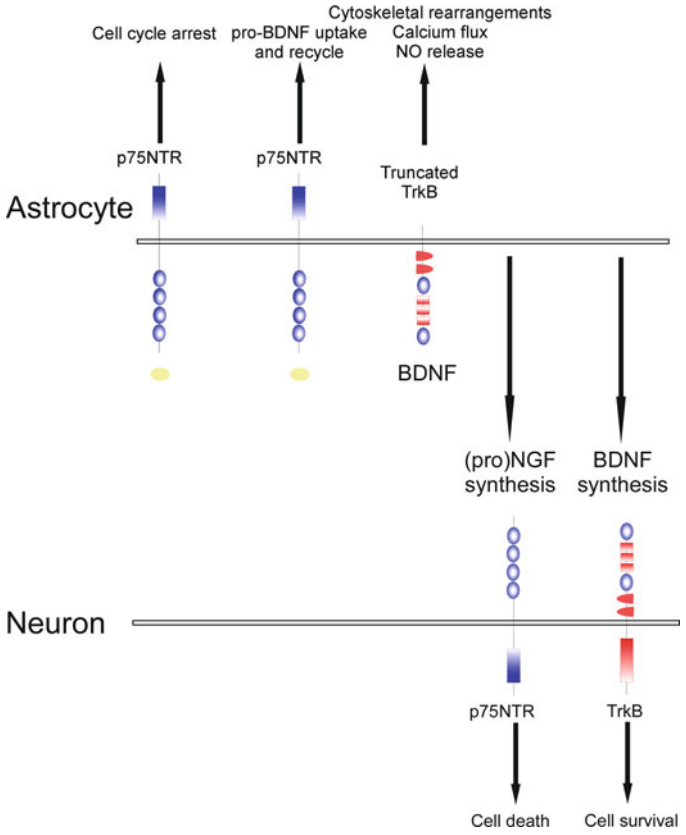

Fig. 7.7 NT system in the interaction between neurons and astrocytes

providing antioxidants such as glutathione, thioredoxin, and metallothioneins (Chiueh et al. 2003), on the other hand upon activation they can contribute to oxidative stress by releasing NO (Colombo et al. 2012) which is then converted into neurotoxic products like peroxynitrite.

Astrocytes are sensitive to neurotrophin action, as they bear NT receptors (Colombo et al. 2012; Condorelli et al. 1994). Astrocyte responses to NT include cell cycle arrest via p75NTR (Cragnolini et al. 2009, 2012), and calcium flux via truncated TrkB (Rose et al. 2003; Colombo et al. 2012) (Fig. 7.7).

The cross talk between astrocytes and neurons via neurotrophins modulates neuronal survival and activity (Fig. 7.7).

Astrocytes upregulate synthesis of NGF in vitro in response to glutamate (Wu et al. 2004) or to peroxynitrite (Vargas et al. 2004), and produce proNGF in vivo following kainic acid-induced seizures (Volosin et al. 2006). Importantly, astrocyte proNGF triggers neuronal apoptosis via activation of p75NTR–sortilin complex (Volosin et al. 2006; Domeniconi et al. 2007). Glutamate induces also BDNF in

astrocytes via binding to glutamate metabotropic receptors and astrocyte BDNF supports activity and survival of cholinergic neurons in vitro (Jean et al. 2008). BDNF release by astrocytes during neuroinflammation protects from neurodegeneration (Linker et al. 2010), probably targeting neurons via full-length TrkB (Linker et al. 2010). By contrast, CNS inflammation upregulates TrkB on astrocytes and astrocyte responsiveness to TrkB ligands in vivo is detrimental to neurons (Colombo et al. 2012; Colombo and Farina 2012). These data clearly indicate that NT may lead to opposite outcomes (neuroprotection vs. neurodegeneration) depending on the cell types and receptors they bind. In fact, differently from the positive signal mediated by full-length TrkB in neurons, activation of truncated TrkB on astrocytes results in NO production, which triggers a secondary NO wave in neurons leading to apoptosis (Colombo et al. 2012; Colombo and Farina 2012) (Fig. 7.7).

Astrocytes may regulate LTP of synaptic transmission by internalizing proBDNF secreted by neurons via p75NTR and storing it so that it is released upon astrocyte activation (Bergami et al. 2008). In this way, glia cells regulate synaptic plasticity by clearing and recycling BDNF. Moreover, astrocytes participate to inhibitory synapse formation by modulating the number of postsynaptic $GABA_A$ receptor clusters, and these effects are mediated by TrkB signalling in neurons (Elmariah et al. 2005).

7.5 Conclusions

Neurotrophins display a Janus-like function in the nervous system as they may promote survival or death, proliferation or differentiation, protection or degeneration. Several efforts led to the definition of the ligands, receptors, and main signalling pathways, and highlighted that timely expression of the neurotrophin system allows system-wide integration of information, so that complex processes as myelination and synaptic plasticity can correctly take place. Future challenges regard the development of appropriate tools and approaches to better refine the conditions that move the balance from one action to the opposite considering that several distinct cell types contribute to the final outcome. Unraveling the settings sustaining neuroprotection, neuroregeneration, and remyelination has great importance for the development of appropriate therapeutic strategies for human neurodegenerative disorders.

Conflict of Interest The author declares she has no conflict of interest. This chapter does not contain any studies with human or animal subjects.

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Chapter 8
Glial Communication via Gap Junction in Neuroinflammation

Hideyuki Takeuchi

Abstract Gap junction is the major intercellular channel that facilitates direct signaling between cytoplasmic compartments of adjacent cells by transferring various small molecules (~1,000 Da) and ions. Gap junction consists of a pair of hemichannels, each of which is a hexameric cluster of protein subunits named connexin. Recent studies have revealed that uncoupled "free" hemichannels also facilitate two-way transfer of molecules between the cytosol and extracellular space. In the central nervous system (CNS), gap junctions and hemichannels form the neuron–glia network and contribute to the maintenance of homeostasis by propagating signals and buffering against toxins. Other evidence suggests that gap junctions and hemichannels—especially in glial cells—are also involved in the initiation and amplification of neuroinflammation in various neurological disorders. The purpose of this review is to summarize recent insights into the roles of gap junctions and hemichannels in the physiologic and pathologic conditions of the CNS.

8.1 Introduction

Gap junctions are the major intercellular channels that directly connect the cytoplasmic compartments of adjacent cells (Yeager and Harris 2007). These channels allow various small molecules (~1,000 Da) and ions to pass freely between cells, although recent evidence suggests that the charge and shape of the molecules can affect the rate of transfer via gap junctions (Goldberg et al. 2004). Gap junction consists of hemichannels docked in a head-to-head configuration; the hemichannel is organized as a hexagonal cylinder with a central pore, and each hemichannel is

H. Takeuchi, M.D., Ph.D. (✉)
Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Japan
e-mail: htake@riem.nagoya-u.ac.jp

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Table 8.1 The connexin family

Human		Mouse	
Protein name	Gene name	Protein name	Gene name
Cx43	*GJA1*	Cx43	*Gja1*
Cx46	*GJA3*	Cx46	*Gja3*
Cx37	*GJA4*	Cx37	*Gja4*
Cx40	*GJA5*	Cx40	*Gja5*
–	–	Cx33	*Gja6*
Cx50	*GJA8*	Cx50	*Gja8*
Cx59	*GJA9*	–	–
Cx62	*GJA10*	Cx57	*Gja10*
Cx32	*GJB1*	Cx32	*Gjb1*
Cx26	*GJB2*	Cx26	*Gjb2*
Cx31	*GJB3*	Cx31	*Gjb3*
Cx30.3	*GJB4*	Cx30.3	*Gjb4*
Cx31.1	*GJB5*	Cx31.1	*Gjb5*
Cx30	*GJB6*	Cx30	*Gjb6*
Cx25	*GJB7*	–	–
Cx45	*GJC1*	Cx45	*Gjc1*
Cx47	*GJC2*	Cx47	*Gjc2*
Cx30.2/Cx31.3	*GJC3*	Cx29	*Gjc3*
Cx36	*GJD2*	Cx36	*Gjd2*
Cx31.9	*GJD3*	Cx30.2	*Gjd3*
Cx40.1	*GJD4*	Cx39	*Gjd4*
Cx23	*GJE1*	Cx23	*Gje1*

made up of a hexameric cluster of protein subunits named connexin (in vertebrates) or innexin (in invertebrates).

Connexins are encoded by a conserved family of genes composed of at least 21 members in mammals. There are 21 connexin genes in the human genome and 20 connexin genes in the mouse genome; 19 of connexins have orthologs in humans and mice (Table 8.1) (Laird 2006; Willecke et al. 2002). Members of the connexin protein family are named using Cx (abbreviation of connexin) followed by a suffix indicating the predicted molecular weight (e.g., the ~43 kDa connexin protein is called Cx43). When different connexins have similar molecular masses, a decimal point is used to distinguish between them (e.g., Cx30 and Cx30.3). Human and mouse connexin genes begin with *GJ* and *Gj*, respectively (abbreviations for gap junction), followed by a Greek letter indicating the subgroup (α to ε), and a number based on the order in which the proteins were discovered. Each connexin contains a short cytoplasmic amino-terminal domain, four transmembrane hydrophobic domains (M1 to M4), one cytoplasmic loop, two extracellular loops (E1 and E2), and a cytoplasmic carboxyl-terminal domain (Fig. 8.1) (Loewenstein 1967; Flower 1977; Peracchia 1980; Flagg-Newton et al. 1979; Schwarzmann et al. 1981; Revel et al. 1971). The extracellular loops E1 and E2 are the most conserved regions in the proteins and mediate hemichannel docking to form gap junctions. The cytoplasmic loop and the cytoplasmic carboxyl-terminal domain are the most divergent regions among the connexins, conferring unique functional or regulatory properties to channels formed by different connexins. The different connexin isoforms structurally

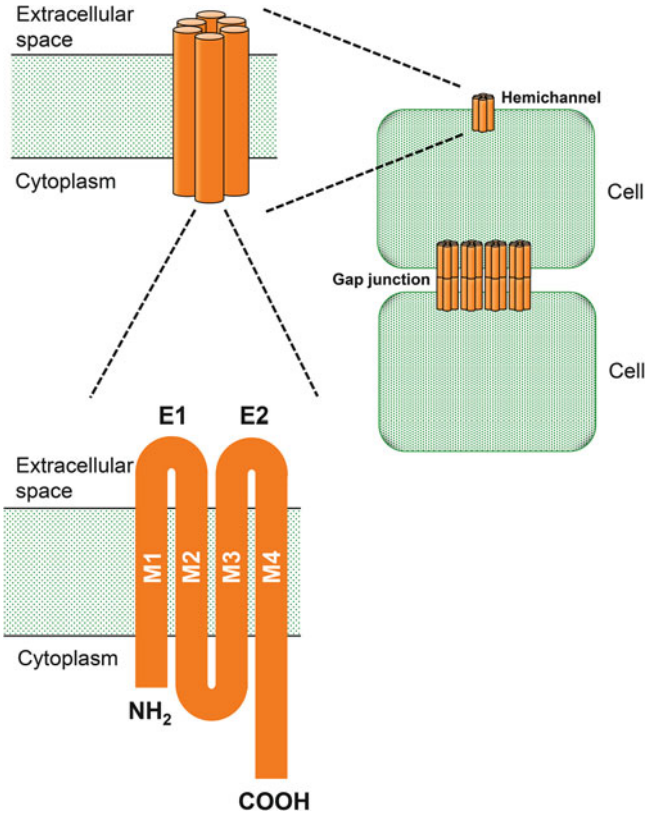

Fig. 8.1 A diagram of connexins, hemichannels, and gap junctions. Connexin contains four transmembrane domains (M1 to M4), one cytoplasmic loop, and two extracellular loops (E1 and E2). The amino terminus and carboxyl terminus (NH_2 and COOH, respectively) are located in the cytoplasm. A hexameric cluster of connexins forms a hemichannel characterized by a hexagonal cylinder with a central pore. Gap junctions consist of hemichannels that have docked together in a head-to-head configuration

interact in various ways. Homomeric hemichannels consist of a single connexin isoform. Heteromeric hemichannels contain two or more different connexin isoforms. Homotypic gap junction channels are composed of two identical hemichannels, whereas heterotypic gap junction channels are formed by two different hemichannels. Thus, compositional patterns of gap junctions can be categorized into four types: homomeric and homotypic; heteromeric and homotypic; homomeric and heterotypic; and heteromeric and heterotypic (Fig. 8.2).

Gap junctions allow direct intracellular propagation of second messengers (e.g., Ca^{2+}, IP_3, cAMP, and cGMP), metabolites (e.g., glutamate, glucose, and glutathione), and nucleotides (e.g., ATP, ADP, and RNA) between adjacent cells (Laird 2006; Saez et al. 2003; Harris 2001, 2007; Goldberg et al. 1999, 2002; Valiunas et al. 2005). Moreover, recent evidence suggests that uncoupled "free" hemichannels can facilitate two-way transfer of molecules between the cytosol and extracellular milieu (Laird 2010; De Vuyst et al. 2007; Retamal et al. 2007).

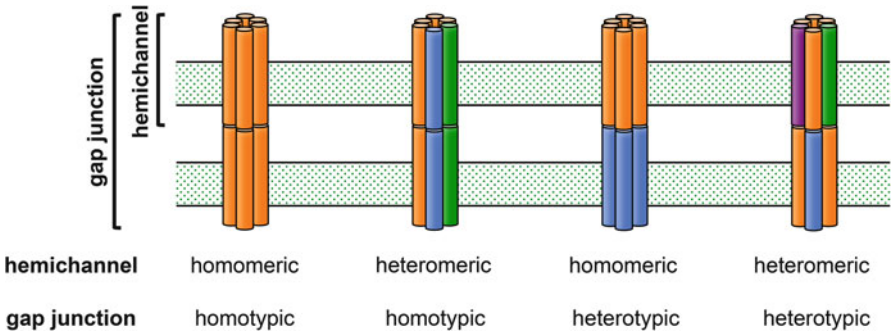

Fig. 8.2 The composition of gap junctions. Each colored column (*orange*, *blue*, *green*, and *purple*) represents a different connexin isoform. Hemichannels may be homomeric (composed of one connexin isoform) or heteromeric (composed of more than one connexin isoform). Gap junction channels may be homotypic (formed by identical hemichannels) or heterotypic (formed by different hemichannels)

Intracellular communication via gap junctions and hemichannels is regulated by such mechanisms as channel gating via chemical, pH, and voltage, and changes in connexin transcription, translation, posttranslational phosphorylation and ubiquitination, membrane insertion, and hemichannel internalization and degradation (Laird 2006; Solan and Lampe 2009; Leithe and Rivedal 2007). The time courses of these changes range from milliseconds to hours and are influenced by the environmental conditions in the cells and tissues.

Whereas connexins form gap junctions and hemichannels in vertebrates, invertebrates use innexins, which lack sequence homology with connexins. A search of the human genome identified three innexin-related genes (Barbe et al. 2006). Because of the occurrence of homologous genes in both vertebrates and invertebrates, the corresponding proteins were termed pannexins, denoted pannexin1 (Panx1), pannexin2 (Panx2), and pannexin3 (Panx3). Pannexins have the same transmembrane topology as connexins, with four transmembrane domains and cytoplasmic amino-terminal and carboxyl-terminal domains. Recent evidence indicates that pannexins also form uncoupled hemichannels in the mammalian cells; however, it is not clear whether they form functional gap junctions (Dahl and Locovei 2006). Therefore, this review is mainly restricted to the connexins.

8.2 Gap Junctions in the Central Nervous System

Various tissues exhibit characteristic connexin expression profiles. Multiple connexins are expressed in the central nervous system (CNS) (Rouach et al. 2002; Nagy and Rash 2000; Takeuchi et al. 2006; Parenti et al. 2002; Bittman and LoTurco 1999; Rash et al. 2001; Chang et al. 1999; Dermietzel et al. 1989, 2000; Eugenin et al. 2001; Altevogt et al. 2002; Odermatt et al. 2003; Teubner et al. 2001) (Table 8.2).

Table 8.2 Connexins in the central nervous system

Neurons	Astrocytes	Oligodendrocytes	Microglia
Cx26	Cx26	Cx29	Cx32
Cx30.2	Cx30	Cx31.3	Cx36
Cx32	Cx40	Cx32	Cx43
Cx36	Cx43	Cx36	
Cx40	Cx45	Cx45	
Cx43	Cx46	Cx47	
Cx45	Cx47		
Cx47			

8.2.1 Neurons

All neurons mainly express Cx36 and Cx45, whereas other neural connexins are expressed with more specific spatiotemporal profiles (Sohl et al. 2005). Electrical coupling between neurons has been implicated in neuronal synchronization in various areas of the CNS (Christie et al. 1989; Wong et al. 1995; Bouskila and Dudek 1993). Neuronal gap junctions composed of Cx36 and Cx45 are thought to be homomeric and homotypic (Teubner et al. 2001; Al-Ubaidi et al. 2000), and play important roles in electrical synapses (Hormuzdi et al. 2001; Deans et al. 2001). Interestingly, some rodent knockout models have shown that other connexins can compensate for the functions of Cx36 and Cx45 despite different conformations or permeabilities (Zlomuzica et al. 2010; Frank et al. 2010). In vitro and in vivo studies have also revealed a critical role for gap junction coupling in neuronal differentiation. Mice lacking Cx43 die as neonates and exhibit abnormal migration in the neural crest and neocortex (Lo et al. 1999; Xu et al. 2001; Fushiki et al. 2003). Blocking gap junctions also suppresses retinoic acid-induced neuronal differentiation of NT2 and P19 cells (Bani-Yaghoub et al. 1999a, b). Moreover, Cx36-containing gap junctions are required for neuronal remodeling and short-term spatial memory in some mature organisms (Hartfield et al. 2011; Allen et al. 2011). In contrast to convincing evidence of neuron–neuron coupling, the existence of functional neuron–glia coupling in the CNS is still a matter of debate (Rash et al. 2001, 2007; Nadarajah et al. 1996; Alvarez-Maubecin et al. 2000).

8.2.2 Astrocytes

Astrocytes are the main type of cells in the CNS that are coupled by gap junctions. Astrocytes mainly express Cx43 and Cx30 (Nagy and Rash 2000; Dermietzel et al. 1991); Cx43/Cx30 double-knockout mice show minimal gap junction communication between astrocytes (Rouach et al. 2008; Wallraff et al. 2006), suggesting that functional astrocytic gap junctions are primarily composed of these connexins. Cx43-deficient astrocytes show reduced gap junctional coupling, although they express

other connexin subtypes such as Cx30, Cx26, Cx40, Cx45, and Cx46 (Dermietzel et al. 2000; Naus et al. 1997; Scemes et al. 1998). Mice lacking Cx30 exhibited only mild abnormalities, including hearing loss due to cochlear degeneration, although Cx30 has been detected exclusively in astrocytes (Teubner et al. 2003). Thus, other astrocytic connexin subtypes do not seem to compensate for a lack of Cx43.

Astrocytic gap junctions facilitate the formation of functional syncytium that buffers extracellular glutamate elevation, pH, and K^+ concentrations that are associated with firing neurons, and propagate intracellular Ca^{2+} waves to modulate neuronal activities (Jefferys 1995; Walz and Hertz 1983; Charles 1998; Ransom et al. 2003; Anderson and Swanson 2000). Moreover, astrocytic gap junctional communication facilitates the trafficking of glucose and its metabolites, thereby mediating interactions between cerebral vascular endothelium and neurons (Goldberg et al. 1999; Giaume et al. 1997; Tabernero et al. 2006). Thus, astrocytic gap junctions play critical roles in modulating neuronal activities and maintaining CNS homeostasis.

Astrocyte–astrocyte coupling can result from any of the allowed combinations of homomeric or heteromeric hemichannels in homotypic or heterotypic configurations. Cx30 and Cx26 form heteromeric and heterotypic channels (Altevogt and Paul 2004; Nagy et al. 2003), whereas Cx43 forms homomeric and homotypic channels (Orthmann-Murphy et al. 2007). A previous report demonstrated that gap junctional coupling in astrocytes results in two distinct subpopulations of cells. Astrocytes expressing glutamate transporters are extensively coupled to each other, whereas astrocytes expressing glutamate receptors are not coupled to other astrocytes (Wallraff et al. 2004), suggesting a role in buffering extracellular glutamate (Anderson and Swanson 2000). Astrocyte–oligodendrocyte coupling will be discussed in the following section.

8.2.3 Oligodendrocytes

Oligodendrocytes mainly express Cx29, Cx32, and Cx47 (Dermietzel et al. 1989; Altevogt et al. 2002; Odermatt et al. 2003). Oligodendrocytic gap junctions facilitate the trafficking of ions and nutrients from somas to myelin layers (Paul 1995). Mice deficient for Cx32 exhibit a reduced volume of myelin and enhanced excitability in the CNS as well as progressive peripheral neuropathies (Sutor et al. 2000; Anzini et al. 1997). Cx32/Cx47 double-knockout mice show abnormal movements and seizures associated with vacuolated myelin and axonal degeneration in the CNS, whereas Cx47-deficient mice display only minimal effects in the CNS (Menichella et al. 2003). Cx32 and Cx47 in oligodendrocytes are critical for spatial buffering of K^+ in response to neuronal activity; failure of this function leads to myelin swelling and following axonal degeneration (Menichella et al. 2006). Oligodendrocyte–oligodendrocyte coupling can result from homotypic configurations with homomeric or heteromeric hemichannels containing Cx32 or Cx47 (Orthmann-Murphy et al. 2007). Oligodendrocytes also couple with astrocytes. Astrocyte–oligodendrocyte coupling may include heterotypic configurations of

Cx43–Cx47, Cx30–Cx32, or Cx26–Cx32 (Altevogt and Paul 2004; Nagy et al. 2003; Orthmann-Murphy et al. 2007). Like astrocyte–astrocyte coupling, astrocyte–oligodendrocyte coupling plays an important role in the formation of the glial syncytium to facilitate the propagation of Ca^{2+} waves and the buffering of extracellular K^+ and neurotransmitter such as glutamate (Jefferys 1995; Walz and Hertz 1983; Charles 1998; Ransom et al. 2003; Anderson and Swanson 2000).

8.2.4 Microglia

Microglia express Cx32, Cx36, and Cx43 (Takeuchi et al. 2006; Parenti et al. 2002; Eugenin et al. 2001; Kielian 2008; Garg et al. 2005). Microglia form few amount of functional gap junctions under resting conditions. The expression of connexins increases in activated microglia although whether upregulated expression of connexins leads to enhanced formation of functional gap junctions with microglia and other CNS cells is still a matter of debate (Eugenin et al. 2001; Kielian 2008; Garg et al. 2005; Takeuchi 2010). Recent evidence demonstrates that uncoupled microglial hemichannels play important roles in bidirectional trafficking of small molecules between the cytoplasm and the extracellular space (Takeuchi et al. 2011; Eugenin et al. 2012).

8.3 Glial Gap Junction in the Pathological Condition

As described above, glial gap junctions contribute to the maintenance of homeostasis in the CNS under the physiological conditions. These structures, however, also contribute to the initiation and propagation of pathologic conditions (Orellana et al. 2009).

8.3.1 Brain Ischemia

A sudden reduction in cerebral blood flow leads to a rapid decrease in intracellular oxygen levels and subsequent drops of ATP synthesis, which are the initial steps of eventual cell death (Kalogeris et al. 2012). Injured cells contain toxic molecules at high concentrations (e.g., Ca^{2+}, K^+, reactive oxygen species (ROS), nitric oxide (NO)). These toxic molecules can be propagated from injured cells to healthier cells through gap junctions. Ischemic conditions also induce uncoupled hemichannels to open, leading to paracrine transfer of toxic molecules (De Vuyst et al. 2007; Thompson et al. 2006). These waves of death signaling activate astrocytes and microglia, causing the release of toxic molecules including glutamate, ROS, NO, and pro-inflammatory cytokines and chemokines. This vicious amplification spiral of signaling could worsen neuroinflammation by recruiting leukocytes and increase

lesion area (Orellana et al. 2009). Moreover, a growing pool of evidence demonstrates that gap junction and hemichannel blockers are therapeutic in experimental models of stroke (Rawanduzy et al. 1997; Takeuchi et al. 2008; Frantseva et al. 2002; Tamura et al. 2011; de Pina-Benabou et al. 2005).

8.3.2 Multiple Sclerosis (MS)

Recent studies have reported abnormal expression of glial connexins in the inflamed lesions of MS patients and experimental autoimmune encephalomyelitis (EAE) animals. Expression of oligodendrocytic Cx32 and Cx47 and astrocytic Cx43 is downregulated in the active lesions of MS patients and EAE mice (Eugenin et al. 2012; Markoullis et al. 2012; Brand-Schieber et al. 2005). Expression levels of Cx47 and Cx32 increase during remyelination but decrease in the relapsing phase, and EAE induced in Cx32 knockout mice results in an exacerbated clinical course with more demyelination and axonal loss (Markoullis et al. 2012). Whereas mice lacking astrocytic expression of Cx43 and Cx30 exhibit white matter vacuolation and hypomyelination, the severity of EAE was similar to that in wild-type mice (Lutz et al. 2012). Thus, oligodendrocytic expression levels of Cx32 and Cx47 appear to be associated with the degree of damage and remyelination, whereas astrocytic expression levels of Cx43 do not. Recent studies, however, indicate that a loss of Cx43 in astrocytes precedes demyelination in the MS-related disorders neuromyelitis optica and Balo's disease (Masaki et al. 2012; Matsushita et al. 2011). Further studies are needed to elucidate the precise role of glial connexins in the pathogenesis of MS.

8.3.3 Neurodegenerative Disease

Scientific and clinical data have implicated neuroinflammation, including accumulating activated astrocytes and microglia, in the pathogenesis of such neurodegenerative diseases as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Glass et al. 2010). Microglial activation followed by astrocytic activation is the earliest pathologic finding in many of these diseases, appearing before the manifestation of overt symptoms. Recent studies have indicated that activated microglia release a large amount of glutamate through Cx32 hemichannels resulting in excitotoxic neuronal death (Takeuchi et al. 2006, 2008; Yawata et al. 2008). Interestingly, microglia-derived glutamate and proinflammatory cytokines induce dysfunction of gap junction and hemichannels in astrocytes (Kielian 2008), which may disrupt homeostasis in the CNS.

Reactive astrocytes at amyloid β (Aβ) plaques show increased levels of Cx43 and Cx30 in the brains of AD patients (Koulakoff et al. 2012). A recent study demonstrated that Aβ peptide induces the release of glutamate and ATP via uncoupled hemichannels in microglia and astrocytes, leading to neuronal death (Orellana et al.

2011). Agreeing with this observation, blocking gap junctions and hemichannels ameliorates memory impairments in a mouse model of AD (Takeuchi et al. 2011). MTPT-treated mice and rotenone-treated rats—two animal models of PD—show increased astrocytic expression of Cx43 in affected areas (Rufer et al. 1996; Kawasaki et al. 2009). A recent report demonstrated that α-synuclein—a main component of Lewy bodies—directly binds to Cx32, and overexpression of α-synuclein inhibits the activity of Cx32 in the SH-SY5Y dopaminergic neuroblastoma cell line (Sung et al. 2007). Other studies have revealed that microglia and astrocytes are determinants of disease progression in ALS (the nonautonomous neuronal death hypothesis) (Yamanaka et al. 2008; Boillee et al. 2006). Activation of microglia and astrocytes is associated with enhanced expression levels of gap junctions and hemichannels. In fact, blocking gap junctions and hemichannels slowed disease progression in a mouse model of ALS (Takeuchi et al. 2011). Few studies, however, have focused on the expression profiles and functions of connexins in these diseases. Therefore, whether gap junctions and hemichannels are involved in the pathogenesis of neurodegenerative diseases remains largely unclear.

8.4 Conclusions

Several lines of evidence have uncovered pathologic roles for gap junctions and hemichannels in various neurological disorders. For example, dysfunction of these structures in glial cells contributes to neuroinflammation in the CNS, often resulting in neuronal damage (i.e., glial cells as "bad neighbors" for neurons) (Block et al. 2007). Despite recent progress in elucidating the pathologic roles of gap junctions and hemichannels, many challenges remain due to the technical limitation. For instance, reagents that are commonly used to block connexin channels are not specific for those channels; connexin channel blockers such as glycyrrhetinic acid, its derivative carbenoxolone, niflumic acid, and octanol also block pannexin channels. Although the most specific gap junction and hemichannel blockers currently are mimetic peptides that reflect specific sequences in the extracellular loops E1 and E2, recent studies showed that mimetic peptides specific for Cx32 (^{32}gap 24 and ^{32}gap 27), Cx43 (^{43}gap 27), or Panx1 (^{10}panx1) nonspecifically block both connexins and pannexins (Wang et al. 2007). The heterogeneity of gap junctions and hemichannels (Fig. 8.2) and the potential for various connexins to compensate for the loss of other isoforms (e.g., in connexin-knockout studies) also complicate analysis of this system. Whereas the development of fluorescently tagged connexins—for instance, with EGFP—has facilitated live cell imaging, tagging and/or overexpression of connexins in cultured cells often produces abnormally large gap junction plaques (Gaietta et al. 2002; Hunter et al. 2003; Lopez et al. 2001). Moreover, tagging the amino termini of connexins results in nonfunctional channels, whereas tagging the carboxyl termini alters the properties of the channels (Contreras et al. 2003; Bukauskas et al. 2000). Therefore, future studies should detail spatiotemporal expression profiles of connexin isoforms under pathologic conditions in the CNS,

which will require the specific blockers and tracers for each connexin isoform, hemichannel, and gap junction. Understanding the precise pathologic roles of gap junctions and hemichannels may lead to new therapeutic approach that can slow and halt the progression of various chronic neurologic disorders.

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Chapter 9
Toll-Like Receptors and Neuroinflammation

Sung Joong Lee

Abstract Inflammatory responses are pathological hallmarks of infectious diseases of the nervous system such as bacterial meningitis and viral encephalitis. Noninfectious neurological disease or injury often accompanies neuroinflammation, although the underlying mechanisms of such "sterile neuroinflammation" are not completely understood. Studies conducted over the past 10 years on the function of Toll-like receptors (TLRs) in the nervous system have shed new light on the molecular and cellular mechanisms of neuroinflammation. TLRs belong to a class of pattern-recognition receptors that play important roles in host defense against pathogens and tissue injury/recovery by recognizing a wide variety of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). In the nervous system, different members of the TLR family are expressed on astrocytes, microglia, oligodendrocytes, and Schwann cells, implicating these glial cells in neuroinflammation in pathological contexts. In this chapter, we summarize recent studies of TLR expression in the cells of the nervous system and discuss its roles in neuroinflammation in the context of infectious diseases as well as noninfectious neurological disorders such as stroke, spinal cord injury, and peripheral nerve injury.

9.1 Introduction: Toll-Like Receptors and Their Ligands

Toll-like receptors (TLRs) are type I transmembrane glycoproteins that are evolutionarily conserved between insects and mammals. The Toll gene was originally identified in *Drosophila* as an essential gene regulating the development of the

S.J. Lee, Ph.D. (✉)
Department of Neuroscience and Physiology, Dental Research Institute, School of Dentistry, Seoul National University, 28 Yeongun-dong, Jongno-gu, Seoul, Republic of Korea
e-mail: sjlee87@snu.ac.kr

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dorsal–ventral axis during embryogenesis (Hashimoto et al. 1988). Later, it was found that Toll also plays a role in the *Drosophila* innate immune response against microbial infection (Lemaitre et al. 1996; Williams et al. 1997). Based on these findings, the quest for a mammalian homologue of Toll was initiated and in 1997, the first human homologue, called Toll-like receptor (TLR), was identified (Medzhitov et al. 1997). Since then, 10 genes have been described in humans and 13 have been described in mice (Uematsu and Akira 2006). Among them, ligands for 10 TLR members (TLR1–9 and TLR11) were identified, leaving others yet to be elucidated. Briefly, TLR2, in association with either TLR1 or TLR6, recognizes a wide array of bacterial-derived pathogen-associated molecular patterns (PAMPs) including peptidoglycan (PGN), lipoteichoic acid (LTA) and lipoproteins of Gram-positive bacteria, and mycoplasma lipopeptide (Aliprantis et al. 1999; Brightbill et al. 1999; Schwandner et al. 1999). In addition, the receptor binds to zymosan, a yeast cell wall component (Underhill et al. 1999). TLR4 binds to lipopolysaccharide (LPS) from Gram-negative bacteria in association with the co-receptor MD-2 (Shimazu et al. 1999). TLR5 was shown to be activated by bacterial flagellin (Hayashi et al. 2001). Mouse TLR11 recognizes uropathogenic bacteria such as uropathogenic *Escherichia coli* or profilin-like protein from *Toxoplasma gondii* (Yarovinsky et al. 2005; Zhang et al. 2004). TLR10 is known to be able to heterodimerize with TLR1 and TLR2, but its ligand remains to be identified (Hasan et al. 2005). Compared to these TLRs that function on the cytoplasmic membrane, other TLR members bind to their ligands on the endosomal membrane. Among them, TLR3 recognizes double-stranded RNA (dsRNA), which is generated as an intermediate product of viral replication within cells (Alexopoulou et al. 2001). TLR7 and TLR8 function as receptors for the GU-rich single stranded RNA (ssRNA) that is also produced during viral infection. TLR9 is also expressed on the endosomal membrane and functions as a receptor for bacterial and viral DNA that is enriched with an unmethylated CpG sequence motif (Latz et al. 2004).

TLR can be activated not only by pathogen-derived molecules but also by endogenous molecules that are exposed during tissue damage. For example, TLR4 can be activated by extracellular matrix components fibronectin, hyaluronan, biglycan, fibrinogen, and soluble heparin sulfate (Smiley et al. 2001; Okamura et al. 2001; Termeer et al. 2002; Johnson et al. 2002; Schaefer et al. 2005). These molecules can be released during enzymatic degradation of the extracellular matrix during tissue injury. Cytoplasmic or nuclear proteins that are not normally exposed to innate immune cells such as heat shock protein (HSP) 60, HSP70, HSP22, HSP72, and high mobility group box-1 (HMGB1) may also activate TLR4 once released extracellularly due to necrotic cell death. In addition, lung surfactant protein A, beta-defensin, tenascin-C, and S100 proteins were implicated as TLR4 endogenous agonists (Guillot et al. 2002; Biragyn et al. 2002; Vogl et al. 2007; Midwood et al. 2009). Among these TLR4 agonists, HSP60, HSP70, HMGB1, hyaluronan, and biglycan were shown to activate TLR2 as well. TLR2 also functions as a receptor for gangliosides and necrotic neurons, though the specific molecular identities of the ligand have not been identified (Kim et al. 2007). TLR3 has been reported to recognize mRNAs or RNAs with hairpin structures (Kariko et al. 2004a, b).

Table 9.1 DAMPs: endogenous TLR ligands

TLRs	Endogenous ligands	References
TLR2	HSP60, 70, Gp96	Asea et al. (2002), Vabulas et al. (2001, 2002)
	HMGB1	Park et al. (2004)
	β-Defensin3	Funderburg et al. (2007)
	Surfactant protein A, D	Ohya et al. (2006), Murakami et al. (2002)
	Eosinophil-derived neurotoxin	Yang et al. (2008)
	Gangliosides	Yoon et al. (2008b)
	Serum amyloid A	He et al. (2009)
	Hyaluronic acid fragment	Termeer et al. (2002)
	Biglycan	Schaefer et al. (2005)
TLR3	mRNA	Kariko et al. (2004a)
	Small interfering RNA	Kariko et al. (2004b)
TLR4	HSP60, 70, 22, Gp96	Asea et al. (2002), Vabulas et al. (2001, 2002), Roelofs et al. (2006)
	HMGB1	Park et al. (2004)
	Fibrinogen	Smiley et al. (2001)
	Fibronectin extra domain A	Okamura et al. (2001)
	Tenascin-C	Midwood et al. (2009)
	Surfactant protein A, D	Guillot et al. (2002), Ohya et al. (2006)
	β-Defensin2	Biragyn et al. (2002)
	S100A8, 9 (MRP8, 14)	Vogl et al. (2007)
	Neutrophil elastase	Devaney et al. (2003)
	Lactoferrin	Curran et al. (2006)
	Gangliosides	Jou et al. (2006)
	Serum amyloid A	Hiratsuka et al. (2008)
	Oxidized LDL	Miller et al. (2003)
	Saturated fatty acids	Shi et al. (2006)
	Hyaluronic acid fragment	Termeer et al. (2002)
	Heparan sulfate	Johnson et al. (2002)
	Biglycan	Schaefer et al. (2005)
TLR7	ssRNA	Barrat et al. (2005), Vollmer et al. (2005)
TLR8	ssRNA	Vollmer et al. (2005)
TLR9	Chromatin–IgG complexes	Leadbetter et al. (2002)
	DNA immune complexes	Barrat et al. (2005)

Similarly, RNAs and small interfering RNAs can induce intracellular signals through TLR7 and TLR8 (Barrat et al. 2005; Vollmer et al. 2005). Chromatin–immunoglobulin G complexes of necrotic cells have been shown to activate dendritic cells via TLR9 (Leadbetter et al. 2002). It is generally believed that in pathological conditions, these various ligands can be either released from injured tissues and dying cells or actively secreted by activated cells in order to serve as "danger signals" in response to tissue damage. Recognition of these damage-associated molecular patterns (DAMPs) by TLRs triggers sterile inflammatory responses, which have relevance in various neurological disorders. The DAMPs identified thus far are summarized in Table 9.1.

For TLRs, the ligand–receptor interaction occurs in the extracellular domain of receptors, which share a common structural framework containing several leucine-rich repeats (LRRs) (Medzhitov et al. 1997). Ligand binding to this LRR induces dimerization of this extracellular domain with other TLR molecules or co-receptors to form an "m-shape" dimer structure. The cytoplasmic portion of TLRs contains a domain that is similar to those of IL-1 receptors and is thus called the TLR/IL-1 receptor homology domain (TIR). Upon binding to its cognate ligands, this TIR domain is utilized to recruit other TIR-containing intracellular signaling adaptor proteins to transmit intracellular signals (Akira and Takeda 2004). Thus far, five TIR-containing adaptor proteins, MyD88, Mal, TRIF, TRAM, and SARM, have been reported. Most TLRs utilize MyD88 to transduce an intracellular signaling cascade, with the exception of TLR3 which uses TRIF for transduction instead. TLR4 signaling may utilize TRAM as an additional adaptor protein to recruit TRIF, thus allowing it to transmit both MyD88- and TRIF-dependent signaling cascade. The recruitment of MyD88 to the cytoplasmic TIR of TLRs leads to the activation of IL-1 receptor-associated kinase (IRAK) and TRAF6. Next, TRAF6 induces the activation of TAK1, which eventually leads to NF-κB activation mediated by IKK. TRAF6 also activates the p38 and JNK MAP kinase pathways through the phosphorylation of MKK3/6 and MKK4, respectively. Meanwhile, TRIF activation by TLR3 or TLR4 recruits TRAF3, which allows activation of TBK1 and IKKε. These kinases are responsible for the phosphorylation of specific transcription factors called IRFs (IRF3 and 7), which induce the transcriptional activation of antiviral genes such as type I interferon (IFNα/β). At the same time, TRIF can mediate NF-κB and MAP kinase activation independently of MyD88 via RIP1 binding.

9.2 TLRs in the Nervous System

There are different types of glial cells in the nervous system, including microglia, astrocytes, oligodendrocytes, and Schwann cells. Among these cell types, microglia are of hematopoietic cell lineage and are considered to be innate immune cells in the central nervous system (CNS). Similar to the innate immune cells in peripheral organs, microglia function as sentry cells that constantly monitor the CNS microenvironment for infection and damage (Ramlackhansingh et al. 2011). Thus, it was anticipated that this glial cell type is well-equipped with receptors for detecting pathogen infection and tissue damage, namely TLRs. To test this hypothesis, we have screened for the mRNA expression of different TLR members in a BV-2 microglia cell line and found that transcripts of all TLR members (TLR1–9) are expressed (Lee and Lee 2002). These findings were later confirmed in primary mouse cultures and in human microglial cells (Jack et al. 2005; Olson and Miller 2004; Bsibsi et al. 2002). Within the TLR family, only TLR1, 2, 3, 4, and 9 have been detected in primary rodent cultures or in human microglial cells at the protein level (Bsibsi et al. 2002; Kielian et al. 2002; Yoon et al. 2008a; Cassiani-Ingoni et al. 2006). These data suggest that there may be distinct posttranscriptional

regulatory mechanisms involved for different TLR members. Still, it is possible that other TLR proteins, namely TLR5, 6, 7, and 8, are also expressed, but their levels were too low to be detected by immunohistochemistry. In microglia, the activation of TLRs by their cognate agonists induces strong inflammatory responses, culminating in the expression of typical proinflammatory genes such as TNF-α, IL-1β, and IL-6 (Olson and Miller 2004; Kinsner et al. 2006). These data indicate that microglia not only express TLRs but are also equipped with intracellular signaling machinery that enables proinflammatory gene induction by TLR engagement.

For astrocytes, TLR2, 3, 4, 5, and 9 were detected in in vitro-cultured cells (Lehnardt et al. 2006; Bowman et al. 2003; Scumpia et al. 2005; Park et al. 2006). However, their TLR expression profile is more limited in vivo. In studies investigating TLR mRNA expression in rat brains using in situ hybridization, TLR2 and TLR4 mRNAs were not detected in astrocytes, but were detected in microglia (Laflamme and Rivest 2001; Laflamme et al. 2001). These data suggest that TLR2 and TLR4 are not expressed in resting astrocytes in vivo, but are only induced in cultured astrocytes that become activated during the culturing process (Passaquin et al. 1994). In support of this notion, TLR2, 4, 5, and 9 can be strongly induced in primary cultured astrocytes upon activation (Bowman et al. 2003). In addition, TLR4 protein expression was detected in astrocytes in areas of brain lesions in multiple sclerosis (MS) patients, but was barely detected in astrocytes from unaffected white matter (Bsibsi et al. 2002). This localization further supports the idea that TLR2 and TLR4 are only expressed in activated astrocytes in vivo. Thus far, there is no clear evidence of TLR5 or TLR9 expression in astrocytes in vivo. Unlike TLR2 or TLR4, TLR3 is constitutively expressed in murine astrocytes in vivo as well as in vitro (Park et al. 2006). Taken together, these data indicate that astrocytes express TLR3 in vivo at the resting state and may express TLR2, 4, 5, and 9 upon activation in pathological contexts.

TLR expression in oligodendrocytes and Schwann cells is limited compared to that of microglia. Initially, the expression of TLR2 and TLR3 mRNAs was reported in primary cultured human oligodendrocytes (Bsibsi et al. 2002). Later, the expression of TLR2 protein was documented in human oligodendrocytes in normal regions of the brain and MS lesion areas (Sloane et al. 2010), and TLR2, 3, and 4 were documented in in vitro-cultured oligodendrocyte precursor cells (OPCs) (Taylor et al. 2010; Bsibsi et al. 2012). Similarly, TLR2 protein expression was documented in human Schwann cells (Oliveira et al. 2003). Later, TLR3 and TLR4 were also detected in murine or rat Schwann cells at both the mRNA and protein levels (Colomar et al. 2003; Karanth et al. 2006; Lee et al. 2007; Hao et al. 2009). Although the TLR expression profile in oligodendrocytes and Schwann cells is similar, the functional outcome of TLR activation in these cell types seems to be distinct. For instance, in oligodendrocytes, the activation of TLRs, especially TLR3, induced an apoptotic oligodendrocyte cell death signal (Bsibsi et al. 2012). In Schwann cells, TLR activation usually resulted in a proinflammatory intracellular signal, inducing TNF-α and IL-1β expression (Colomar et al. 2003; Cheng et al. 2007) as well as NO production (Zhang et al. 2010). The proinflammatory TLR signal in Schwann cells implies that they not only function as myelin-forming cells but may also play a role

Table 9.2 TLR expression in the nervous system

Cells	TLRs	Species	References
Neuron	TLR3, 4, 7, 9	H	Prehaud et al. (2005), Qi et al. (2011)
	TLR2–4, 6–8, 11–13	M	Tang et al. (2007), Mishra et al. (2006, 2008), Barajon et al. (2009), Qi et al. (2011)
Astrocytes	TLR3, 4	H	Jack et al. (2005), Bsibsi et al. (2002)
	TLR2–5, 9	M	Bowman et al. (2003); Park et al. (2006), Carpentier et al. (2005), El-Hage et al. (2011)
Microglia	TLR1–4	H	Jack et al. (2005), Bsibsi et al. (2002), Cassiani-Ingoni et al. (2006)
	TLR2, 4, 9	M	Yoon et al. (2008a), Kielian et al. (2005a), Lehnardt et al. (2007)
Oligodendrocytes	TLR2	H	Sloane et al. (2010)
	TLR2–4	M, R	Lehnardt et al. (2006), Taylor et al. (2010), Bsibsi et al. (2012)
Schwann cells	TLR2	H	Oliveira et al. (2003)
	TLR3, 4	R	Karanth et al. (2006), Lee et al. (2007)

H: human, M: mouse, R: rat

in detecting pathogen infection or tissue damage in nerves and thereby initiate inflammatory responses or tissue repair in the PNS.

Recent studies show that certain TLR members are also expressed in neurons. Among the different members of the TLR family, TLR3 and TLR8 were first reported in human neuronal cell lines and primary cultured mouse cortical neurons, respectively (Ma et al. 2006; Prehaud et al. 2005). In addition, primary cultured murine cortical neurons were reported to express TLR2 and TLR4 at both the mRNA and protein levels (Tang et al. 2007). TLR11, 12, and 13 were constitutively expressed in neurons in a murine model of neurocysticercosis (Mishra et al. 2008). In the PNS, TLR3, 4, 7, and 9 were detected in dorsal root ganglion (DRG) sensory neurons and cells of the enteric nervous system (Barajon et al. 2009; Qi et al. 2011). These data suggest that neurons may also be involved in neuroinflammation or that PAMPs or DAMPs released during infection and injury may directly affect neurons via TLRs. The TLR protein expression profile in the cells of nervous system is summarized in Table 9.2.

9.3 TLRs in Pathogen Infection

Studies of the expressions of TLRs in the cells of the nervous system and their function as receptors for PAMPs suggested that TLRs may detect pathogen infection in the nervous system and thereby trigger inflammatory responses. Indeed, studies for the past decade using TLR-deficient mice have accumulated data supporting a pivotal role of TLRs in infectious neuroinflammatory diseases such as bacterial meningitis, abscess, and viral encephalitis, which are briefly summarized in this section.

9.3.1 Bacterial Meningitis

Bacterial infection of the leptomeninges and subarachnoid space results in bacterial meningitis, of which the morbidity and mortality rates remain high. The leading cause of bacterial meningitis is *Streptococcus pneumonia* followed by *Neisseria meningitides* and group B streptococci (Schuchat et al. 1997). A series of in vitro studies indicate that TLR2, 4, and 9 are involved in detecting these bacteria by host immune cells. Initially, it was shown that TLR2 can be activated by *S. pneumonia* via pneumococcal LTA (Yoshimura et al. 1999). Later, it was shown that TLR4 responds to the pneumolysin from ethanol-killed *S. pneumonia* (Malley et al. 2003) and TLR9 can be activated by the genomic DNA of *S. pneumonia* (Mogensen et al. 2006). These TLRs are also implicated in the recognition of *N. meningitides* (Mogensen et al. 2006). The ability of TLR2 to detect *N. meningitides* involves the recognition of the neisserial porin PorB, the major outer membrane protein of this bacteria (Massari et al. 2002). In addition, TLR4 and TLR9 recognize meningococcal LPS (Pridmore et al. 2001) and DNA (Mogensen et al. 2006), respectively. These in vitro data suggest that these TLRs may play an important role in the pathogenesis of meningitis.

The first in vivo study implicating TLR in meningitis was carried out by Echchannaoui et al. (2002), in which they induced pneumococcal meningitis in mice by directly injecting *S. pneumonia* in the CNS. In their study, TLR2 knockout (KO) mice had more severe clinical symptoms and more bacterial accumulation in the brain than wild-type control mice. In addition, TNF-α level was significantly up-regulated in the cerebrospinal fluid (CSF) of TLR2 KO mice compared to wild-type mice, indicating that the enhanced inflammatory gene expression may have resulted in the exacerbated clinical symptoms in the KO mice. However, in a later study by Koedel et al., an enhanced proinflammatory cytokine expression was not observed in TLR2 KO mice (Koedel et al. 2003). This suggests that the enhanced clinical symptoms observed in the TLR2 KO mice are probably due to impaired bacterial clearance. Nevertheless, these reports suggest that TLR2 is required for the successful defense from pneumococcal meningitis, at least in an experimental animal model. This hypothesis was further supported by experiments using MyD88-deficient mice (Echchannaoui et al. 2005). In these mice, pneumococcal infection led to increases in the mortality rate, indicating an impaired host defense to bacterial infection. In TLR2 and TLR4 double KO mice, the host immune response to pneumococcal bacterial infection was further impaired compared to single KO of these TLRs (Klein et al. 2008). These data demonstrate that the concerted action of both TLR2 and TLR4 plays an important role in host defense and the innate immune response to pneumococcal meningitis. However, contrary to the in vitro data, TLR9 seems to play a minimal role in pneumococcal meningitis in vivo, since TLR2, 4, and 9 triple KO mice show comparable phenotypes to those of TLR2 and 4 double KO mice (Klein et al. 2008). Although it was not demonstrated in these in vivo studies, it is conceivable that TLRs expressed on the macrophages and dendritic cells in the leptomeninges and subarachnoid space may at least in part contribute to the phenotypes observed in the KO mice.

9.3.2 Bacterial Brain Abscess

Localized bacterial infection in brain parenchyma results in abscesses in the brain that are characterized by suppurative lesion formation. Brain abscesses can be caused by a variety of microorganisms including bacteria and fungi (Mathisen and Johnson 1997), yet the leading etiologic agent is *Staphylococcus aureus*. The pathogenic process of human brain abscesses can be recapitulated by an animal model by injecting *S. aureus* directly into mouse brain parenchyma (Kielian et al. 2001). In this model, *S. aureus* infection initially triggers microglia and astrocyte activation, which later leads to macrophage and lymphocyte infiltration. A series of in vitro studies indicate that TLR2 is involved in the activation of glial cells against *S. aureus*. First, primary cultured microglia can be activated by intact *S. aureus* to express the proinflammatory cytokines IL-1β and TNF-α, chemokines, and costimulatory molecules (Tallini and Stoner 2002). This activation of proinflammatory microglia was recapitulated by the stimulation of *S. aureus*-derived PGN, and these effects were abrogated in TLR2-deficient microglia (Kielian et al. 2005a). Likewise, *S. aureus* and its PGN induce proinflammatory gene expression in primary astrocytes, which is also dependent on TLR2 (Esen et al. 2004). These findings suggested that these glial cell types may play a key role in the initial antibacterial innate immune response in the CNS through the engagement of TLR2. However, an initial study using a mouse brain abscess model has revealed a limited role of TLR2 in the induction of inflammatory responses (Kielian et al. 2005b). In this study, proinflammatory gene induction after *S. aureus* injection was delayed, but not inhibited in TLR2 KO mice compared to wild-type mice. In addition, the bacterial titer was not much different in the TLR2 KO mice compared to the control. Instead, TLR2 influenced adaptive immune responses against *S. aureus* in the later phases (Nichols et al. 2009). There was a significant increase in IL-17-producing T cell-, NKT cell-, and γδT cell-infiltrates following CNS *S. aureus* infection in the TLR2 KO mice (Nichols et al. 2009; Vidlak et al. 2011). However, in a later study by Stenzel et al., it was shown that brain abscess size was exacerbated and bacterial clearance was impaired in TLR2 KO mice (Stenzel et al. 2008). Taken together, these reports indicate that TLR2 indeed plays an important role in defending the host against *S. aureus* infection in the CNS, with functions not limited to initial pathogen recognition, but also encompassing the complex regulation of both innate and adaptive immune responses. Of particular interest, one study has shown that the host immune response to *S. aureus* is also compromised in TLR4 KO mice, which have enhanced bacterial burdens and mortality rates compared to wild-type mice (Stenzel et al. 2008). Considering that the Gram-positive bacteria *S. aureus* do not activate TLR4, it is likely that certain certain endogenous agonist of TLR4 released during brain tissue damage may regulate antibacterial immune responses via TLR4, the mechanism of which needs to be clarified in the future studies.

9.3.2.1 Viral Encephalitis

Viral infection in the brain parenchyma can elicit devastating inflammatory responses in the CNS that are usually called viral encephalitis. A series of RNA and DNA viruses, including herpes simplex virus-1 (HSV-1), West Nile virus (WNV), rabies virus, and HIV, can cause brain encephalitis. Endosomal TLRs (TLR3, 7, 8, and 9) are known to function as pattern-recognition receptors for these viral RNA and DNA, so the involvement of these TLRs in viral encephalitis has long been suspected. Among these TLRs, TLR3 was first implicated in CNS virus infection by Wang et al. (2004). In their study using TLR3-deficient mice, they demonstrated that TLR3 is required for the entry of WNV into the brain. Compared to wild-type control mice, TLR3 KO mice were more resistant to viral infection-induced morbidity, and their CNS viral load was also reduced. However, a later study by Daffis et al. argued that TLR3 has antiviral effects in WNV-induced brain encephalitis (Daffis et al. 2008). In this study, TLR3-deficiency enhanced WNV mortality and increased the viral burden in the brain. Thus far, it is not clear why TLR3 shows conflicting roles in these two studies.

Contrary to its role in WNV encephalitis, it seems clear that TLR3 signaling is vital for the successful antiviral responses against HSV-1 encephalitis in humans. In a study characterizing patients with HSV-1 encephalitis and dominant-negative mutant TLR3, researchers found high levels of viral replication and cell mortality in the fibroblasts from these patients (Zhang et al. 2007). In addition, these fibroblasts had impaired antiviral inflammatory gene (IFN-α/β) expression. These data support the hypothesis that humans with defects in TLR3 signaling have an elevated susceptibility to HSV encephalitis. One study with TLR3-deficient mice in HSV-2 encephalitis shows that the TLR3 expressed in astrocytes plays an important role in controlling the viral infection in the CNS (Reinert et al. 2012). In this study, TLR3 KO mice were hypersusceptible to HSV infection in the CNS, with their astrocytes most frequently infected. In wild-type mice, astrocytes respond to HSV infection by the production of type I IFN, which was defective in TLR3-deficient astrocytes. Thus, the TLR3 on astrocytes is likely to sense HSV-2 infection after entry into the CNS, preventing HSV from spreading by IFN production. Studies of the role of other TLR members, namely TLR2 and TLR9, in HSV encephalitis reported conflicting results. In one study, TLR2 KO mice had reduced mortality compared with wild-type mice, and HSV-induced MCP-1 induction in the CNS was significantly reduced in these mice (Kurt-Jones et al. 2004). These data seem to indicate that TLR2 facilitates and contributes to HSV encephalitis. However, in a more recent study, HSV viral load in the brain was much higher in TLR2/9 double KO mice compared to wild-type mice (Sorensen et al. 2008). In addition, antiviral gene expression in the CNS was reduced in either TLR2 or TLR9 KO mice. These studies argue that TLR2 and TLR9 synergistically stimulate innate antiviral activities, thereby protecting against HSV infection in the brain. Although TLR7 and 8 have the potential to recognize virus infections as well, their involvement in viral encephalitis has not been formally addressed and needs to be investigated in future studies.

9.4 TLRs in Sterile Neuroinflammation

As the receptors for DAMPs, TLRs have been suggested to play a role in sterile neurological diseases involving tissue damage. For the past several years, increasing amounts of evidence have supported the pivotal role of TLRs in the initiation, progression, and resolution of various acute neurological diseases including strokes, traumatic spinal cord/brain injuries, and peripheral nerve injuries. In this section, we will briefly summarize the studies which implicate TLRs in these acute neurological disorders.

9.4.1 TLRs in Stroke

Strokes are one of the leading causes of death and severe long-term disability in adults in developed countries. The interruption of blood supply to the brain and the rupture of cerebral blood vessels lead to ischemic and hemorrhagic strokes, respectively (Donnan et al. 2008). In both types, the primary insult elicits an inflammatory response in the CNS that contributes to secondary brain damage. The inflammatory responses are likely initiated by the activation of local glial cells, microglia, and astrocytes by the DAMPs released due to the initial insult (Wang et al. 2011). Therefore, it has been speculated that TLRs might be involved in secondary neuroinflammation during stroke injury. This hypothesis was formally addressed using TLR KO mice in several different studies. In ischemic/reperfusion (I/R) injury models, there were significant decreases in infarct volume and neurological deficit in both TLR2- and TLR4-deficient mice when compared to wild-type control mice (Tang et al. 2007; Ziegler et al. 2007; Lehnardt et al. 2007; Cao et al. 2007; Caso et al. 2007). Although these reports all agree on the detrimental role of TLR2 and TLR4 in I/R-mediated secondary damage, the precise mechanisms underlying these effects remain to be fully elucidated. In TLR4-mutant mice, the expression of potentially neurotoxic mediators that are induced in the CNS parenchyma upon I/R insult, including TNF-α, IL-6, iNOS, and MMP-9, was significantly reduced when compared to wild-type mice (Cao et al. 2007; Caso et al. 2007). In this study, TLR4 was detected mainly in activated microglia and astrocytes in the brain parenchyma, suggesting that TLR4 activation in these cells augments the expression of the above genes. TLR2 is also mainly found in the microglia in post-ischemic brain tissue (Ziegler et al. 2007), and contributes to the expression of post-ischemic proinflammatory genes in the brain (Ziegler et al. 2011). These studies suggest that TLR2 and TLR4 expressed on microglia or astrocytes may recognize DAMPs released during ischemic brain injury, thereby mediating the inflammatory responses in the CNS that result in secondary brain damage.

The role of TLRs in intracerebral hemorrhagic (ICH) injuries has been also documented. Intracerebral injections of autologous blood result in hematoma and inflammatory responses in the brain, a process which is utilized for animal models of ICH injury. In TLR4-deficient mice, there is markedly reduced macrophage and neutrophil infiltration in perihematoma tissues, and lower neurological deficits upon

autologous blood injection (Sansing et al. 2011). In one study, the TLR4 on leukocytes and platelets within the hemorrhage contributed to perihematomal leukocyte infiltrations and neurological deficits (Sansing et al. 2011). However, in a study by Lin et al., a significant increase in TLR4 expression was detected mainly in reactive microglia (Lin et al. 2012), suggesting that microglial TLR4 may trigger and/or potentiate the inflammatory response in the damaged brain. Therefore, the relative contribution of these cell types in ICH injury needs to be characterized in future studies. Thus far, the putative role of TLR2 in ICH injury has not been characterized. Considering its pivotal function in I/R brain injury, it will be interesting to see if TLR2 plays a comparable role in ICH.

9.4.2 TLRs in Traumatic Spinal Cord and Brain Injuries

Traumatic injury in the spinal cord may cause massive tissue destruction and cellular damage at and around the injury site. The primary tissue injury is usually followed by secondary inflammatory injury cascades that are featured with the infiltration of blood-derived immune cells, activation of resident glial cells, and an increase in proinflammatory and cytotoxic gene expression in the spinal cord. This often accompanies delayed neuronal death, demyelination, and axonal degeneration in the penumbrae of the injury site (Profyris et al. 2004; Tator and Fehlings 1991; Popovich and McTigue 2009). It was first reported by Kigerl et al. that the mRNAs of several TLRs, including TLR1, 2, 4, 5, and 7, were expressed in spinal cord tissue after injury (Kigerl et al. 2007). Among these receptors, the levels of TLR2 and TLR4 are massively increased in the spinal cord after injury. TLR4 expression is induced mainly in activated macrophages and microglia in the spinal cord, while TLR2 expression has been detected in astrocytes as well. It is suspected that TLR2 and TLR4 expression in spinal cord glia and tissue-infiltrating immune cells may contribute to the inflammatory response in the spinal cord after injury. However, in TLR4-mutant or TLR2-deficient mice, the locomotor deficits due to injury were sustained for longer periods of time compared to wild-type control mice. In addition, injury-related pathological changes such as demyelination, astrogliosis, and microglia and macrophage activation were further aggravated in TLR4-mutant mice. These reports demonstrate that TLR2 and TLR4 play a neuroprotective role in spinal cord injury (SCI). This is contrary to their roles in I/R injuries, in which TLR2 and TLR4 potentiate tissue damage and neurological deficits after the injury. It is plausible that the endogenous TLR agonists released during SCI may be distinct from those of I/R injury, which induce delayed tissue repair responses in the SCI-induced tissue microenvironment. Recently, one study proposed that HMGB1 may function as an endogenous agonist for TLR2 and TLR4 in SCI (Chen et al. 2011). In this study, HMGB1 was induced in macrophages and neurons immediately after SCI and preceding proinflammatory gene expression. HMGB1 also co-localized with TLR2 or TLR4 on glial cells and macrophages, suggesting that HMGB1 may directly activate these

TLRs and induce inflammatory gene expression. These data posit a HMGB1–TLR2/4 interaction as being a major player in the acute phase proinflammatory responses in SCI. It will be interesting to test if depletion of HMGB1 in the context of SCI would mimic the pathological phenotype of SCI observed in TLR2-deficient or TLR4-mutant mice.

Similar to SCI, traumatic brain injuries (TBIs) cause acute inflammatory responses that result in secondary neuronal damage. These inflammatory responses are characterized by proinflammatory cytokine and chemokine expression and the recruitment of leukocytes in the brain parenchyma (Morganti-Kossmann et al. 2007; Harting et al. 2008; Ziebell and Morganti-Kossmann 2010). In an animal model of TBI, the expression of TLR2 and TLR4 was up-regulated in the areas with lesions (Chen et al. 2008; Zhang et al. 2012). While the TLR4-expressing cells were mostly infiltrating leukocytes, TLR2 protein expression was observed on macrophages and microglia in the lesion areas as well as in the astrocytes in the subcortical white matter (Zhang et al. 2012). Studies of the role of TLRs in TBI have shown that TLR2 is required for microglia and astrocyte activation around the lesion site of a stab wound (Park et al. 2008). In addition, TLR2-deficient mice had significantly ameliorated neurological deficits, which were associated with decreased expression of inflammatory cytokines compared with wild-type counterparts (Yu and Zha 2012). These results suggest that TLR2 expressed on the activated glia may function as a receptor for DAMPs released during TBI and contribute to secondary brain injuries possibly by regulating neuroinflammation.

9.4.3 TLRs in Peripheral Nerve Injury and Neuropathic Pain

Traumatic peripheral nerve injuries commonly cause Wallerian degeneration in damaged axons. In this process, Schwann cells first respond to the nerve injury and become dedifferentiated or activated (Stoll et al. 2002). Next, the activated Schwann cells express various inflammatory mediators including cytokines and chemokines (Bolin et al. 1995; Chattopadhyay et al. 2007; Levy et al. 1999; Takahashi et al. 2004; Tofaris et al. 2002; Wagner and Myers 1996). The chemokines, specifically MCP-1 and LIF, then recruit monocytes/macrophages to the injury site (Tofaris et al. 2002). These peripheral immune cells, in turn, remove the myelin debris and degenerating axons, completing the cleaning process. In addition, Schwann cells and recruited macrophages regulate axonal regeneration by producing various growth factors such as NGF, CNTF, GDNF, and BDNF (Hammarberg et al. 1996; Meyer et al. 1992; Smith et al. 1993). Schwann cell activation thus plays a central role in regulating inflammatory responses in injured nerves. However, it is not well-known how Schwann cells are initially activated and subsequently regulate these neuroinflammatory responses. Recent studies of the expression of TLRs on Schwann cells in the PNS and the critical role of TLRs in sensing tissue damage have suggested a putative role for TLRs in this process. Previously, we have reported that necrotic neurons induced the expression of various proinflammatory genes, including TNF-α, MCP-1, and LIF, all of which were detected in Wallerian degeneration in Schwann

cells, which were completely blocked in TLR2-deficient Schwann cells and partially blocked in TLR3-deficient Schwann cells (Lee et al. 2006). These data suggest that TLR2 and TLR3 are involved in the activation of Schwann cells following nerve injury. Similarly, blocking TLR4 with neutralizing antibodies inhibited the induction of Schwann cells by nerve injury (Karanth et al. 2006). In an in vivo study, TLR2- and TLR4-deficient mice showed delayed Wallerian degeneration, axonal regeneration, and reduced locomotor recovery after sciatic nerve injury compared with wild-type mice (Boivin et al. 2007). Taken together, these data support the possibility that the TLR2, 3, and 4 that are expressed on Schwann cells may, at least partly, function as receptors for DAMPs released from the damaged nerve and thereby trigger subsequent inflammatory responses in the PNS.

Injuries in peripheral nerves often result in abnormal chronic pain sensations called "neuropathic pain" (Marchand et al. 2005). Studies of the mechanisms of these diseases have revealed that spinal cord microglia activation plays a critical role in the development of neuropathic pain. Proinflammatory cytokines and growth factors secreted from the activated microglia such as TNF-α, IL-1β, IL-6, and BDNF may induce pain hypersensitization, either by directly enhancing the excitability of pain-transmitting neurons or by attenuating inhibitory synaptic transmission (McMahon et al. 2005; Coull et al. 2005). However, it is not clear how nerve injury induces spinal cord glial cell activation. So far, several studies have proposed that glial TLRs may function as receptors for sensing peripheral nerve injury. TLR4 was found to be up-regulated in spinal cord microglia after L5 nerve transection injury (Tanga et al. 2004), and the expression of TLR4 was determined to be required for injury-induced spinal cord microglia activation and neuropathic pain (Tanga et al. 2005). Similarly, TLR2 is also required for injury-induced spinal cord microglia activation and the subsequent development of neuropathic pain (Kim et al. 2007). This TLR2-dependent activation of microglia in the spinal cord after nerve injury is mediated by intracellular Nox2 expression and ROS production in the microglia (Kim et al. 2010). Interestingly, nerve injury-induced inflammatory gene expression and macrophage infiltration in the DRG were significantly reduced in TLR2-deficient mice (Kim et al. 2011). In this study, TLR2 expression was detected in satellite glial cells in the DRG. Taken together, these data suggest that TLR2 in satellite glial cells and spinal cord microglia may trigger inflammatory responses in the DRG and spinal cord, respectively, that eventually lead to the development of neuropathic pain (Kim et al. 2011). At present, it is not clear whether microglial TLR2 or DRG TLR2 contributes independently to nerve injury-induced pain hypersensitivity. Dissecting the relative contribution of TLR in these two cell types would greatly advance our knowledge on the mechanisms of neuropathic pain.

9.5 Summary

Neuroinflammation is an integral component of infectious and sterile neurological diseases. Studies for the past decade have demonstrated that many TLR members including TLR2, 3, 4, and 9 are expressed in the cells of the nervous system.

Recent studies using KO or mutant mice of these TLR members are beginning to reveal the in vivo roles of these pattern-recognition transmembrane receptors in the development or regulation of neuroinflammation in the context of various neurological diseases. In bacterial meningitis caused by *S. pneumonia* and *N. meningitides* infection, TLR2 and TLR4 play neuroprotective roles by clearing CNS-infecting bacteria and thereby reducing meningitis. The same TLR members function to defend against *S. aureus*-induced brain abscesses. It is conceivable that the proinflammatory signals transmitted by TLR2 and TLR4 during bacterial infection are prerequisite for successful bacteria clearance. Such bactericidal effects of TLRs seem to outweigh the putative detrimental effects of TLR-triggered inflammation in the brain, thus implicating the neuroprotective roles of TLRs in meningitis and abscess. Studies of animal models of viral encephalitis suggest that TLRs play a more complex role in the host response. In many studies, TLR3 exerted antiviral and neuroprotective effects on WNV- and HSV encephalitis. Nevertheless, other studies show that TLR3 contributes to WNV infection into the brain parenchyma. In addition, both beneficial and detrimental roles of TLR2 have been reported in models of HSV encephalitis. Studies of the in vivo role of TLRs in sterile neurological damage also show both beneficial and detrimental roles of TLR as well. TLR2 and TLR4 exacerbate neuroinflammation after stroke or TBI. Similarly, TLR2 and TLR4 contribute to the inflammatory response and the induction of neuropathic pain after nerve injuries. However, TLR2 and TLR4 have been shown to exert neuroprotective or regulatory effects after SCI and to facilitate recovery from nerve injury. It is obvious that each TLR plays distinct roles depending on the injury model. Even in the same injury context, different receptors may induce unique effector functions in specific injury microenvironments. The response can be affected by the location of the infection or injury, as well as the type and concentration of the DAMPs. In addition, differences in the TLR-expressing cell types involved in pathogen- or injury-recognition may differentially direct the in vivo role of TLR. It is conceivable that the TLRs on brain-resident glial cells are involved in bacterial abscess but have limited roles in meningitis. Likewise, the TLRs on spinal cord glial cells may behave differently compared to those in brain glial cells. Therefore, the molecular and cellular mechanisms of TLR functions in these neuroinflammatory diseases need to be more thoroughly investigated in future studies. Nevertheless, based on the studies so far, it is indisputable that TLRs play an important role in the development and resolution of various neuroinflammatory diseases, and thus could be utilized as a therapeutic target.

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Chapter 10
The Blood–Brain Barrier in Neuroinflammation

Fumitaka Shimizu and Takashi Kanda

Abstract The blood–brain barrier (BBB) is brain-specific capillary barrier that restricts the movement of soluble mediators and leukocytes from the blood to the central nervous system (CNS). The pathological breakdown of the BBB may be the initial key step of various neuroinflammatory CNS diseases including multiple sclerosis, bacterial meningitis, and neuroAIDS. This review describes an update of the biology of the cell comprising the BBB, and highlights the pathology and pathomechanisms of BBB breakdown in neuroinflammatory diseases. The human immortalized cell lines of BBB origin established in our laboratory will facilitate the future development of BBB research.

10.1 Introduction

The presence of the blood–brain barrier (BBB) restricts the movement of soluble mediators and leukocytes from the blood content to the central nervous system (CNS). Since the CNS homeostasis protected by the BBB is a prerequisite for the proper function of the CNS, pathological breakdown of the BBB may be a key event in the induction of various neuroinflammatory diseases, such as multiple sclerosis (MS), neuromyelitis optica (NMO), bacterial meningitis, and neuroAIDS. The aims of this chapter are to review the recent progress made in cell biology research regarding BBB-composing cells, and to discuss the importance of BBB breakdown in neuroinflammatory diseases to provide a better understanding of their pathogenesis and for the future development of novel therapies against these intractable disorders.

F. Shimizu, M.D., Ph.D. • T. Kanda, M.D., Ph.D. (✉)
Department of Neurology and Clinical Neuroscience, Yamaguchi University Graduate School of Medicine, 1-1-1, Minamikogushi, Ube, Yamaguchi 7558505, Japan
e-mail: fshimizu@yamaguchi-u.ac.jp; tkanda@yamaguchi-u.ac.jp

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10.2 Structure and Function of the BBB

The BBB is primarily composed of microvascular endothelial cells, which are surrounded by basement membranes, pericytes, and astrocytes (Fig. 10.1) (Abbott et al. 2006). The highly differentiated endothelial cells comprising the BBB are completely surrounded by basement membrane with embedded pericytes (Fig. 10.1) (Man et al. 2007). In the BBB, the entire abluminal aspect of this endothelial cell/pericyte/basement membrane complex is further ensheathed by a unique structure called the glia limitans perivascularis, consisting of a basement membrane composed of laminins, which is distinct from that of the endothelial basement membrane (Fig. 10.1) (Sixt et al. 2001) and of a layer of astrocytic endfeet. The endothelial cells composing the BBB are normally nonfenestrated and contain few pinocytotic vesicles, and adjacent endothelial cells are connected by complex and continuous tight junctions (TJs) (Gloor et al. 2001; Kniesel and Wolburg 2000). In addition, endothelial cells forming BBB express various receptors and transporters which remove toxic metabolites to maintain CNS homeostasis and help to incorporate necessary compounds into CNS parenchyma (Begley and Brightman 2003). Thus, BBB is not just a "barrier" or "wall," but a competent interface which actively exchanges materials between the brain microenvironment and blood contents. The infiltration of mononuclear cells and leakage of soluble factors across the BBB is the key step for the development of neuroinflammatory disorders including MS, NMO, bacterial meningitis, neuroAIDS, and so on (Larochelle et al. 2011; Shimizu et al. 2012a; Bencurova et al. 2011).

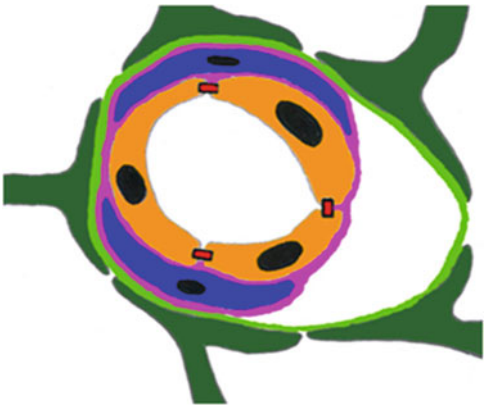

Fig. 10.1 The cellular structure of the blood–brain barrier (BBB). Endothelial cells (*orange*) have luminal tight junctions and form the capillaries and the barrier. There is a basement membrane (*pink* and *yellow-green*) that surrounds the pericytes (*blue*) and astrocytes (*green*) outside the endothelial cells. Astrocytic endfeet are in close proximity to all of these structures. Another basement membrane, which is distinct from that of the endothelial basement membrane (*pink*) and of the layer of astrocytic endfeet (*yellow-green*), is called the glia limitans perivascularis

10.3 Cellular Biology of the BBB

Brain microvascular endothelial cells (BMECs) directly mediate BBB function. The endothelial cells forming the BBB express many tight junction-associated molecules, including occludin, claudin-5, claudin-3, claudin-12, ZO-1, ZO-2, and JAM-A (Hawkins and Davis 2005; Harhaj and Antonetti 2004). These molecules form tight junctions and limit the paracellular permeability in order to maintain the brain microenvironment, thus exerting barrier properties. Occludin is a 60–65 kDa protein with a carboxy (C)-terminal domain that is capable of binding to ZO-1 (Yu et al. 2005). The main function of occludin appears to be in tight junction regulation. In the BBB, the expression of the claudin-3, claudin-5, and, possibly, claudin-12 proteins appears to contribute to the high TEER (Wolburg and Lippoldt 2002). The uptake of essential molecules occurs through specific carrier and transport systems, including the glucose transporter 1 (GLUT1) glucose carrier, and several amino acid carriers (including large neutral amino acid transporter-1 (LAT1), system L for large neutral amino acids) (Begley and Brightman 2003).

Astrocytes are important components of the BBB. Astrocytic endfeet ensheath 99 % of the surface of brain microvessels, from which their endfoot processes are separated only by a thin basal membrane (Hawkins and Davis 2005). There is now strong evidence, particularly from studies in in vitro models, that astrocytes regulate various aspects of BBB physiology via secreted factors, and influence particular BBB features, such as the permeability, leading to tight junction formation and expression in endothelial cells (Alvarez et al. 1812). Astrocytes are able to secrete several growth factors, including transforming growth factor-β (TGF-β), glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF), and angiopoietin 1 (Ang-1), thus playing a major role in BBB maintenance (Dohgu et al. 2005; Igarashi et al. 1999; Reuss et al. 2003).

Pericytes are morphologically, biochemically, and physiologically heterogeneous and they may have distinctive characteristics in different organs (Armulik et al. 2005). Pericytes are localized at the abluminal side of the microvascular endothelium and are completely enveloped by a basement membrane (Shepro and Morel 1993). The extensive pericyte coverage is found around microvessels in organs that have a barrier system, and the pericyte-to-endothelial ratios are 1:1–3 in the brain and approximately 1:10 in the lung (Shepro and Morel 1993; Dalkara et al. 2011). It is not clear why the nervous system requires a larger degree of pericyte coverage than other organs, but one possibility is that brain pericytes contribute to the barrier maturation and maintenance in the BBB. Several in vitro studies demonstrate that pericytes can increase vascular stability and regulate the BBB by secretion of paracrine growth factors including bFGF, TGF-β, Ang-1, VEGF, GDNF, and BDNF (Shimizu et al. 2008, 2011). Pericytes as well as astrocytes are contributors to barrier induction in the BBB.

10.4 Cell Lines for BBB Experiments

Primary cultures of BMECs, pericytes, and astrocytes derived from rat, cow, and pig have been used for in vitro investigation of the physiological roles of BBB (Deli et al. 2005). The use of human BMECs is rare and limited due to the restricted availability of human brain tissue partly due to the difficulty in isolation and culture of primary human BMECs (Bernas et al. 2010). Unfortunately, most primary BMECs lose their specific characteristics in culture within limited passages and rapidly become less useful as in vitro models of the human BBB (Nakagawa et al. 2009). To address these issues, we established the immortalized human brain microvascular endothelial cell line (TY08) and human brain pericytes cell line (Sano et al. 2010). These cell lines were isolated from human brain tissue, which harbored a temperature-sensitive SV40 large T-antigen (tsA58) protein. Human primary astrocytes were also immortalized via transfection by retroviral vectors harboring the tsA58 gene. The gene product of tsA58 is in an active conformation and binds to p53 at 33 °C, thus facilitating the immortalization of the cells, whereas the conformation of the gene product can change, leading to its degradation and the release of p53 when the cells are grown at 37 °C. These cells are thus conditionally immortal, thus retaining BBB-like differentiated characteristics. The TY08 expressed all key tight junctional proteins, such as occludin, claudin-5, ZO-1, and ZO-2, and had low permeability to inulin across monolayers and high expression of influx and efflux transporters (Sano et al. 2010). We demonstrated that the brain pericytes secreted several growth factors, including bFGF, TGF-β, Ang-1, VEGF, GDNF, and BDNF, and that the barrier function in TY08 was increased through the up-regulation of claudin-5 by soluble factors released from brain pericytes (Shimizu et al. 2011). These cell lines might therefore be useful tools for understanding the BBB function under both physiological and pathological conditions.

10.5 BBB Alterations in Neuroinflammation

Because the healthy CNS is tightly sealed by the BBB, the intrusion of pathogenic T cells, as well as humoral factors, including immunoglobulin, into the CNS parenchyma needs to follow BBB impairment. Although elevated levels of soluble adhesion molecules, chemokines, and matrix metalloproteinases (MMPs) in serum and cerebrospinal fluid (CSF) observed in patients with neuroinflammatory diseases such as MS may be indicative of T-cell migration across the BBB (Alvarez et al. 1812; Larochelle et al. 2011), only magnetic resonance imaging (MRI) and pathological examination can be the reliable methods so far to assess whether there are BBB derangements in these neuroinflammatory diseases. Enhancement of the brain parenchyma in the T1-weighted MRI with gadolinium enhancement is occasionally observed in neuroinflammatory diseases, including MS, NMO, or immune-mediated encephalitis, and is interpreted as a hallmark of BBB breakdown (Waubant 2006).

10.6 Blood-to-Brain Leukocyte Trafficking Across the BBB is a Multistep Process

The CNS exhibits strictly controlled inflammatory reactions, in part because the BBB restricts the exchange of inflammatory cells and mediators. Leukocytes have to perform various actions before being allowed to move across the endothelial layer (into the perivascular space) and to subsequently find a way through the glia limitans (into the brain parenchyma). Most studies about the passage of immune cells across the BBB have not been in normal animals, but in models of multiple sclerosis (MS). The most commonly used animal models in the study of MS are those with experimental autoimmune encephalomyelitis (EAE), which is induced by generating a T-cell-mediated autoimmune response against CNS antigens. As shown in MS patients and the EAE animal models, the main routes of entry for leukocytes into the CNS are through postcapillary venules, to which many properties of the BBB extend (Engelhardt and Ransohoff 2005; Engelhardt 2010). Such entry of leukocytes into the brain parenchyma requires the sequential interaction and activation of different signaling and adhesion molecules on the surfaces of both the brain endothelial cells and leukocytes. These steps include the capture of the immune cells by the brain endothelium, the cessation of rolling, firm adhesion to the endothelium, and subsequent passage across the capillary wall of the BBB (Ley et al. 2007) (Fig. 10.2). The details of the five steps concerning transendothelial

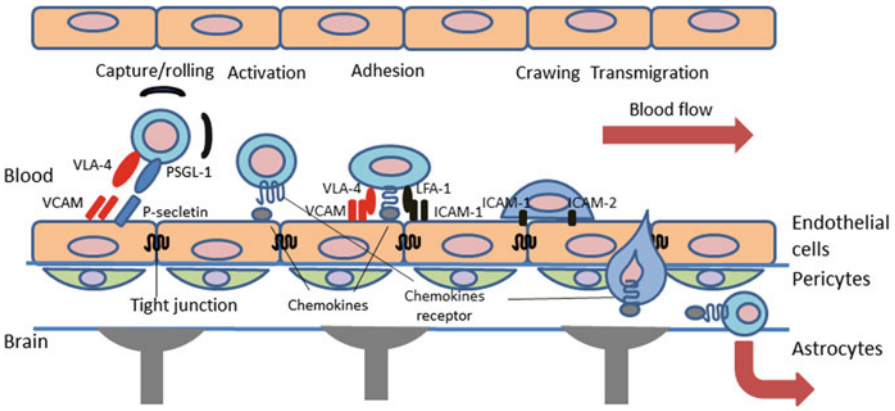

Fig. 10.2 Multistep recruitment of leukocytes into the central nervous system (CNS). Capture/rolling: the binding of P-selectin and PSGL-1 in leukocytes and VCAM1 and VLA-4 in leukocytes allows the leukocytes to slow on endothelial cells. Activation: chemokines on the endothelial cells activate the rolling leukocytes. Adhesion: activated leukocytes upregulate their expression of VLA-4 and LFA-1. Binding to VCAM-1 and ICAM-1 on the endothelial cells allows the activated leukocytes to attach to endothelial cells. Crawling: arrested leukocytes crawl to the preferred sites for migration. Transmigration: crawling leukocytes migrate across the endothelial cells via the paracellular or transcellular pathway. Abluminal chemokines stimulation induces the migration of leukocytes to the CNS across the glia limitans

leukocyte migration across the BBB are described below (Fig. 10.2). (1) Rolling—This first step involves an initial, relatively weak interaction between the leukocytes and endothelium that occurs through two mechanisms in the CNS during EAE. The first mechanism requires the expression of selectins on the vascular surface, which can then bind to the carbohydrate ligand, PSGL-1, on the lymphocyte surface (Kerfoot and Kubes 2002). The second mechanism requires the binding of α4-integrins expressed on the lymphocyte surface to endothelial VCAM-1, which can also mediate the initial interaction between the activated leukocytes and brain vessels (Kerfoot et al. 2006; Engelhardt 2008). This results in lymphocyte "rolling" across the vessel. (2) Activation—The second step involves the activation of integrins on the leukocyte surface (Engelhardt 2008). This occurs when chemokines bind their receptors, which results in G-protein signaling within the leukocyte. The activation of G-protein signaling pathways then causes conformational changes in surface-expressed leukocyte integrins (Ward and Marelli-Berg 2009). The result is an increased affinity and avidity of integrins for their cell adhesion molecule receptors on the endothelial cell surface. (3) Arrest—The third process involves firm adhesion of leukocytes to the vasculature. In EAE, the interactions between ICAM-1 and LFA-1 or VCAM-1 and α4 integrins result in firm adhesion between leukocytes and brain vessels (Greenwood et al. 2011; Correale and Villa 2007). (4) Crawling—This fourth step involves leukocytes seeking preferred sites of transmigration across the endothelium, with a probable involvement of endothelial ICAM-1 and ICAM-2 (Engelhardt and Ransohoff 2005). (5) Transmigration—The final step involves the migration of leukocytes across the CNS endothelial cells into the perivascular space in a LFA-1/ICAM-1- and ICAM-2-dependent manner, leaving tight junctions morphologically intact (Lyck et al. 2003). After penetrating the endothelial monolayer, T cells have to migrate across the endothelial basement membrane and encounter antigen-presenting cells in the CSF drained from the perivascular space. Entry into the CNS parenchyma requires local digestion of the glia limitans perivascularis, composed of a second basement membrane and astrocytic endfeet. Currently, there are no suitable in vitro BBB models for analyzing this transmigration of cells. Therefore, this process remains incompletely understood, including which chemokines and chemokine receptors are critical for the process.

After considering the steps outlined above that are involved in leukocyte trafficking across the BBB in EAE, it was clear that lymphocyte-expressed α4 integrin plays an important role (Miller et al. 2003). This conclusion led to the development of drugs to block α4 integrin on the lymphocyte surface, in the hope of preventing the pathological entry of leukocytes into the brain observed in MS patients. It was initially shown that a monoclonal antibody blocking α4 integrin could prevent leukocyte trafficking across the BBB in vitro, and could also reverse the disease in a murine model of chronic EAE (Coisne et al. 2007). A humanized form of this antibody, natalizumab, was developed based on its demonstrated efficacy in treating EAE. Following success in clinical trials, natalizumab was approved by the FDA in 2004 for the treatment of relapsing remitting MS (Engelhardt and Kappos 2008). As of May 3, 2012, approximately 99,600 MS patients have received natalizumab worldwide (Biogen Idec, 2012). The magnitude of both the clinical and radiological efficacy of this agent had not previously been documented with any other

disease-modifying strategy (Miller et al. 2007). However, the use of natalizumab for the treatment of MS patients has been associated with the development of progressive multifocal leukoencephalopathy (PML), with 264 PML cases reported as of Aug 15, 2012 (Biogen Idec, 2012). Further knowledge concerning the pathological process of blood-to-brain leukocyte trafficking across the BBB may be able to provide information that would be helpful for the development of the novel, more highly selective and specific, immunomodulatory therapies.

10.7 Role of Soluble Immune Mediators in the Disruption of the BBB

Neuroinflammatory disorders, including MS and EAE, are immune CNS-mediated disorders characterized by immune cell infiltration and up-regulation of proinflammatory cytokines and chemokines such as IL-1β, IL-17, IL-22, IFN-γ, and CCL2, among others (Kebir et al. 2009; Mahad et al. 2003; Badovinac et al. 1998). Most of these immune mediators are released by leukocytes during transmigration, and they can also affect the integrity of the BBB within the CNS by promoting and expanding the immune cell activation. However, some cytokines and MMPs produced by peripheral blood mononuclear cells can directly disrupt components of the BBB or act on receptors expressed by BBB comprising endothelial cells. Some in vivo and in vitro studies have demonstrated that IL-1β indirectly destabilizes the BBB by inducing the production of MMP-9 (Bolton et al. 1998; Sozen et al. 2009), which degrades tight junction proteins, including occludin, ZO-1, and claudin-5 (Asahi et al. 2001; Bauer et al. 2010). IL-17 and IL-22 have also been shown to destabilize the BBB by inducing alterations in the BBB permeability that coincide with decreased expression of occludin and ZO-1 (Kebir et al. 2007). TNF-α can also affect the barrier permeability by up-regulating the expression of NF-κB, which induces the transcription of myosin light chain kinase (MLCK), a factor known to induce the internalization of TJ proteins (Nusrat et al. 2000). IFN-γ induces actin restructuration and decreases the protein levels and changes the subcellular localization of ZO-1 in the BBB (Youakim and Ahdieh 1999; Blum et al. 1997). VEGF was able to induce BBB impairment by increasing the phosphorylation of occludin and ZO-1, or by decreasing the expression of claudin-5 (Argaw et al. 2009). Chemokines can also affect the stability of the BBB by modifying the phosphorylation state of the proteins in TJs. For example, the chemokine CCL2 (formerly known as MCP-1) affects the permeability of the BBB by inducing the phosphorylation of occludin, ZO-1, ZO-2, and claudin-5 (Stamatovic et al. 2006, 2009).

In contrast to the detrimental effect of proinflammatory cytokines, other soluble immune mediators and growth factors, such as IFN-β, IL-25, bFGF, and GDNF, promote BBB integrity and impermeability. IFN-β is a type I IFN with immunomodulatory effects that has been used for the treatment of MS for more than 15 years (Hohlfeld and Wekerle 2004; Yong et al. 1998). Several studies have shown that IFN-β increases the transendothelial resistance, reduces permeability, stabilizes the barrier function (Kraus et al. 2004, 2008), and induces cell surface shedding of

adhesion molecules on BMECs (Calabresi et al. 2001; Graber et al. 2005) in vitro and in vivo. In addition, IL-25 is a member of the IL-17 family and is expressed in BMECs. The stimulation of BMECs with inflammatory cytokines such as TNF-α, IL-17, IFN-γ, and IL-1β reduces the IL-25 expression, a pattern also observed in active MS lesions and in EAE (Sonobe et al. 2009). IL-25 has a protective effect on the BBB, because the expression of claudin-5, JAM-A, and occludin which is decreased by treatment with TNF-α can be reversed by treatment with IL-25 (Sonobe et al. 2009). In addition, bFGF and GDNF are also candidate agents for increasing the BBB properties. GDNF is a member of the TGF-β superfamily, and its neurotrophic action is mediated by a unique multicomponent receptor system consisting of the GDNF-family of receptors (GFRa1–4) (Sariola and Saarma 2003). We have previously demonstrated that brain pericytes produce bFGF or GDNF, and that those secreted from brain pericytes increase the transendothelial resistance, reduce permeability, and increase the expression of claudin-5 in the BBB (Shimizu et al. 2008, 2012b). Taken together, these studies provide strong evidence that numerous cytokines or growth factors play important roles in regulating multiple aspects of TJ proteins and, ultimately, BBB permeability.

10.8 Bacterial Translocation Across the Blood–Brain Barrier

Several bacterial species have been shown to be common causes of CNS infections. *S. pneumoniae*, *N. meningitidis*, and *H. influenzae* type b remain the most common causes of meningitis in adults, while *S. pneumonia* and *N. meningitidis* are the most prominent pathogens that cause meningitis in children (Hart and Thomson 2006). How these bacteria cross the BBB and cause meningitis is still incompletely understood (Fig. 10.3). Recent studies have shown that successful crossing of the BBB by circulating bacteria requires: (1) a high degree of bacteremia; (2) bacteria binding to

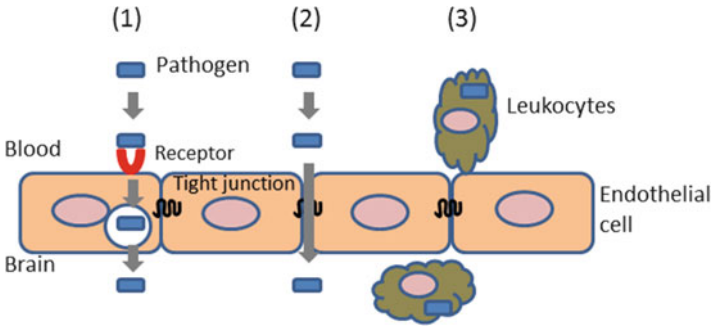

Fig. 10.3 The cellular pathways through which pathogens cross the BBB. (1) Transcellular penetration via a receptor-mediated mechanism without evidence of tight junction disruption. (2) Paracellular entry between cells with or without disruption of tight junctions. (3) The Trojan horse mechanism (transmigration with infected leukocytes)

and invading the BMECs (Fig. 10.3); (3) rearrangements of the BMEC actin cytoskeleton; and (4) traversal of the BBB by live bacteria.

Studies in humans and experimental animals have suggested a relationship between the level of bacteremia and the development of meningitis due to *Escherichia coli* (Kim 2002), group B *Streptococcus* (Ferrieri et al. 1980), and *S. pneumoniae*. However, while a high level of bacteremia is necessary, it is not sufficient for the development of meningitis, and binding to and invasion of BMECs is a prerequisite for bacteria penetration of the BBB in vivo (Kim 2006). Recent studies have suggested that bacteria invade human BMECs via ligand–receptor interactions (Fig. 10.3). For example, *S. pneumoniae*, *N. meningitidis*, and *H. influenza* adhere to the BMECs via the 37/67-kDa laminin receptor (LR), which binds *pneumococcal* CbpA, *meningococcal* PilQ and PorA, and OmpP2 of *H. influenza* (Orihuela et al. 2009), thus suggesting that these pathogens use the same strategy for targeting a BBB receptor. The invasion of *S. pneumonia* into BMECs is also promoted by cytokine activation, which increases the amount of surface-expressed platelet-activating factor receptor (PAFr) on the endothelial membrane (Cundell et al. 1995; Swords et al. 2001). The binding of bacterial phosphorylcholine to PAFr leads to the activation of β-arrestin-mediated endocytosis of the bacteria into BMECs (Radin et al. 2005). In addition, the outer membrane protein, Opc, and the pili type IV proteins have an important role in the *meningococcal* invasion of the BBB (Pron et al. 1997; Nassif 2000). Opc binds to fibronectin and vitronectin, which anchor the bacterium to the endothelial α5β1-integrin (the fibronectin receptor) and αVβ3-integrin (the vitronectin receptor) (Unkmeir et al. 2002; Sa et al. 2010). Pili type IV proteins interact with CD46, a human cell surface protein involved in the regulation of complement activation (Kallstrom et al. 1997; Kirchner et al. 2005). The involvement of Pili in the adhesion to BMECs contributes to the formation of microvilli-like cell membrane protrusions underneath bacterial colonies, which help the bacteria to form microcolonies on the BMEC surface and to destabilize cellular junctions (Mairey et al. 2006; Coureuil et al. 2009).

E. coli invades the BMECs by interacting various bacterial proteins with endothelial receptors, including type 1 fimbriae (FimH), outer membrane protein A (OmpA), Ibe proteins (IbeA and IbeB), YijP, AslA, and cytotoxic necrotizing factor 1 (CNF-1). IbeA interacts with the specific receptor vimentin, which causes the activation of focal adhesion kinase (FAK) and paxillin leading to cytoskeletal rearrangements and thus allowing *E. coli* to cross the endothelial monolayer (Chi et al. 2010). IbeB and OmpA interact with different receptors on BMECs, although the effects of these interactions are additive. OmpA interacts with glycoprotein gp96 of BMECs via *N*-glucosamine epitopes and leads to the FAK-dependent invasion of bacteria (Khan et al. 2002; Wang and Kim 2002).

Listeria monocytogenes invasion of BMECs has been shown to be mediated by internalin B (Greiffenberg et al. 1998). Two receptors for internalin B have been identified; gC1q-R (the receptor for the globular head of the complement component, C1q) and Met tyrosine kinase (Braun et al. 2000; Shen et al. 2000). Host–pathogen protein interactions during the penetration and invasion of the BBB are summarized in Table 10.1. Further studies are needed to understand the contribution

Table 10.1 Host–pathogen protein interactions during the penetration and invasion of the BBB

Pathogen	Possible route of pathogen translocation	Ligand (pathogen)	Ligand (host)	Reference
Streptococcus pnemoniae	Transcellular	Phosphorylcholine	Platelet-activating factor receptor	Ring et al. (1998)
Neisseria meningitidis	Transcellular	Opc (outer membrane protein)	Fibronectin (anchoring to the α5β1 receptor)	Unkmeir et al. (2002)
		Pili (Pil A and PilB)	CD46	Kallstrom et al. (1997)
Escherichia coli	Transcellular	CNF1 (cytotoxic necrotizing factor 1)	37LRP	Chung et al. (2003)
		FimH	CD48	Prasadarao et al. (2003)
		OmpA	Gp96	Wang and Kim (2002)
Listeria monocytogenes	Transcellular	Internalin B	qC1q-R (receptor for the globular head of the complement component C1q)	Greiffenberg et al. (1998)
	Trojan horse mechanism	Vip	Met receptor tyrosine kinase gp96 (glycoprotein 96)	Shen et al. (2000)
Group B *Streptococci*	Transcellular	Glycosyltransferase	ND	Doran et al. (2005)
Candida albicans	Transcellular	Enolase	Plasmin	Jong et al. (2001, 2003)
Cryptococcus neoformans	Transcellular	Hyaluronic acid	CD44	Chang et al. (2004)
HIV	Trojan horse mechanism	Tat protein	ND	Rappaport et al. (1999)
	Transcellular	ND	CD4 and CCR5	András et al. (2003)
		ND	ND	Kramer-Hämmerle et al. (2005)

of these ligand–receptor interactions to BMEC invasion and the traversal of the BBB by meningitis-causing bacteria.

Previous studies revealed that internalized bacteria are found within membrane-bounded vacuoles of BMECs and transmigrate without multiplication and are protected from fusion with lysosomes (Kim 2003, 2006). Electron microscopy studies have shown that *E. coli* and group B *Streptococcus*, as well as *M. tuberculosis* invasion, are associated with microvilli-like protrusions at the entry site on the surface of human BMECs (Nizet et al. 1997), suggesting that there is a rearrangement of the host cell actin cytoskeleton. Actin cytoskeleton rearrangements are necessary for BMEC invasion by meningitis-causing bacteria, but the signaling mechanisms involved in actin differ among the meningitis-causing bacterial species. These include FAK, paxillin, phosphatidylinositol 3-kinase (PI3K), Src kinase, Rho GTPases, cytosolic phospholipase A2 (cPLA2) and ezrin, radixin, and moesin (ERM) (Reddy et al. 2000; Das et al. 2001; Khan et al. 2002; Kim et al. 2005). For instance, *E. coli* K1 invasion of human BMECs depends on the activation of FAK, Src kinase, PI3K, and cPLA2. In contrast, group B *streptococcal* invasion of human BMECs was found to be independent of Src kinase and cPLA2 activation and *L. monocytogenes* invasion of human BMECs was independent of FAK and cPLA2 activation.

Another crucial factor for the development of meningitis is the ability of pathogens to cross the BBB as live bacteria. No free bacteria are found in the cytoplasm of BMECs (Kim 2003), and transcytosis of BMECs by meningitis-causing bacteria is shown to occur without any change in the integrity of BMEC monolayers (Nizet et al. 1997; Ring et al. 1998), suggesting that meningitis-causing bacteria, such as *E. coli* and group B *Streptococcus*, traverse the BBB using a transcellular mechanism involving ligand–receptor interactions.

10.9 Transloaction Across the Blood–Brain Barrier by Fungi and Parasites

Several fungi have been shown to cause CNS infections in humans. *Cryptococcus neoformans* (Gordon et al. 2000) are the most frequently isolated yeasts from patients with CNS involvement. In the case of *Cryptococcus neoformans*, brain invasion does not require the recruitment of host inflammatory cells (Chrétien et al. 2002; Chang et al. 2004), which eliminates the possibility that these penetrate into the brain using a Trojan horse mechanism via transmigration of infected phagocytes (Fig. 10.3). *Cryptococcus neoformans* can traverse BMECs without any obvious change in their integrity. Transmission and scanning electron microscopy studies have revealed that *C. neoformans* induces the formation of microvilli-like protrusions to initiate entry into BMECs. These findings indicate that *C. neoformans* uses a transcellular mechanism for entry into the CNS (Chang et al. 2004).

Malaria is another major public health problem in many parts of the tropical world. Cerebral malaria is the most severe pathology caused by the malaria parasite, *Plasmodium falciparum* (Schofield and Grau 2005). The pathogenic mechanisms

leading to cerebral malaria are still poorly defined, as studies have been hampered by limited access to human tissues. Nevertheless, the histopathology of postmortem human tissues and mouse models of cerebral malaria have indicated the involvement of the BBB in cerebral malaria (Rénia et al. 2012).

In cases of cerebral malaria, the endothelial barrier is often compromised by interactions with parasitized red blood cells (PRBCs), as well as innate and adaptive immune responses. One of the early steps in cerebral malaria is the sequestration of PRBCs in the brain microvasculature. *Plasmodium falciparum* erythrocyte membrane protein (PfEMP-1) mediates endothelial binding and affects the barrier integrity. PfEMP-1 binds to ICAM-1, CD36, chondroitin sulfate, and other trypsin-sensitive binding determinants (Chang et al. 2004; Craig and Scherf 2001; Tripathi et al. 2007). ICAM-1 ligation of the endothelium was demonstrated to cause stress fiber formation through cytoskeletal rearrangements (Etienne-Manneville et al. 2000), providing another potential mechanism by which PRBC adherence alters the vascular permeability. The adherence of *P. falciparum* PRBCs also induces the apoptosis of endothelial cells via Rho kinase signal transduction in human endothelial lines (Pino et al. 2003; Taoufiq et al. 2008). PRBCs thus amplify the immune response and utilize multiple pathways to begin the process of vascular breakdown during cerebral malaria.

10.10 Mechanisms of the Blood–Brain Barrier Disruption by HIV-1 Infection

NeuroAIDS is becoming a major health problem among AIDS patients and long-term HIV survivors. The two main CNS complications in individuals with HIV are encephalitis and dementia, which are characterized by leukocyte infiltration into the CNS, microglial activation, aberrant chemokine expression, BBB disruption, and eventual damage and/or loss of neurons (McArthur 2004; Gendelman 2005). One of the major mediators of neuroAIDS is the transmigration of HIV-infected leukocytes across the BBB into the CNS. The molecular mechanisms by which HIV enters the CNS and contributes to both acute and chronic inflammatory processes are still unclear. HIV can access the CNS due to increased trafficking of HIV-infected CD4+ T cells or circulating monocytes (Ivey et al. 2009). Inflammatory responses within the brain vasculature also have critical significance for the development of the neuropathology associated with neuroAIDS, because the lymphocytes and monocytes/macrophages can gain access to the CNS by increased migration through the BBB under inflammatory conditions, although leukocyte trafficking toward the CNS is very low in healthy individuals (Tardieu 1999). For instance, the viral Tat protein, which is secreted by infected cells, induces the expression of adhesion molecules on endothelial cells and chemokine secretion by astrocytes and microglial cells, possibly enhancing leukocyte trafficking toward the CNS (Weeks et al. 1995; Wu et al. 2000). In addition, the viral protein gp120, which can be detected in the brain of

HIV-1-infected patients, triggers the release of MCP-1 (CCL2), a potent chemoattractant for monocytes (Jones et al. 2000; Fantuzzi et al. 2003). Interestingly, an increased risk of HIV-associated dementia (HAD) has been shown to be linked to a mutant MCP-1 allele (Gonzalez et al. 2002). Finally, the proinflammatory cytokine levels are elevated in the CSF and brain parenchyma of HAD patients. These can alter the BBB integrity, and increase the expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectins on endothelial cells, thus facilitating leukocyte adhesion, rolling, and estravasation into the brain (Yadav and Collman 2009).

This recruitment allows for subsequent infection of these resident cells and the spread of HIV within the CNS. HIV infects perivascular macrophages and microglia productively, while astrocyte infection is restricted, resulting in the formation of viral reservoirs within CNS cells in which replication competent viral genomes persist in a stable state (Lavi et al. 1998). There is no evidence of direct HIV infection of neurons; therefore, the neuronal cell damage and death that occurs in HAD must be mediated by the production and release of neurotoxic factors by other infected or uninfected cells within the CNS. In fact, neuronal dysfunction correlates more closely with inflammation and activated monocytes/microglia than with the viral load (Sevigny et al. 2004).

10.11 Inflammatory Response of the Blood–Brain Barrier in Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by a gradual loss of memory, orientation, judgment, and reasoning (Roses 1996; Zlokovic 2008). The inflammation and BBB dysfunction in the adult brain are considered to play an important role in the pathogenesis of AD, because inflammatory reactions around the cerebral microvessels are frequently observed in the brain sections from AD patients (D'Andrea 2003; Sardi et al. 2011; Stewart et al. 1992; Ujiie et al. 2003; Stolp and Dziegielewska 2009). The primary neuropathology of AD patients is characterized by the extracellular deposition of amyloid β (Aβ) peptide in senile plaques, and intracellular neurofibrillary tangles composed of phosphorylated tau, within the brain (Trojanowski et al. 1995; Wenk 2003). Aβ is a series of proteolytic by-products of the amyloid-β precursor protein (AβPP) that vary in length from 39 to 43 amino acids. According to the "amyloid hypothesis," the increased Aβ in the brain of AD patients induces the hyperphosphorylation of tau and synaptic dysfunction and leads to neuronal cell death (Deane et al. 2009). The inflammation in AD is generally considered to be a consequence of Aβ accumulation, and the number of perivascular macrophages increases and hypertrophy of astrocytes and microglia is observed in brain section of AD patients (Minagar et al. 2002). Several proinflammatory substances could be involved in the development of AD; IL-1β is expressed by activated microglia surrounding plaques, and seems to promote the production and metabolism of amyloid precursor protein (APP),

increasing the amyloid deposition and plaque formation (Griffin et al. 1998). Increased levels of IL-6 and MCP-1 in the plasma and CSF of AD patients may also be good candidates as biomarkers for monitoring the inflammation process in AD (Sun et al. 2003). The VEGF concentration is higher in the cortex of affected brains, thus causing increased vascular permeability, neuronal loss, and amyloid deposition (Sardi et al. 2011). Some studies have proposed a protective role of anti-inflammatory drugs, including NSAIDs (Gupta and Pansari 2003; Zotova et al. 2010), although trials in AD patients did not lead to encouraging results, in spite of good outcomes in a murine model (Sardi et al. 2011).

The dysfunction of the BBB is implicated in the pathogenesis of AD (Zlokovic 2011). The first report about the BBB disruption in the AD brain described the observation that IgG and complement proteins aggregated near plaques, thus indicating focal or subtle changes in BBB permeability (Alafuzoff et al. 1987). Subsequent studies confirmed the notion that the BBB disruption is a pathological feature of AD; albumin concentrations are enhanced in the CSF of patients with AD at the early onset of AD, apparently resulting from an increased permeability of the BBB (Algotsson and Winblad 2007; Bowman et al. 2007). Subtle abnormalities in endothelial tight junctions in brain biopsies from patients with AD were also observed, and suggested that they may be responsible for the damage of the BBB (Zipser et al. 2007; Farkas and Luiten 2001; Bailey et al. 2004). The reduced staining of endothelial markers, CD34 and CD31, observed in AD brains suggested that there was an extensive degeneration of the endothelium during the disease progression (Kalaria and Hedera 1995). The loss of integrity of the BBB can cause abolition of the immunological privilege of the CNS and lead to a penetration of circulating Aβ in the brain, where it can bind astrocytes, starting a degenerative and inflammatory process. Furthermore, the disruption of the BBB also allows T lymphocyte infiltration, and the number of T lymphocytes is increased, especially in the hippocampus and temporal cortex, of the brains of AD patients (Sardi et al. 2011). Currently, BBB dysfunction is considered to be one of the earliest pathological events underlying the development of AD.

It is considered that significant amounts of Aβ are produced by the peripheral sources, including the blood, platelets, and skeletal muscle (Tang et al. 2006; Kuo et al. 1999, 2000). The BBB dysfunction could affect AD pathogenesis by decreasing the Aβ clearance and increasing the Aβ production. The clearance of Aβ is controlled in part by an intact and functional BBB that transports soluble Aβ from the blood to the brain, mainly via the receptor for advanced glycation end-products (RAGE) and from the brain to the blood via the low-density lipoprotein receptor-related protein (LRP-1) (Deane et al. 2003, 2004), and depends on the apolipoprotein E (ApoE) isoform, the Aβ chaperone proteins (Deane et al. 2008). Thus, altered BBB function could cause the accumulation of Aβ within the brain due to inadequate Aβ efflux induced by the decreased expression of LRP-1 and accelerated Aβ influx via the increased expression of RAGE (Zlokovic 2008; Bell and Zlokovic 2009). The ApoE4 allele, which is considered to be genetic risk factor for sporadic

AD, slows Aβ clearance from brain in an isoform-specific manner (Deane et al. 2008). In addition, Aβ can be produced locally in and around the BBB; AβPP is expressed in endothelial cells and pericytes, and Aβ production has been demonstrated in isolated brain microvessels of AD patients (Kalaria et al. 1996; Natté et al. 1999). Perivascular inflammatory reactions during AD can cause Aβ production in the BBB. Therefore, the impaired clearance of Aβ from the brain and increased Aβ production by the cells of the neurovascular unit may lead to its accumulation in blood vessels and in the brain parenchyma.

10.12 The Blood–Brain Barrier in Parkinson's Diseases

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disorder characterized by abnormal motor symptoms, including tremors, bradykinesia, rigidity, and postural instability (Obeso et al. 2000). The pathological feature of PD is a loss of dopaminergic neurons, mainly in the substantia nigra pars compacta (Obeso et al. 2000). The cellular and molecular mechanisms underlying the pathogenesis of PD are currently unclear, but it has increasingly been considered to be linked to neuroinflammation and oxidative stress (Stolp and Dziegielewska 2009).

Similar to AD, BBB disruption may play an important role in the pathogenesis of PD. Rite et al. found that increased permeability in the BBB induced by the injection of VEGF into the substantia nigra of experimental animals could induce a subsequent loss of dopaminergic neurons, thus suggesting a pathogenic link between BBB disruption and the degeneration of dopaminergic neurons (Rite et al. 2007). Pathological alterations in endothelial cells within the substantial nigra are also found in the brain sections of PD patients (Faucheux et al. 1999). The results of this pathological study are ambiguous when examined alone, because it was unclear how to interpret the reported increases in endothelial nuclei within the vasculature (Faucheux et al. 1999). However, combined with the results of the recent experimental study of Rite et al. (Rite et al. 2007), it can be hypothesized that increased angiogenesis within the substantia nigra induced by VEGF may result in altered function of the BBB, and may play an important role in the pathogenesis of PD (Stolp and Dziegielewska 2009). Moreover, there is increasing interest in inflammation as a pathogenic mechanism in PD, and a mechanism involving alterations in BBB properties has been suggested by many different experimental methods (Faucheux et al. 1999; Whitton 2007). Kortekaas et al. provided the first evidence of increased dysfunction of the BBB in PD patients using a PET study (Kortekaas et al. 2005). They showed a significantly increased uptake of ^{11}C-verapamil in the midbrain of PD patients relative to controls, which they suggested was due to a decreased function of P-glycoprotein (Kortekaas et al. 2005). It was also speculated that an increased passage of metals, such as iron or manganese, through the BBB may be involved in the pathogenesis of PD (Toescu 2005).

10.13 Conclusion

BBB dysfunction, often referred to as "BBB opening," has long been known to constitute a key feature of the progression of several CNS diseases, as a consequence of neuroinflammation. It is likely that the recently acquired knowledge about TJ regulation and leukocyte transmigration at the BBB will pave the way to novel therapeutic strategies for neuroinflammatory CNS diseases. Just like anti-VLA-4 antibody treatment has recently appeared as a beneficial approach to limit leukocyte infiltration to the brain in MS, the identification of various active transport systems or other membrane proteins at the BBB may allow for the development of new brain-targeted strategies to efficiently deliver drug to the CNS. Finally, obtaining a complete understanding of the microbial–human BMEC interactions that are involved in translocation of the BBB will provide the first step in the development of novel strategies for the prevention and treatment of CNS infections.

Conflict of Interest Fumitaka Shimizu declares that he has no conflict of interest. Takashi Kanda declares that he has no conflict of interest.

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About the Editors

Dr. Kazuhiro Ikenaka is Professor at the National Institute for Physiological Sciences in Okazaki, Japan since 1992. He graduated from Osaka University, Faculty of Science majoring in Biochemistry. His current research interest is in glial biology. He is studying the development of glial cells (oligodendrocyte and astrocyte) and their function in the adult brain. He is also interested in pathophysiology of demyelinating diseases, especially multiple sclerosis. He developed a sensitive way to analyze N-linked sugar chain structure and is willing to find a new function of N-glycans in glial cells. He is currently an Associate Editor of *Journal of Neuroscience Research*, *Neurochemical Research*, and *Developmental Neuroscience*, and Senior Editor for *ASN Neuro*.

Akio Suzumura, **M.D.**, **Ph.D.**, graduated from Gifu University, School of Medicine in 1975. After getting Ph.D. degree at Department of Neurology, Nagoya University, School of Medicine in 1983, he had worked for Department of Neurology, University of Pennsylvania as a postdoctoral fellow until 1987. There, he established the methods for isolation of oligodendrocytes and microglia and had studied immunological functions of glial cells. He became an assistant professor in Department of Neurology, Fujita Health University in 1987, and an associate professor in Department of Neurology, Nara Medical University in 1995. He became Professor and Chairman in Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University in 2001, and is currently a vice president of the Institute. He had been a president of Japanese Society of Neuroimmunology (2008–2012). He is currently an Editor-in-Chief of *Clinical and Experimental Neuroimmunology*, an Academic Editor of *PLoS ONE*. His current interest is neuron–glia interactions in both physiological and pathological conditions.

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