Phillip K. Peterson · Michal Toborek
Editors

Neuroinflammation and Neurodegeneration

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The editors dedicate this book to their wives, children, and mentors.

Preface

> As long as our brain is a mystery, the universe, the reflection of the structure of the brain, will also be a mystery. —Santiago Ramón y Cajal

Over a century has passed since Santiago Ramón y Cajal, the father of modern neuroscience, was awarded the Nobel Prize for Physiology or Medicine. Yet the brain, like the universe, remains a mystery. But as is true in astrophysics, discoveries in neuroscience continue to astound us. We stand in awe of both these outer and inner worlds. Designation of the 1990s as "The Decade of the Brain" by President George H.W. Bush and the recent launching of the BRAIN (Brain Research through Advancing Innovative Technologies) Initiative by President Barack Obama reflect a universal curiosity about the workings of the nervous system.

The story behind this book, however, begins in the early years of the 1980s—what one might consider the end of the Dark Ages of neuroimmunology. At that time, two highly interdisciplinary scientific fields—psychoneuroimmunology and neuroimmunopharmacology—were just beginning to emerge. In contrast to the prevailing view that the nervous system and the immune system functioned more or less autonomously, research in these fields clearly demonstrated that these two systems are connected. The nervous system, via stress-responsive hormones and neuropeptides, impacted on the immune system. And cells and mediators of the immune system could profoundly affect the brain.

Fast forward to the chapters in this book: state-of-the-art reviews of the neuroimmunological mechanisms underlying some of the most crippling and challenging diseases of the twenty-first century, including Alzheimer's disease, Parkinson's disease, multiple sclerosis, Huntington's disease, stroke, cerebral malaria, neuroAIDS, meningitis, encephalitis, and substance abuse. The reader will find that the authors of these chapters and of the introductory chapters on mechanisms of brain defense and neuropathogenesis are talking the same language. This language—activated brain endothelial cells, microglia, and astrocytes and their mediators (cytokines/chemokines and free radicals)—did not exist in the Dark Ages. In that unenlightened era, the brain was regarded simply as an "immunologically privileged" organ.

Now, as you will read, cells of the peripheral immune system (neutrophils, monocytes, and T and B lymphocytes) are not only known to provide critical defense of the nervous system but also implicated in neuroinflammation-induced neurodegeneration.

A concept shared by many of the contributors to this book is that the healthful relationship of the cells of the nervous system and immune system is harmonious. It is when the yin-yang balance is tipped that neurodegenerative processes ensue. Having worked in the field of neuroimmunopharmacology, both before and after the Dark Ages, our main purpose in pulling this book together was to foster communication between neuroscientists, immunologists, and pharmacologists who are dedicated to discovering more about the yin-yang relationship of the nervous and immune systems. But our ultimate goal, which is shared by all the contributors, is to find better treatments and prevention strategies for the diseases highlighted in the book. Several chapters deal with such innovative approaches.

The number of stars in the universe is mind-boggling—current estimate of about one septillion (10^{24}). But so too with the brain, which contains just shy of 100 billion neurons and ten times as many glial cells (totaling over one trillion cells). And when one considers an estimated 100 trillion synapses of neurons and a countless number of neuronal and glial cell receptors and signals, the brain is an incomprehensible organ. While enormous scientific progress has been made in the past quarter century, we remain at the threshold of understanding of how the brain functions and of how the immune system affects both neurophysiological and neuropathological processes. We hope that the readers of this book, like the editors, will share the same sense of awe inspired by the authors of the chapters in this book.

Somewhere, something is waiting to be known. —Carl Sagan

Minneapolis, MN, USA — Phillip K. Peterson
Miami, FL, USA — Michal Toborek

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Part I
Mechanisms of Brain Defense and Neuropathogenesis

The Blood-Brain Barrier

Victor Castro and Michal Toborek

Abstract The blood-brain barrier (BBB) is the anatomophysiological unit that interfaces between the blood and the brain. It is composed of brain vascular endothelial cells and their surrounding astrocytes and pericytes. These cells interface with neurons to form a functional unit that regulates blood flow in the brain and the traffic of substances between blood and brain parenchyma. The proper function of the BBB requires specialized roles for each of the cell types that compose it; thus, the endothelial cells form a proper biological barrier by expressing tight junctions (TJ) that seal the intercellular space while forming paracellular ion pores. The expression of TJ brings an additional benefit to the endothelial cells as they are determinants of membrane polarization; the resulting cell polarity is crucial for the proper expression of membrane transporters and ion channels responsible for the transcellular exchange of substances across the endothelium. The physiological properties of endothelial cells, however, are regulated by their interaction with astrocytes and pericytes that in turn interact with each other and nearby neurons. This chapter explores the cellular structure of the blood-brain barrier and provides an introduction to the molecular characteristics of tight junctions and electrophysiological properties of the brain vascular endothelium.

Keywords Blood-brain barrier • Microvasculature • Endothelium • Astrocytes • Pericytes • Transporting phenotype • Tight junctions

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1 Introduction

The blood-brain barrier (BBB) is a paramount determinant of brain homeostasis. It is a modulated anatomophysiological interface which separates and controls substance exchange between the blood and the brain parenchyma. Its discovery dates to the late nineteenth century, when Paul Ehrlich observed that if aniline-based dyes were injected into the vasculature of a living animal, most tissues would be readily stained, but not the brain or the spinal cord [1]. In 1898, Max H. Lewandowsky demonstrated that neurotoxins were able to affect brain functions if administered directly into the brain, but not if delivered through the vasculature [2]. Subsequently, in 1913, Edwin Goldman showed that the central nervous system (CNS) could be stained if the dyes were injected directly into the cerebrospinal fluid instead of systemic circulation; however, this staining was restricted to the brain, and not found in other organs [3], suggesting that the aniline compounds were not admitted into the circulating blood flow. This showed the existence of compartmentalization between the brain and the blood, and since no obvious separating membrane was found, it was suggested that the barrier resided directly in the bran microvessels. Later, with the introduction of electron microscopy, the barrier function was correlated to endothelial cells of brain capillaries.

2 Brain Vascular Endothelium

Epithelia are biological barriers that separate and maintain the physicochemical homeostasis between two biological compartments. They constitute a membrane formed by one or more layers of epithelial cells that adhere and communicate with each other, interact with the extracellular matrix, and rest on a basal lamina of connective tissue. To maintain homeostasis, epithelia are subspecialized to fulfill specific needs depending on their topological localization. Nevertheless, two features are common to all epithelial tissues regardless of their specialization: they are capable of vectorial transepithelial transport and they regulate the intercellular ionic flux (Fig. 1).

Endothelia are a subset of epithelia which form the interior lining of the heart, blood and lymph vessels, capillaries, and the serous cavities of the body. Endothelial cells are distinguished from epithelial cells by having different protein expression and molecular behavior patterns. However, similar to epithelia, endothelial cells are polarized, specialize in molecular transport, and regulate the intercellular flux of substances. In the circulatory system, the vascular endothelium ensures laminar blood flow, interacts with blood cells and plasma molecules, secretes hormones, expresses surface immunological recognition molecules, prevents thrombosis, and modulates the passage of immunological cells, water, and substances between the blood and the underlying tissues. In specific tissues (e.g., hepatic sinusoids and the

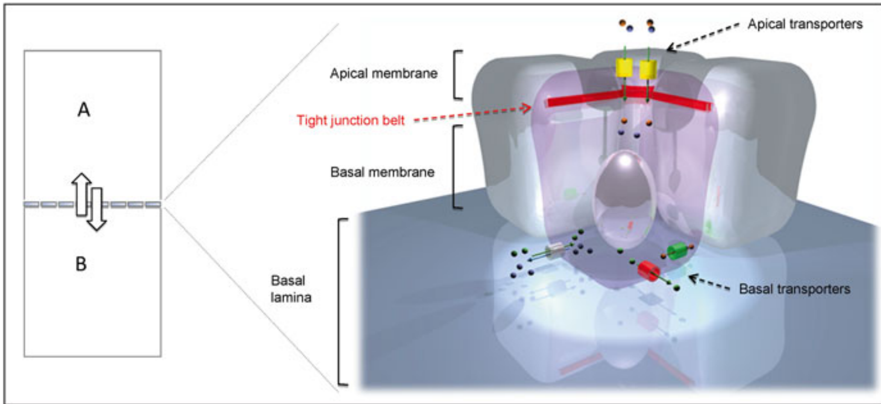

Fig. 1 Epithelia and endothelia are biological membranes that separate two biological compartments and are responsible for maintaining the physicochemical homeostasis between them. These two compartments are generally known as apical (A) and basal (B). The membrane of epithelial/endothelial cells is divided accordingly into apical and basal domains, separated by a molecular belt formed by tight junctions. Different sets of membrane receptors and transporters are expressed in each of these domains. As a result, the cell membrane is physically and functionally polarized

choroid plexus), the vascular endothelium is paired with an underlying epithelial membrane to form a dual transporting unit.

The vascular endothelium has paramount physiopathogenic roles in the onset and perpetuation of atherosclerosis and hypertension, and is involved in systemic inflammation processes, edema, and thrombosis. Furthermore, systemic viral and bacterial infections, and most therapeutic and recreational drugs disseminate through the blood, posing a challenge to the normal vascular endothelial physiology. Endothelial dysfunction is also involved in the progression of systemic metabolic diseases like diabetes mellitus or hypercholesterolemia.

The brain capillaries represent a special case of endothelial specialization and adaptation. Given the particular physicochemical isolation required by the brain, the capillary endothelial function must be regulated with precision, and proper mechanisms must be in place to ensure that brain functions are maintained during vascular growth and/or remodeling. To achieve this degree of regulation, the brain capillary endothelial cells rest on a basal lamina and form a cellular network with the surrounding pericytes and astrocytes that, in turn, are in contact with neurons. This cellular arrangement forms a neurovascular unit and constitutes the BBB, which is embedded in a thin layer of extracellular matrix and strictly regulates the transport of water, ions, glucose, nutrients, and other molecules to and from the brain, playing a major role in the removal of drugs and toxins from the brain parenchyma (Fig. 2).

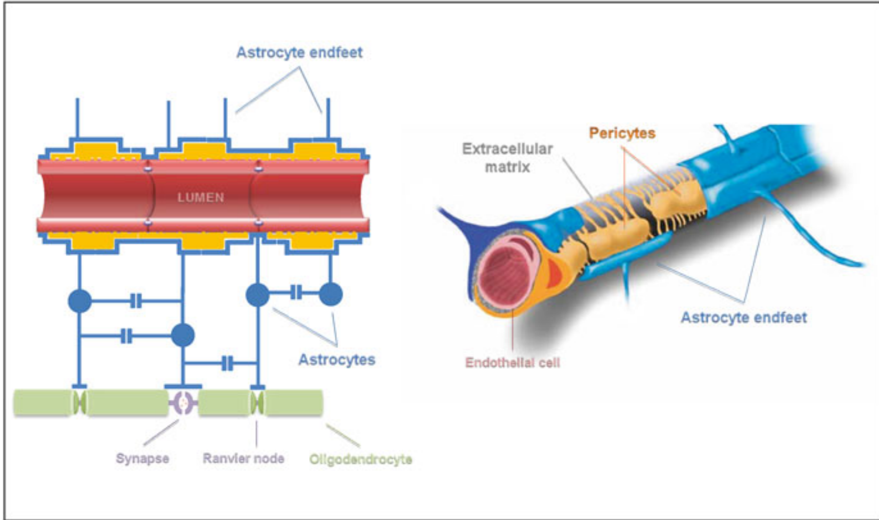

Fig. 2 The blood-brain barrier (BBB) is the regulated anatomophysiological interface that controls homeostasis between the blood and the brain parenchyma at the capillary level. Here, the brain vascular endothelium (*pink*) is surrounded by pericytes (*yellow*) and the end-feet of astrocytes (*blue*). They form a functional unit that is regulated by blood flow and neuronal activity. Astrocytes and pericytes play an important role in this regulation. Pericytes are in direct contact with endothelial cells, embracing them with multiple podocytes, to regulate blood flow and transduce signals to and from astrocytes and endothelial cells. Astrocytes form an extensive and well-organized network that interconnects capillaries with neurons and synapses. Specialized astrocyte end-feet are thus in direct contact with the synaptic space, Ranvier nodes, and brain capillaries

2.1 Transporting Phenotype

Electrophysiology experiments performed in the middle of the nineteenth century showed that electrical current measures in semi-intact frog-leg muscle preparations were consistently lower when the skin was conserved as compared to skin-free preparations [4]. These were the first published experiments that hinted the possibility of the skin modulating the electrical properties of the underlying tissues and prompted the first formal epithelial electrophysiological studies. Shortly after, it was discovered that the frog skin exhibited spontaneous electrical activity if placed between two compartments filled with ionic solutions, a condition that invariably resulted in the solution bathing the inner side of the skin (basal) becoming more electropositive than the solution bathing the outer side (apical) [5]. It was then shown that such spontaneous potential depended on the sodium and lithium ions present in the solutions used, so it was proposed that the electric activity of the frog skin resulted from the asymmetric epithelial permeability to those ions [6, 7].

Further studies demonstrated unambiguously that the skin is indeed capable of transporting Na^+ and that this transport occurs preferentially from the outer side to the inner side of the skin regardless of electrochemical gradient.

Based on these observations, epithelia and endothelia became represented as an equivalent electrical circuit that was originally represented as a "two-membrane" model, where the net transepithelial transport was equal to the net flux of Na^+ across the outer and the inner cell membranes [8]. To explain this phenomenon, it was proposed that the luminal membrane allowed Na^+ to passively enter the cell while the basal membrane transported it out while being exchanged by K^+. Thus, the model required a mechanism to pump Na^+ ions out of the cell through the basal membrane against their electrical and concentration gradients [9]. Incidentally, in a series of unrelated experiments, a ubiquitous membranal protein that actively transported Na^+ ions against their concentration gradient was described [10]. This protein, later identified as the Na^+/K^+-ATPase, was proposed to be the Na^+ pump required in the model. A direct functional implication was that two different sets of membrane transporters had to be expressed differently in the apical and basal regions of the cell membrane in order to explain the different transporting capabilities of both regions.

Further studies demonstrated that in the absence of paracellular free diffusion of ions (electric paracellular leakage), the apical and basal membranes of epithelial and endothelial cells had independent electrical properties and their combined voltage could be added, as if they were in a serial circuit. Thus, the intercellular space had to be closed to prevent the transported ions from freely diffusing back along their concentration gradients.

Biological barriers transport not only ions but also water and other solutes (e.g., glucose and amino acids) that are needed to maintain the homeostasis between the two biological compartments they separate. Endothelial transport occurs by transcellular and/or paracellular routes. Transcellular transport is determined by the presence or absence of specific membrane transporters and channels, whereas the paracellular route is regulated by tight junctions (TJs), protein complexes sealing the paracellular space and forming paracellular ion channels.

TJs are expressed as a continuous belt around the lateral borders of cells, dividing the cell membrane into apical and basal domains, preventing free diffusion of membrane receptors, transporters, and ion channels from one domain to the other, Effectively polarizing the cells. The vectorial transport of Na^+ from the apical to the basal side of the epithelium/endothelium generates a transepithelial/endothelial electrochemical gradient that constitutes the driving force for all other membrane transporters to work [11]. Thus, the transcellular exchange of glucose, amino acids, water, ions, toxins, drugs, etc. is driven by the transport of Na^+, whose vectoriality is determined by the expression of TJs. These two features, namely, the vectorial transport and TJ expression, reflect the current physiological understanding of the two-membrane model and define the transporting phenotype characteristic of the BBB endothelium and, in general, all mature epithelia (Fig. 3).

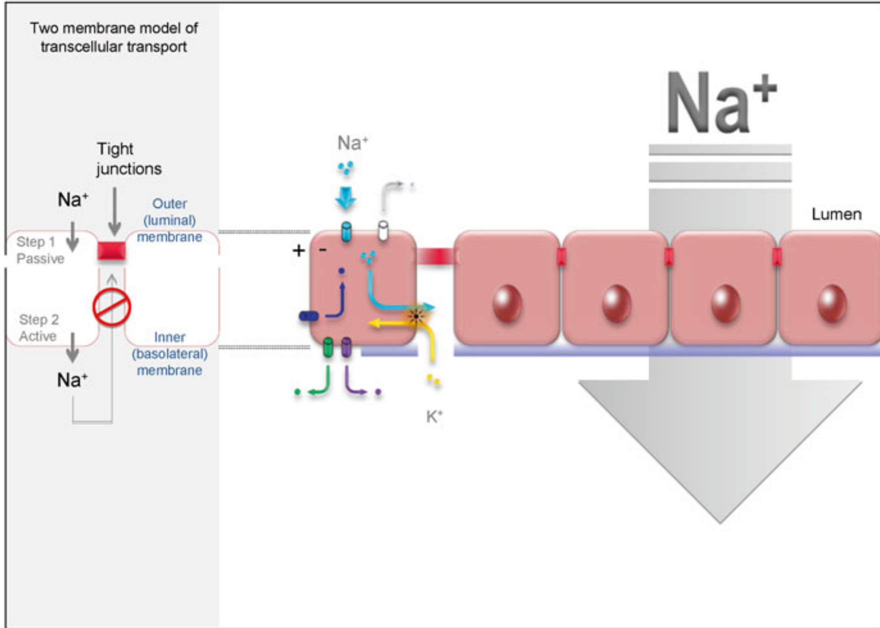

Fig. 3 All endothelia and epithelia share a common transporting phenotype characterized by the expression of tight junctions and vectorial transcellular transport. The classical description of this phenotype established the need of a polarized expression of transporters in the apical (outer/luminal) or basolateral (internal/abluminal) membrane. In order for this model to work as a transporting mechanism, sodium (the most abundant electrolyte in the extracellular fluid) must passively enter the apical membrane, following its concentration gradient, and be actively exchanged with potassium in the basolateral membrane. The sodium pump (Na+/K+-ATPase) is the enzyme that fulfills this role, and its polarized expression is paramount in maintaining the transporting properties of the brain endothelium. In order to prevent sodium backflow, the paracellular space is sealed with tight junctions (*red boxes*). The resulting biochemical effect is the formation of an electrochemical gradient between the intracellular and extracellular sides of the luminal and abluminal membranes. Due to its implications in cell metabolism, this gradient is canonically considered the driving force that allows other membrane transporters to work and ultimately maintain the proper electrochemical environment for cellular metabolism to occur

2.2 Electrical Representation

The introduction of the two-membrane model represented a milestone in understanding the transport characteristics of epithelia and endothelia. However, the observed change in ionic concentration between the apical and basal compartments was not consistent with the total change in voltage across the epithelium/endothelium. This condition was inferred to be caused by transepithelial leakage of ions that could not be explained by the two-membrane model alone, suggesting the existence of an "electrical shunt" pathway. Further studies demonstrated that ionic diffusion through the paracellular space was an important contributor to the electrical shunt [12], and different paracellular conductivities were identified for a number of tissues, leading

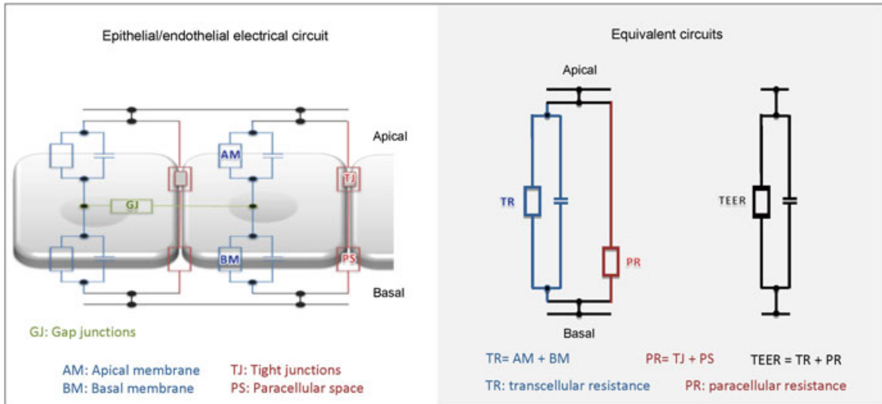

Fig. 4 Electrical gradients arising from the transendothelial ionic transport generate voltage across the apical and basal membranes, tight junctions, and the paracellular space. The particular localization and conductivity of these elements to transport specific ions results in the generation of electrical resistance, creating an electrical circuit with two sets of resistors in parallel: apical and basal membrane (representing transcellular transport), and the tight junctions and paracellular space (representing paracellular diffusion). In addition, direct cell-cell communication via gap junctions allows ions to move between cells, interconnecting adjacent circuits and creating an additional intercellular resistance that has a practical role when the ionic equilibration rate between adjacent cells needs to be accounted for. The transepithelial/transendothelial electrical resistance (TEER) is obtained by obtaining the summatory of the transcellular and paracellular resistances

to the distinction between "tight" (low-conductivity) and "leaky" (high-conductivity) epithelia [13]. It was later proposed that the molecular composition of the TJs was responsible for determining the low or high conductivity of epithelia. These concepts were integrated into the two-membrane model, ultimately representing epithelia/endothelia as an electrical circuit with four resistors: the apical and basal cell membranes form two serial resistors representing the transcellular route, while parallel to them, TJs form a resistor serially coupled to the electrical resistance of the paracellular space, representing the paracellular route (i.e. the shunt pathway) [14] (Fig. 4).

The most sensitive approach to determine the transport function of the BBB endothelium is thus to measure the transendothelial electrical resistance (TEER), which results from the mathematical integration of the transcellular and paracellular resistances. Since TEER is directly proportional to the paracellular resistance, it constitutes an accurate determination of the ionic permeability of TJs.

In general, TJs are selectively permeable to cations, depending on the size and charge; however, their selectivity can be altered by changes in pH, osmotic load, by applying an electrical current [15], or if the molecular structure of the TJ changes. Thus, different ionic selectivities found in different types of epithelia and endothelia depend on the particular molecular composition of TJs [16]. Therefore, it is not surprising that alterations in the molecular composition of TJs of the brain endothelium can influence ionic selectivity and tightness of capillaries influencing the overall BBB permeability.

3 Endothelial Cells and Tight Junctions

Mature endothelial cells are characterized by low height, low number of caveolae in their luminal surface, and numerous mitochondria. Brain endothelial cells (Fig. 5) differ from those outside the brain by the absence of fenestrations, low pinocytic activity, and extensive TJs. The morphology of TJs has been intensively studied by freeze-fracture electron microscopy [17] where it appears as a network of particles organized into multibranched and interconnected fibrils, known as strands. These strands associate with other strands from opposing membranes, forming Velcro-like molecular seal that closes the paracellular space. The number of strands and their branching frequency vary notably among different types of epithelia and correlate with TJ ionic permeability [14] (Fig. 6).

The biochemical nature of TJs started to be characterized when a TJ-enriched membrane fraction obtained from mouse liver was used to generate a monoclonal antibody that detected a previously unknown cytosolic protein of ~225 kDa [18], this was the first TJ protein discovered and named "zonula occludens-1" (ZO-1).

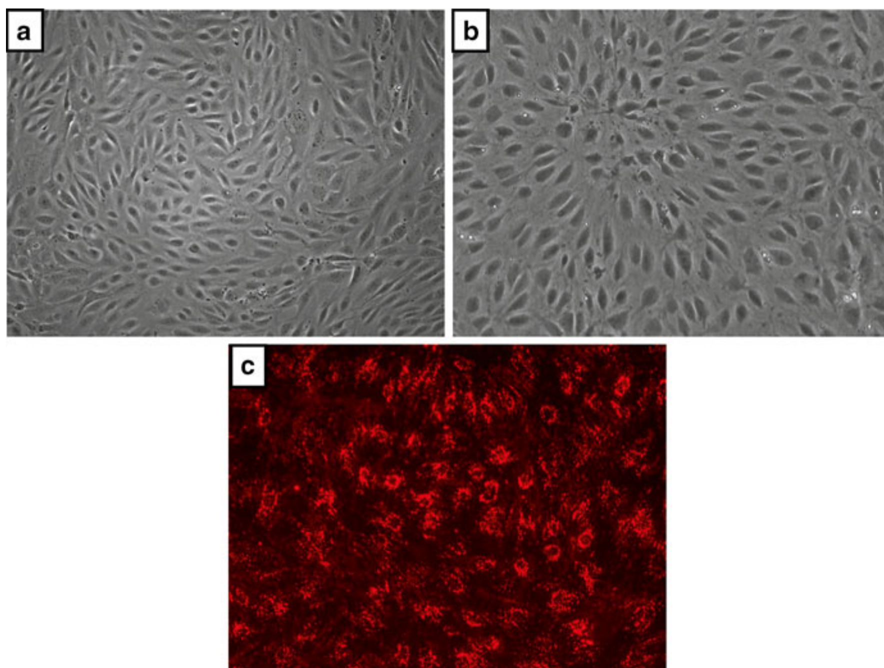

Fig. 5 Brain capillary endothelial cells cultured in vitro. (**a**) Confluent monolayer of frequently used human brain endothelial cells hCMEC/D3. (**b**) Confluent monolayer of primary mouse brain endothelial cells. In (**a**) and (**b**) notice typical morphology of brain endothelial cells, such as a fusiform shape and cobblestone appearance. (**c**) Positive uptake of acetylated low-density lipoprotein are one of the markers of endothelial cells

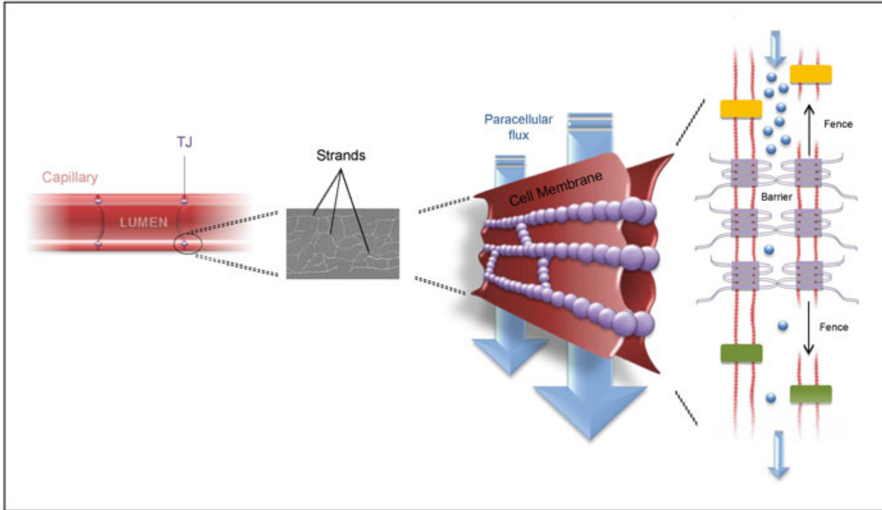

Fig. 6 One of the most prominent features of brain endothelial cells, as well as those from other endothelia or epithelia, is the expression of tight junctions. When studied by freeze-fracture electron microscopy, they appear as a series of filaments, collectively known as strands. Higher number of strands and complex branching patterns have been associated with reduced paracellular ionic permeability (high TEER, i.e. tight paracellular space), whereas fewer strands and simple branching correlate with increased paracellular permeability (low TEER, i.e. leaky paracellular space). These strands are formed by transmembranal proteins that associate laterally in the same membrane (*cis*) to form a fence preventing the free diffusion of proteins and transporters between the apical and basal membrane domains, and frontally with their homologues in a neighboring membrane (*trans*) to form a paracellular barrier that restricts ionic diffusion through the paracellular space

A second molecular component of TJs was later identified as a transmembranal protein of ~65 kDa that received the name "occludin" [19]. Shortly after, two other transmembranal proteins were found claudin-1 and 2, the first of a novel protein family responsible for sealing the intercellular space [20] and forming paracellular ion pores. With the advent of novel protein-protein interaction and molecular screening techniques, the discovery of many additional TJ proteins followed in a short period of time. Currently, more than 40 proteins have been identified as part of TJs [21]. Although the list is large and growing, the key roles in defining the structure and function of TJs reside in a small number of these proteins.

Structurally, TJs consist of a set of transmembranal proteins that interact laterally with each other (*cis*) and across the paracellular space with those expressed in an opposing membrane (*trans*). The *cis*-interaction patterns determine the formation and branching of TJ strands, while the *trans*-interaction arrangements define the paracellular space sealing and formation of ion pores. The transmembranal proteins are scaffolded and attached to the underlying cytoskeleton by a set of submembranal adaptors that, in turn, form a link between transmembranal proteins, regulatory molecules, and transcription factors (Fig. 7).

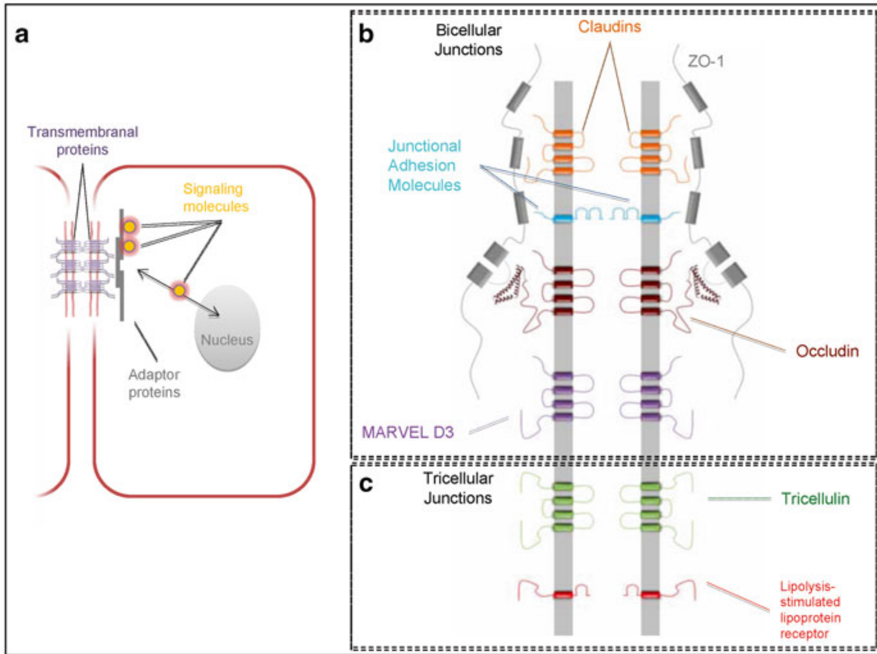

Fig. 7 (**a**) Tight junction complexes are formed by a set of transmembrane proteins anchored and stabilized by a large number of adaptor proteins that, in turn, are associated to the cytoskeleton and numerous signaling molecules that elicit changes in cell metabolism and gene expression. (**b**) The known transmembranal constituents of tight junctions are shown anchored to the submembranal adaptor ZO-1. The figure depicts these proteins in isolation, but they complex to form homo- and hetero-oligomers in living cells. Bicellular contacts are the regions where the membranes of two opposing cells are in contact and the streamlined descriptions of the tight junctions refer to the bicellular junctions. (**c**) A special set of proteins are required in those places where three cells contact each other. Tricellulin and the lipolysis-stimulated lipoprotein receptor organize tight junctions and the tricellular contacts

Epithelia/endothelia are tridimensional structures; therefore, the paracellular space formed where three cells meet together (tricellular contacts) must be subjected to the same sealing and regulatory mechanisms as the bicellular contacts. When the bicellular TJ belt reaches the tricellular contacts, the most apical strands turn down and extend toward the basal membrane, elongating the TJ perpendicularly to the bicellular TJ belt (Fig. 8). Tricellular and bicellular TJs are thus interconnected and form a continuous complex. The vertically oriented strands interact with each other and form an extracellular tubular channel of small diameter (~10 nm) known as the central tube which, similar to the bicellular TJs, regulates paracellular flux (Fig. 8).

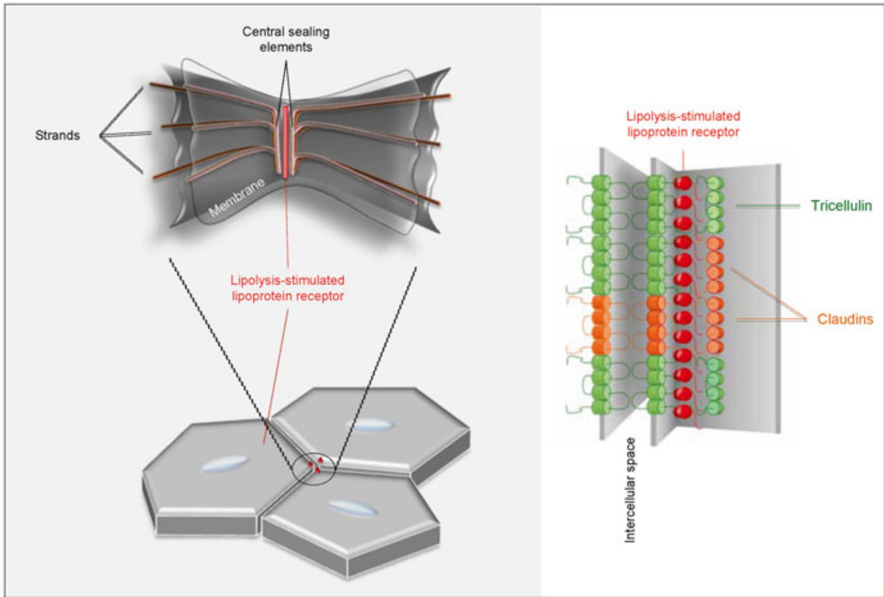

Fig. 8 At the tricellular contacts, the lipolysis-stimulated lipoprotein receptor functions as a beacon for tricellulin to find the cellular corners. Once in place, tricellulin organizes the incorporation of claudin-containing vertical strands to the tricellular borders, forming the central sealing elements. Similar to claudins that form paracellular pores to regulate ionic conductivity, tricellulin controls the ionic environment at the tricellular junctions

3.1 Molecular Structure of the Tight Junctions

3.1.1 Transmembranal Proteins

TJ transmembranal proteins are grouped into three main families: claudins, TJ-associated MARVEL (*m*yelin/lymphocyte *a*nd *r*elated proteins for *ve*sicle trafficking and membrane *l*ink) proteins (TAMPs), and adhesion molecules of the immunoglobulin superfamily. Claudins and TAMPs are tetraspanins, having four transmembranal domains, two extracellular loops, and intracellular N- and C-terminal domains. On the contrary, junctional adhesion molecules (JAMs) are single spanning molecules with a large extracellular domain and a short intracellular tail (Fig. 7).

Claudins

Claudins are the main functional constituents of the TJ strands, seal the paracellular space and form ion pores. Currently, there are 27 known human claudin molecules that, according to their amino acid sequence, can be organized into classic

(claudin-1 to 10, -14, -15, -17, and -19) and nonclassic (claudin-11 to 13, -16, -18, and 20–27) [22]. The precise functional role of many of them is still unknown. Some claudins exhibit a clear paracellular sealing function (claudin-1, -5, -11, and -14), while others are better known for their capacity to form cation pores (claudin-2, -7, -10B, -15, and -16), increasing TJ permeability and decreasing TEER [23]. Claudin-2 is involved in forming pores for monovalent cations (Na^+, K^+, Rb^+, Li^+, Cs^+), claudin-7 and -15 for Na^+, claudin-10B for cations, and claudin-16 for mono- and divalent cations. The cationic permeability is decreased if members of another subset of claudins (claudin-4, -5, -8, -11, -14, and -19) are incorporated into TJs. The formation of anion pores is less understood. Claudin-10A and -7 may be involved in forming Cl^- pores.

Although claudin-1, -3, -5, and -12 are major players in forming TJs between brain microvascular endothelial cells, the involvement (or absence) of other claudin types in the BBB has not been sufficiently clarified. The functions of claudin-6, -9, -12, -13, -17, -18, and 20–27 are still unidentified. Claudin-6, -9, and -13 are thought to be involved in kidney maturation [69]. Claudin-12 and -18 have been found in epithelia and endothelia of the intestine, inner ear, and brain endothelial cells. Claudin-17, -20, -22, and -23 have been found in the kidney, colon, stomach, and placenta, while claudin-21 and 24 have only been identified by genomic analysis.

Different claudins can interact with each other in *cis* and *trans*, homo- and heterotypically. They are anchored in the plasma membrane by adaptor proteins like ZO-1, ZO-2, ZO-3, and the PALS-1-associated tight junction protein PATJ [17, 24]. In most cases, at least two types of claudins are simultaneously expressed; thus, the cell-type-specific variation of claudin isoforms determines the differences in TEER and paracellular permeability [17, 25, 26].

Occludin

Occludin is another constituent of the TJ strands and the first transmembranal protein of TJs to be identified [19]. Although occludin is also a tetraspanin, it does not share similarities with any of the known claudins. It belongs to the TAMP family [27] for which it represents the archetype. It has seven known isoforms generated by alternative splicing. The classical description of occludin refers to the isoform 1, which weighs ~60 kDa, has a very short N-terminal domain, and has a very large C-terminal domain accounting for almost 50 % of its weight. Its MARVEL domain encompasses the four transmembranal domains, the loops in between, and the most N-proximal region of the C-terminal domain. The C-terminal domain has similarities to the RNA polymerase II elongation factor ELL and is thought to mediate protein-protein interactions. Isoform 2 lacks the fourth transmembranal domain, presumably causing the C-terminal domain to become extracellular. Isoform 3 lacks the last 47 amino acids on the C-terminal region, corresponding to half the coiled-coil domain that normally binds to the adaptor protein ZO-1. Isoform 4 is formed by the fourth transmembranal and the full C-terminal domain. Isoform 5 is comprised of the C-terminal domain, excluding a stretch of 57 amino acids on its N-terminal region, and lacks any transmembranal domains, being presumably a cytosolic

protein. Nevertheless, this isoform maintains the full binding site for ZO-1. Isoform 6 is 69 amino acids long, conserves the first 49 amino acids of the N-terminal domain, and presumably is cytosolic. Isoform 7 is 70 amino acids long, conserves the first 50 amino acids of the N-terminal domain, and is also presumed to be cytosolic.

The functions of occludin have proven to be elusive to determine. Most of our current knowledge comes from studying the isoform 1 which has been suggested to be a part of a system that regulates the expression of other TJ molecules and the function of the TJ itself. Occludin may be involved in the activation of the TJ-associated guanine nucleotide exchange factor GEF-H1/Lfc [28] and in targeting the tumoral growth factor-ß (TGF-ß) receptors to the TJs. On the other hand, the interaction of occludin with ZO-1 links it indirectly with other transmembranal TJ molecules (e.g., claudins and JAMs) that also attach to ZO-1. The C-terminal domain is also rich in serines, threonines, and tyrosines that can be differentially phosphorylated [29–31]. In intact cells, occludin is highly phosphorylated on threonine and serine residues, while tyrosines are dephosphorylated. When threonines and serines are dephosphorylated, and tyrosines phosphorylated, occludin delocalizes from TJs. The differential phosphorylation of occludin has been linked to changes in its ability to interact with itself and with ZO-1, as well as modulation of TJ permeability.

Tricellulin

Tricellulin (MARVEL D2) is also a TAMP family member. It is found almost exclusively at the tricellular contacts, where it functions as a master molecule directing organization of the tricellular TJs (Fig. 8). It has an ELL domain and also binds ZO-1 [32, 33]. There are four known isoforms of tricellulin which, similarly to occludin, are formed by alternative splicing. Tricellulin-a is the classical isoform, weighing 64 kDa. Tricellulin-a1 lacks a small number of amino acids in the C-terminal domain, but maintains the ELL domain. Tricellulin-b lacks the ELL domain, and tricellulin-c is predicted to have only two transmembrane domains.

Tricellulin is regulated by phosphorylation, particularly by a PKC-signaling pathway that has been linked to the activity of peroxisome proliferator-activated receptor gamma (PPARγ). The presence of tricellulin is required for the adequate development of TEER and molecular organization of TJ molecules in both tricellular and bicellular contacts. At tricellular contacts, tricellulin selectively seals the paracellular space against macromolecules without altering the ionic permeability of the tricellular TJs [34]. Furthermore, expression of tricellulin has been suggested to protect against viral and transepithelial penetration of foreign antigens. Interestingly, in the absence of occludin in epithelial kidney cells, tricellulin migrates from the tricellular to the bicellular contacts [70], suggesting that tricellulin may partially compensate for some occludin functions. On the other hand, the presence of tricellulin in bicellular TJs reduces strand discontinuities and improves their paracellular barrier function.

MARVEL-D3

MARVEL-D3 is a TAMP member whose association with TJs was recently discovered [35]. There are two known isoforms (~45 and ~46 kDa) widely expressed in epithelia and endothelia. Currently, little is known about its function. MARVEL-D3 expression is not required for functional TJs; interestingly, its depletion results in enhanced TEER. Thus, it has been suggested that MARVEL-D3 modulates the paracellular barrier properties of TJs. MARVEL-D3 was also suggested to have an overlapping function with tricellulin and occludin, although the precise nature of this function needs to be clarified.

Lipolysis-Stimulated Lipoprotein Receptor (LSR)

LSR is a receptor for triacylglyceride-rich lipoproteins that binds chylomicrons and low- and very-low-density lipoproteins in the presence of free fatty acids, allowing their subsequent cellular uptake. It is a single spanning molecule of ~71 kDa with an extracellular N-terminal Ig-like domain, a single transmembranal domain, and a large cytosolic C-terminal domain. There are four known isoforms with slightly different molecular weights. Its recent identification as a TJ molecule led to the proposal of a model that explains the formation of tricellular TJs. LSR is suggested to define a topographic landmark for cellular corners at tricellular contacts [36]. The cytosolic C-terminal domain of LSR binds to tricellulin, suggesting that LSR can be directly involved in recruiting it to the tricellular borders, where in turn, tricellulin directs the organization of the tricellular TJs (Fig. 8).

Junctional Adhesion Molecules (JAMs)

JAMs are members of the immunoglobulin superfamily and, similarly to occludin, are not exclusive to epithelial/endothelial cells, as they are also found in leukocytes and platelets. They are formed by a single transmembranal domain, and their large extracellular domain has two Ig-like motifs. The family is comprised of four members: A, B, C, and 4/L (4 in mouse, L in human). While JAM-A and -C are localized at the TJs, JAM-B is expressed along the whole lateral membrane of endothelial cells.

JAMs are involved in cell adhesion by *trans*-interacting with themselves and integrins; thus, they have been proposed to play a role in adhesion of leukocytes to endothelial cells. Their involvement in TJs also contributes to endothelial barrier function [37], but the mechanisms of such regulation are still not defined [38]. JAM-A, -B, and -C have PDZ-binding motifs that allow them to bind a number of TJ-associated adaptor proteins, such as ZO-1, MAGI-1, or MUPP-1. The serine protein kinase CASK/Lin2 and the cell polarity-related/G protein-coupled receptor Par3 are also known binding partners of JAMs. Therefore, it has been suggested that JAMs have a double role in endothelial cells, regulating leukocyte/platelet/endothelial cell interactions and TJ formation during the acquisition of cell polarity.

Coxsackievirus and Adenovirus Receptor (CAR)

CAR was originally identified as a protein that enables group B coxsackievirus and different types of adenoviruses to attach to the cell surface. It is a single spanning molecule formed by an extracellular N-terminal domain that contains two Ig-like domains, a single transmembrane domain, and a smaller cytosolic C-terminal domain. Five isoforms have been identified, but their functions in TJs are yet to be clarified [39]. CAR-1 has a binding region for ZO-1 and its overexpression leads to an increase in TEER. Since it can bind to IgG and IgM in serum and is overexpressed at sites of inflammation, it has been speculated that, similarly to JAMs, CAR-1 may be involved in the transepithelial transmigration of immune cells.

3.1.2 Submembranal Proteins

These adaptors bind to the transmembrane proteins and allow them to acquire a proper organization in cell membranes, linking them to the cytoskeleton and signaling molecules. Some of these adaptors are big scaffolds (i.e. ZO-1) that allow multiple proteins to be bound simultaneously. The most studied proteins in this group are members of the membrane-associated guanylate kinase (MAGUK) family, MAGUK-inverted proteins (MAGIs), and cingulin.

Membrane-associated Guanylate Kinases

MAGUK proteins are characterized by having one or more PSD95/Disk-large/ZO-1 (PDZ) domains, a Src homology-3 (SH3), and a non-catalytic guanylate kinase homology (GuK) domain. Most of them are scaffolding proteins and localize to cell-cell contacts, where they interact with numerous structural and signaling proteins via their PDZ, SH3, and GuK domains. Based on their size and domain distribution, four MAGUK subfamilies are known: DLG-like, ZO-1-like, p55-like, and LIN2-like. ZO-1-like proteins have the most relevance for TJs. ZO-1, -2, and -3 play a key role in regulating membrane protein assembly, clustering of receptors and ion channels, and regulation of cell differentiation [40].

ZO-1 is a ~220 kDa protein that anchors claudins, occludin, JAMs, and tricellulin in TJs. ZO-1 is thus considered the main TJ scaffolding protein. It also binds the Y-box transcription factor ZONAB, the adherens junction protein β-catenin, the signaling proteins ($G\alpha_{12}$ and $G\alpha_{i2}$), the β-subunit of the L-type Ca^{2+} channel, actin, CAR, afadin, and the desmosomal protein AHNAK [41, 71]. Similar to occludin, expression of ZO-1 is not restricted to TJs and epithelial/endothelial cells. The amino acid sequence of ZO-1 contains nuclear localization signals. In fact, ZO-1 localizes to the cell nucleus in sparse cultures of epithelial or endothelial cells. In brain capillary endothelial cells, the small GTPase Rho is involved in the nuclear localization of ZO-1 by inducing phosphorylation of the transcription factor cAMP response element-binding protein (CREB) [42]. The nuclear localization of ZO-1 has also been observed in proliferating corneal fibroblasts and HEK293T cells.

There are five known isoforms of ZO-1 formed by alternative splicing. The most studied are a+, which corresponds to the classical description, and a−, which lacks 80 amino acids in its C-terminal domain. They appear to have different roles; a+ is predominant in epithelia, while a− is in endothelia. In addition, a+ seems to correlate with the establishment of functional TJs and a− is associated with structurally dynamic TJs that undergo active remodeling, e.g., in Sertoli cells or podocytes [43]. The other isoforms are b1 and b2, about which little is known.

ZO-2 is a 160 kDa molecule that binds to ZO-1, claudins, occludin, cingulin, α-catenin, and actin. In sparse epithelial cultures, it is conspicuously located in nuclear speckles, where it co-localizes with the splicing factor SC35. It also associates with the transcription factors Fos, Jun, and C/EBP. ZO-3 is a 130 kDa protein that binds to ZO-1/ZO-2 complexes and seems to mediate the assembly of TJs by associating with PATJ, cingulin, and occludin [44].

The MAGUK-inverted (MAGI) group of proteins is a subset of MAGUK molecules that, inversely to the archetypical MAGUKs, have most of their PDZ domains located N-terminally to the SH3-GuK domains [40]. MAGI-1 co-localizes with ZO-1 and the GTP exchange protein GEP at TJs. MAGI-2 and -3 form a complex with the phosphatase PTEN that catalyzes the dephosphorylation of phosphatidylinositol 3,4,5-triphosphate, which is involved in apoptosis suppression by activating AKT/PKB.

The protein associated with Lin-7 (PALS-1) is also a MAGUK protein. It is recruited to TJs by the PALS-1-associated tight junction protein PATJ and functions as an adaptor that links PATJ to CRB-1, a molecular scaffold that participates in the development of cell polarity. The resulting PALS1/PATJ/CRB-1 complex plays a paramount role in establishing apico-basal polarity and TJ biogenesis. PALS-1 is also involved in adherens junction formation and the trafficking of E-cadherin.

Other Relevant Proteins

The partitioning-defective protein PAR-3 is associated at the TJ level with JAMs and forms a complex with PAR-6 and the atypicial (a) kinases PKC-λ and PKC-ξ. PAR-6, a binding partner of the Rho GTPases Cdc42-GTP and Rac1, is a key molecule that mediates the association of Rac1, Cdc42, and the atypical PKCs to PAR-3. The PAR-3/PAR-6/Cdc42/Rac1 complex is directly involved in cell polarity during the acquisition of the epithelial phenotype and is thought to be recruited to TJs by the association of PAR-3 with JAMs. PAR-6 also interacts with PALS1, linking PALS1/PATJ/CRB with the PAR3/PAR6/aPKC complex [45].

Afadin, also known as AF-6, is a 205 kDa protein that interacts with ZO-1, cingulin, JAMs, profilin, F-actin, and Fam. The binding between ZO-1 and afadin is mediated by the Ras-binding domains of afadin, and activation of members of the small GTPase family Ras (e.g., Ras, Rap1A, Rit, Rin, and M-Ras) inhibits this binding and disrupts cell-cell contacts [46]. JAMs and ZO-1 are mutually exclusive in their association with afadin, which is a critical regulator of cell-cell junctions during development [47]. Afadin is also located at the adherens junctions, where it binds nectin, a Ca^{2+} independent Ig-like molecule, and ponsin, a molecule that links afadin and

vinculin to adherens junctions. Through its association with profilin, afadin is thought to participate in the cortical actin assembly and cytoskeletal remodeling [48].

The multi-PDZ domain protein 1 (MUPP-1) is a large molecule that is associated, at the TJ level, with claudins, JAMs, and PALS-1 [49]. MUPP-1 is not needed for TJ establishment or polarization, but has instead been proposed to modulate cell proliferation [50].

Cingulin is a ~150 kDa protein that localizes to TJs and interacts with ZO-3, afadin, JAM-A, F-actin and myosin, and forms complexes with ZO-1. Cingulin is known to modulate activity of RhoA by interacting and inactivating its exchange factor GEF-H1, particularly in mature epithelia where it regulates gene expression and cell proliferation [51].

3.2 Transcellular Transport

While endothelial cell TJs limit the paracellular flux of hydrophilic molecules, small lipophilic molecules (e.g., anesthetics, O_2, or CO_2) can diffuse freely across plasma membranes following their concentration gradients. The polarized expression of receptors and transporters embedded in the membranes of endothelial cells, ensures the vectorial transport of nutrients such as glucose and amino acids, and the uptake of larger molecules like insulin or leptin. In endothelial cells, transcellular permeability is largely mediated by endocytic and transcytotic processes [52]. Clathrin plays a major role in the formation of polyhedral lattices that surround and coat endocytotic and transcytotic vesicles. These coated vesicles allow endothelial cells to acquire and transfer nutrients, import signal receptors and growth factors, mediate immune responses, and provide an alternate mechanism to remove pathogens and toxins. However, clathrin-mediated endocytosis can also provide an entry pathway for pathogenic agents.

Whereas conserving a proper barrier function is essential to maintain brain homeostasis, delivery of vital molecules from the blood into the brain is essential to preserve brain metabolism. To achieve this, brain endothelial cells express a large number of transporters in their membranes; however, the expression and activity levels of many of them are controlled by astrocytes [53]. In specific cases a transporter may not be expressed in endothelial cells, as is the case of dopamine, nevertheless, L-DOPA can be transported into the cells, where it is enzymatically transformed to dopamine. Transporters for glucose, galactose, amino acids, monocarboxylic acids, purines, nucleosides, amines, and ions have been identified in the membranes of endothelial cells.

Molecular transport, however, does not occur only from the blood into the brain parenchyma. Non-required metabolites, toxic substances, and drugs must be removed from the brain, and endothelial cells express a large variety of carriers to actively transport these molecules into the blood. P-glycoprotein (Pgp), an ATP-binding cassette (ABC) efflux transporter, is one such major carrier. Along with multidrug resistance-associated proteins (MRPs), Pgp is responsible for the active efflux of a wide range of nonpolar molecules out of endothelial cells.

The plasma membranes of a large variety of cells incorporate microdomains composed of glycosphingolipids and glycolipid-associated proteins. These glycolipoprotein domains, known as lipid rafts, influence membrane fluidity, membrane protein and receptor trafficking, and constitute organizing centers for the assembly of signaling molecules. They have three to five times more cholesterol than other parts of the plasma membranes and are rich in sphingolipids and low in phosphatidylcholine. Because their lipid content is more organized and tightly packed than the surrounding cell membrane, these lipid microdomains float in plasma membranes similarly to a raft in water [54]. Their molecular composition makes them relatively insoluble in nonionic detergents (e.g., Triton X-100) at low temperatures.

Caveolae are small (50–100 nm) lipid rafts, forming invaginations of the plasma membrane induced by the oligomerization of caveolins, of which caveolin-1 forms very high molecular weight oligomers while binding to cholesterol and fatty acids. Caveolae have functional effects on signal transduction and play a role in endocytosis, oncogenesis, and the uptake of pathogenic bacteria and viruses [55]. They have been suggested to be a docking site for glycolipids and glycosylphosphatidylinositol-linked proteins. Caveolae contain numerous receptors and transporter systems, including receptors for low- and high-density lipoproteins, insulin, albumin, transferrin, advanced glycation end products, ceruloplasmin, interleukin-1, and vesicle-associated membrane protein-2 (VAMP-2). Caveolin-1, besides its role in the structural arrangement of caveolae, forms signaling complexes with endothelial nitric oxide synthase (eNOS), heterotrimeric G proteins, members of the membrane-associated protein kinase (MAPK) pathway, src tyrosine kinase, and protein kinase C. Caveolin-1 also regulates TJ protein expression. For example, cytoskeletal rearrangements due to actin depolymerization can cause TJ proteins to be internalized by caveolae-mediated endocytosis [56].

4 Astrocytes

Astrocytes are the most abundant cell type in the human brain and one of the major types of glial cells. They are morphologically characterized by star-shaped bodies and histologically identified by their content of intermediate filaments constituted by glial fibrillary acidic protein (GFAP) (Fig. 9). They express a large number of G-coupled receptors, which transduce neurotransmitter, neuromodulator, and hormonal signals into Ca^{2+} and cAMP signaling cascades. According to their morphology, astrocytes are classified as fibrous, protoplasmic, or radial. Fibrous astrocytes are found mainly in the white matter, contain a small number of organelles, and have many long and unbranched fiber-like processes. Protoplasmic astrocytes are the most abundant and localize in the gray matter, they contain a larger quantity of organelles and exhibit fewer and relatively shorter multibranched processes. Radial astrocytes are located perpendicular to the axis of the ventricular system, having one of their processes immersed into the pia mater and the other buried in the gray matter.

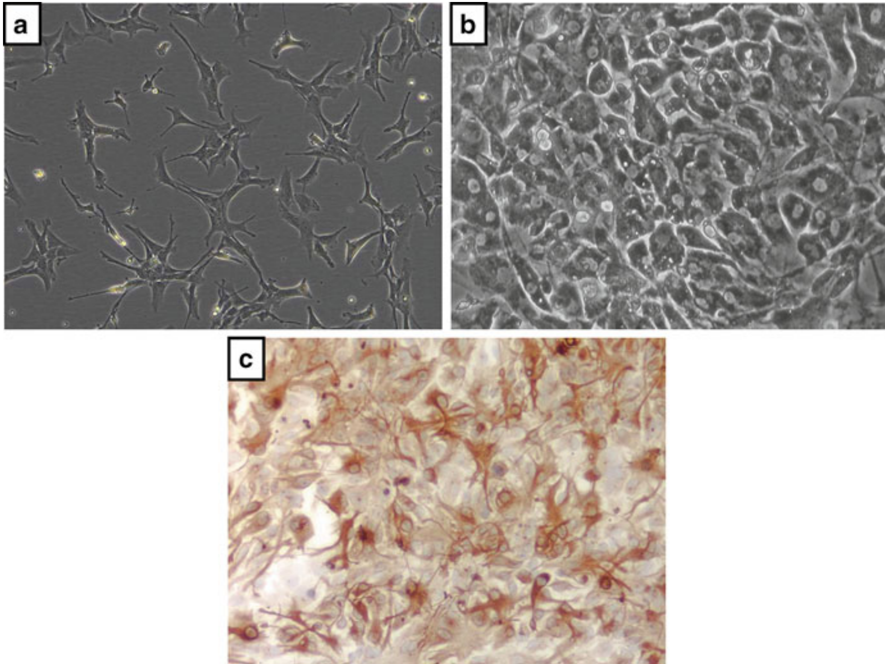

Fig. 9 Cortical astrocytes cultured in vitro and visualized by light microscopy. (**a**) Spare culture of human astrocytes; notice the characteristic star-shaped morphology. (**b**) Confluent culture of mouse astrocytes isolated from neonatal mice revealing typical features of confluent cultures, such as multipolar shape and cell overlapping. (**c**) GFAP-positive immunoreactivity of a confluent culture of mouse astrocytes

They are involved in neuronal migration during CNS development but decrease in number in the adult brain [57]. Regardless of their type, all astrocytes emit processes (vascular feet) that make extensive contact and surround the neighboring capillaries; however, fibrous astrocytes contact the nodes of Ranvier, while their protoplasmic counterparts envelop the synapses. Gap junctions are found in the distal regions of their processes, enhancing astrocyte-astrocyte communication directly in these regions. When in close proximity to the pia mater, astrocytes also emit processes towards it, forming the pia-glial membrane.

Astrocytes populate the CNS in a well-organized and nonoverlapping manner. Under normal conditions, protoplasmic astrocytes maintain well-limited and nonoverlapping domains in the gray matter in such way that only the most distal region of their end-feet interdigitates with one another. Similar domain organization seems to exist in the white matter. There is a considerable structural and molecular diversity among astrocytes at local and regional levels [58], and the complexity and diversity of astrocytes associated with neurons seem to have increased in different

species at different evolutionary stages. In the human brain, an average of 1.4 astrocytes per neuron in the cortex has been proposed [59].

Functionally, astrocytes express potassium and sodium channels, can exhibit evoked inward currents (but cannot trigger or propagate action potentials along their bodies), and show controlled fluctuations in their intracellular calcium concentration that are related to astrocyte-astrocyte and astrocyte-neuron communication. These fluctuations result from calcium release from intracellular stores triggered by neurotransmitters and can, in turn, induce the release of neurotransmitters (e.g., glutamate) from astrocytes into the extracellular space and trigger neuronal activity. Calcium signaling can also propagate to neighboring astrocytes via gap junctions, eliciting responses across their large intercommunicated cellular network. As gap junctions exist between astrocytes, pericytes, and endothelial cells, astrocyte signaling can elicit effects at the capillary level in their respective topographical domains. In fact, astrocytes produce and release various molecular mediators like prostaglandins, nitric oxide, and arachidonic acid that can increase or decrease vessel diameter and thereby control blood flow and intravascular hydrostatic pressure.

The astrocytic end-feet that surround all synapses maintain the homeostasis of the synaptic interstitial fluid. This is important as proper ionic concentration and pH are paramount for synaptic transmission. End-feet have a high content of potassium influx transporters and a number of proton-transporting mechanisms such as Na/H exchanger, HCO_3 and monocarboxylic acid transporters, and the vacuolar-type H-ATPase. GABA, glycine, and glutamate transporters are also enriched in these areas and contribute to clear the synaptic space of these neurotransmitters. Once retrieved, neurotransmitters are interconverted into other metabolites and send back to the synaptic space for their recycling into active transmitters. The large astrocytic networks are responsible for the uptake of potassium and glutamate from the synaptic spaces, preventing the deleterious effect of their synaptic accumulation. Astrocytes can also modulate synaptic transmission by releasing synaptic active molecules such as glutamate, purines (i.e., ATP and adenosine), GABA, and D-serine, in response to changes in synaptic activity. They also produce neurosteroids like estradiol and progesterone and release growth factors that can have long-term effects by influencing synaptic remodeling [60].

On the capillary side, end-feet-contacting endothelial cells are enriched in aquaporins, in particular aquaporin-4. This water transporter regulates water content in the paracellular space and has special clinical relevance since its altered function plays a role in the formation of brain edema [62]. Glucose transporters are also expressed in this location, and glucose transport is elicited upon glutamate stimulation [63]. Glucose can be taken from endothelial cells and extracellular space and is stored as glycogen. In fact, astrocytes constitute the main glycogen storage in the CNS, and the density of astrocytic glycogen granules correlates with synaptic density. Astrocyte-derived glycogen can sustain neuronal activity during hypoglycemia and is used when high neuronal activity occurs. Both the content of glycogen in astrocytes and the gap junction-mediated exchange of glucose metabolites are regulated by neuronal activity (Fig. 10).

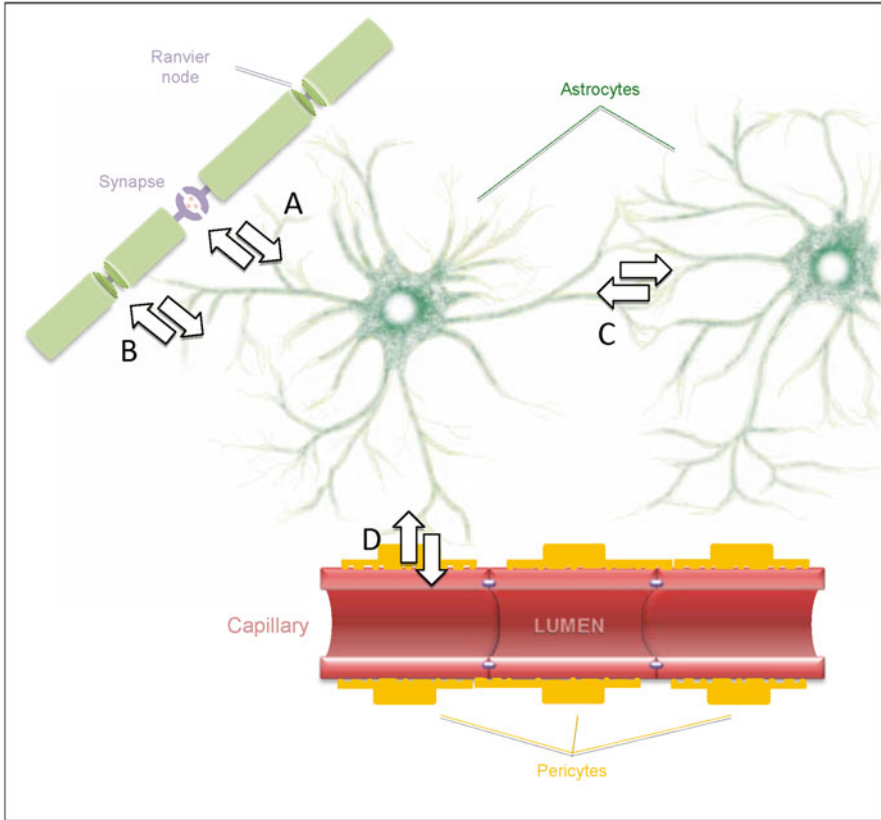

Fig. 10 Astrocytes play paramount roles in maintaining the homeostasis of the brain parenchyma, synaptic function, and transcapillary transport. (*A*) At synapses, their end-feet release energy substrates such as lactate, precursors for neurotransmitters, and neurotransmitters such as glutamate, purines (e.g., ATP or adenosine), growth factors, and neurosteroids. They also remove potassium, water, and neurotransmitters like GABA, glutamate, and glycine. (*B*) At the nodes of Ranvier, astrocytes also exchange energy substrates and electrolytes. (*C*) The astrocyte end-feet are rich in gap junctions that allow the exchange of neurotransmitters, glucose, and ions from one cell to the other and ultimately transport them between the brain capillaries and neurons, or across different astrocyte territories. (*D*) At the capillary level, astrocytes take up glucose and water and release ions, nitric oxide, and amino acids influencing blood flow

5 Pericytes

Pericytes are cells located in the perivascular space and wrapped around endothelial cells to which they provide structural support while conferring vasodynamic capacity to the capillaries. They are also necessary for BBB maturation and maintenance of its properties as they support angiogenesis and prevent endothelial cell apoptosis [64]. Capillary vasoconstriction was the first function identified for pericytes [65]

and can occur in response to vasoactive substances and neurotransmitters. This function has implications for brain activity, as increased local neuronal depolarization is accompanied by incremented regional blood flow. Biochemically, pericytes express a number of receptors for chemical mediators like catecholamines, angiotensin II, vasoactive intestinal peptides, endothelin-1, and vasopressin.

The precise structural and molecular identity of pericytes is still controversial. There is also controversy considering their cell lineage, as pericytes are often classified together with periendothelial smooth muscle cells, fibroblasts, macrophages, sometimes even confused with even endothelial cells. There are no known molecular markers that can be used to specifically identify pericytes and distinguish them from other mesenchymal cells. Furthermore, markers that are commonly found in pericytes are not stably expressed. Consequently, pericytes are often defined by considering their perivascular location, morphology, and gene expression patterns (Fig. 11).

In the BBB, pericytes are in direct contact with the abluminal side of endothelial cells. They extend cytoplasmic processes that often span several endothelial cells and occasionally bridge between neighboring capillary branches where the main body of pericyte resides directly at the branching point and its cytosolic processes extend along each branch. Their density in the human CNS is approximately one pericyte per 1–3 endothelial cells. Pericytes cover approximately 30 % of the abluminal surface of endothelial cells [66, 67]. At the molecular level, platelet-derived growth factor receptor-beta, chondroitin sulfate proteoglycan-4 (NG2), CD13, and alpha-smooth muscle actin are consistently expressed in pericytes [64], although these markers are also found in other mesenchymal cells.

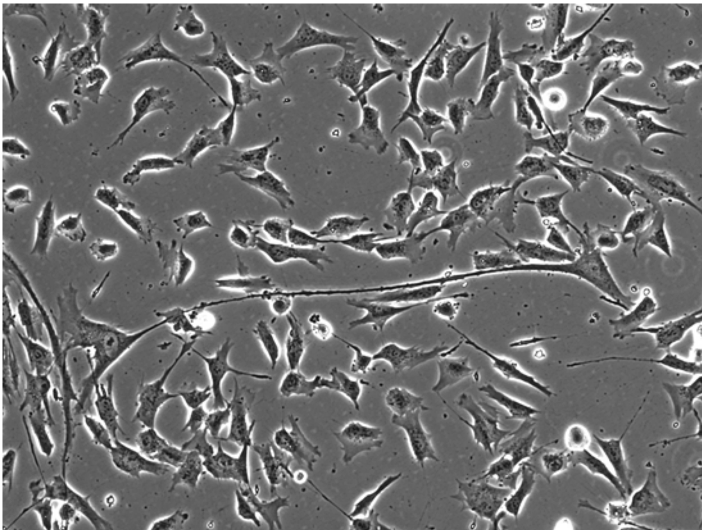

Fig. 11 Spare culture of human brain microvascular pericytes visualized by light microscopy

5.1 Extracellular Matrix

The extracellular matrix (ECM) in the CNS has a distinct composition and organization than that found in other tissues. Since the brain is mechanically protected and enjoys a physicochemically privileged location, there is no need to maintain the high levels of tensile or elastic strength usually present in other tissues. Therefore, the brain ECM, which is predominantly represented by the vascular basal membranes and meninges, has very little amounts of collagen types I and III or fibronectin and has a reduced content of glycosaminoglycan-proteoglycans and hyaluronan. Instead, the thin (20–200 nm thick) and tightly interwoven layers of ECM are predominantly composed of laminins, collagen type IV, heparan sulfate proteoglycans, and nidogens, all of them occurring in different isoforms that generate an ECM with distinct biochemical and functional properties [68]. Laminins in the endothelial cell basal membrane contain chains a4 and a5 combined with b1 and g1 to respectively form the isoforms 411 and 511, while the outer parenchymal basal membranes found at the postcapillary vessels and venules have chains a1 and a2 combined with b1 and g1 to form laminins 111 and 211. Laminins a4 and a5 are produced by endothelial cells, laminin a1 is produced by the leptomeningeal cells and laminin a2 is produced by the astrocyte end-feet. The predominant heparan sulfate proteoglycan in endothelial cell basal membranes is perlecan, while agrin is the predominant counterpart in the parenchymal ECM [61]. Endothelial cell membranes also have additional components such as osteonectin; fibulin-1 and -2; collagen VIII, XV, and XVIII, and thrombospondin-1 and -2. At the capillary level, the endothelial and parenchymal basal membranes are combined and contain laminins a2, a4, and a5, perlecan, and agrin.

6 Conclusions

The BBB constitutes one of the most impregnable mammalian interfaces and is responsible for maintaining the brain homeostasis. It is composed by closely interacting functional multicellular units called the neurovascular units. The properties of the BBB are defined by the unique characteristics of brain capillary endothelial cells that are sealed together by TJ proteins. TJ complexes are formed by transmembranal proteins closely interacting with specific anchor and adaptor proteins. The permeability of the BBB can be modulated by various pharmacological interventions. Importantly, disruption of the BBB integrity is associated with numerous chronic and acute CNS disorders, in which it contributes to the development of neuroinflammatory changes.

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Immune Cell Trafficking in the Central Nervous System

Beena John, Christopher A. Hunter, and Tajie H. Harris

Abstract For many years, it was assumed that cells of the immune system were excluded from the central nervous system (CNS) and thus immune reactions did not occur at this site. Currently, it is widely accepted that the immune system can gain access to and function within the CNS. A growing body of work now supports that the immune system is present in the brain in the steady state. Beyond serving a role in immune surveillance, the immune cells appear to promote neurological function. Under inflammatory conditions, immune cells enter the CNS and, depending on the context, may provide protection or cause tissue pathology. For example, following several infections, the immune system is required to control pathogen replication within the CNS and prevent disease. On the other hand, autoimmune reactions in the CNS, such as multiple sclerosis, cause debilitating tissue destruction. Beyond infection and autoimmune disease, the immune system appears to be involved in many neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease. Thus, the factors that support lymphocyte entry and function in various settings of neuroinflammation are of great interest. In the following chapter, immune cell entry and behavior within the brain will be discussed, with a focus on the role of adhesion molecules and chemokines in this process.

Keywords Neuroinflammation • Lymphocyte trafficking • Chemokine • Adhesion molecules • Integrins

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1 Introduction

For nearly 100 years, the unique immunological status of the brain has been appreciated. The presence of the blood-brain barrier (BBB) and isolation of the central nervous system (CNS) from circulating immune cells and antibodies led to the classification of the CNS as an immune privileged site [1–3]. Subsequently, the detection of small numbers of leukocytes, particularly memory T cells, in the CSF suggested that immune surveillance of these unique sites occurs in the absence of inflammation [4–6]. While it is clear that the nervous system can directly impact immune function, there is also evidence that the presence of a low number of T cells within the brain promotes normal neurological function [7, 8]. Nevertheless, the recruitment of immune cells to the brain is associated with a wide variety of neurological conditions, which, depending on the context, is beneficial or pathological. For example, the immune system is required to limit the replication of a variety of viral, bacterial, and parasitic organisms within the CNS [1, 9]. While the ability to control these pathogens is critical for host survival, the accompanying inflammation can lead to life-threatening disease [10]. Similarly, the development of autoreactive T and B cells specific for antigens present in the CNS accounts for the tissue destruction and disruption of normal neurological function observed in conditions such as multiple sclerosis and limbic encephalitis [11–13]. Inflammatory processes have also been implicated in many neurodegenerative disorders, such as Parkinson's disease and Alzheimer's disease, and in response to sterile injuries that include stroke or trauma [14–18].

A common pathological feature that links many of these conditions is the influx of leukocytes into the CNS [1, 9, 11, 19]. The infiltration of immune cells also triggers changes in resident populations such as microglia and astrocytes, which can amplify the inflammatory response that causes collateral damage which impacts on neuronal survival and function [15, 20]. Thus, understanding the mechanisms that govern the trafficking of immune cells into and within the CNS and their interaction with resident glia is critical for the design of strategies to augment protective immune responses to pathogens and tumors and to prevent the deleterious effects of neuroinflammation. In this chapter, the events involved in the entry of immune cells into the CNS and how this impacts immune surveillance will be discussed, with an emphasis on recent reports that visualize the behavior of these populations in real time.

2 Licensing of Cells for Entry into the CNS

One hallmark of neuroinflammation is the infiltration of immune cells into the multiple compartments of the CNS. In the steady state, small numbers of perivascular macrophages (pericytes) and T cells are present in the meninges and perivascular spaces [21, 22]. In the context of various inflammatory stimuli, the composition of these sites can be dramatically altered to include almost every type of immune cell,

but most commonly T cells, B cells, monocytes, macrophages, and dendritic cells. Immune cells can gain access to the CNS at several sites, including the blood-brain barrier (BBB) present along the capillaries in the brain parenchyma, the choroid plexus, meningeal vessels that extend into the brain parenchyma, and postcapillary venules [1, 9, 23–26]. Local and systemic inflammation can induce changes at these sites that make them more permissive to immune cell infiltration [19, 24, 27, 28]. Consequently, understanding the mechanisms by which diverse inflammatory populations access the CNS may lead to therapeutics that can be useful to manage the inflammatory conditions that affect this site [19].

Our current understanding of how immune cells enter the brain is arguably most developed for T lymphocytes. In current paradigms, naïve T cells do not readily enter the normal or inflamed CNS. Rather, T cells that enter the brain need to be highly activated, suggesting that these cells are primed in the periphery prior to gaining access to this site [9]. These observations raise many questions about the specific conditions that support T cell entry into the CNS, and a recent report has highlighted the importance of peripheral sites where T cells are "licensed" to enter the CNS [29]. In these studies, T cells specific for the CNS autoantigen myelin basic protein (MBP) were activated in vitro and transferred into mice to induce experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis. In the first days following transfer, the T cells were present in the spleen and other tissues, including the lung, but few reached the CNS. However, T cells isolated from the spleen or lung 60 h following transfer were capable of trafficking to the CNS of naïve recipient animals within hours, suggesting that these lymphocytes acquire the ability to traffic to the CNS at these peripheral sites. Microarray analysis of the different T cell populations demonstrated that the T cells that are initially transferred have an activation and replication program, whereas "licensed" T cells express a migratory program characterized by the ability to respond to inflammatory chemokines and increased expression of adhesion molecules [29]. These findings highlight that T cell activation alone is not sufficient for entry into the CNS and that additional events outside the CNS are required to make these cells responsive to the adhesion molecules and chemokine signals that facilitate access to the brain. In the remainder of this chapter, the role of some of the major adhesion molecules and chemokines that influence inflammatory processes in the CNS will be highlighted.

3 Leukocyte Extravasation and the Role of Adhesion Molecules

In many cases, local tissue damage within the CNS and perhaps even systemic insults initiate core processes similar to those employed in other tissue sites that lead to the extravasation of leukocytes across the blood-brain barrier or blood-CSF barrier [22]. However, there are some aspects of this process that appear to be unique to the CNS, and the identification of selective trafficking determinants should lead to the design of strategies to limit or promote leukocyte infiltration into the CNS. The adhesion cascade for leukocyte extravasation involves four canonical steps which

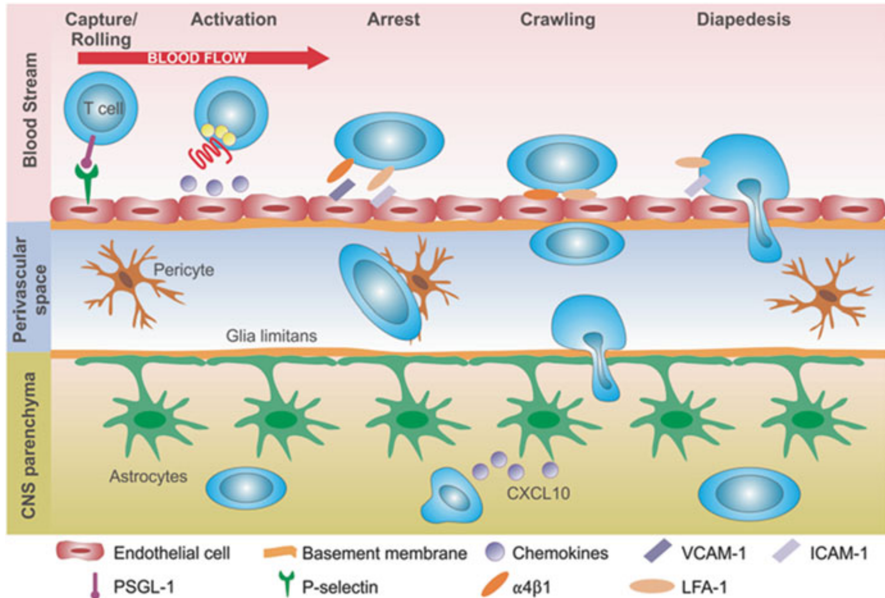

Fig. 1 T cell entry to the CNS through the blood-brain barrier. Activated T cells in the bloodstream interact with activated endothelial cells within the brain vasculature in a stepwise process. First, T cells interact with endothelial cells by binding to adhesion molecules, including PSGL-1, that induce capture or rolling. Next, chemokine signals activate integrin molecules (including $\alpha_4\beta_1$) leading to T cell arrest. Then, T cells crawl along endothelial cells before crossing the endothelium into the perivascular space in a process called diapedesis or extravasation. Within the perivascular space, T cells may interact with perivascular macrophages or pericytes. In order for T cells to gain access to the brain parenchyma, the cells must cross an additional basement membrane laid down by astrocytes, termed the glial limitans. Once T cells reach the brain parenchyma, chemokines provide signals to enhance the migration of T cells

are shared across various tissues (depicted in Fig. 1) and includes (1) capture and rolling of leukocytes regulated by selectins/mucins, (2) activation and change in conformation of integrins mediated by chemokines through Gαi receptor signaling, (3) firm arrest controlled by the integrins and their counter receptors on the endothelia, and (4) diapedesis or transmigration across the endothelial layer. After these events, the major obstacle to the entry of leukocytes is the BBB, which consists of endothelial cells with tight intercellular junctions and endothelial and glial basement membranes. The BBB limits entry from blood vessels in the parenchyma of the brain as well as the postcapillary venules in the meninges [22]. Similarly, the epithelial cells of the choroid plexus have tight junctions, which form the anatomical basis of the blood-CSF barrier. During local and systemic inflammation, endothelial cells of the BBB and epithelial cells of the choroid plexus increase expression of several adhesion molecules that support the recruitment of T cells and other immune cells [5, 19, 25, 26, 30]. Some of the important adhesion molecules required for immune cell recruitment during neuroinflammation are discussed below.

3.1 L-, P-, and E-Selectins

Selectins (L, P, and E) are surface glycoproteins involved in cell adhesion that are crucial for leukocyte rolling and capture on blood vessels. The P- and E-selectins are constitutively expressed on the cerebrovascular endothelium and are upregulated during inflammation [31]. Both P- and E-selectins can bind to PSGL-1, a mucin-type glycoprotein that is expressed on the surface of myeloid cells, activated lymphocytes, and inflamed endothelial cells. The ability of P-selectin to engage PSGL-1 leads to signaling that activates LFA-1, suggesting an indirect role for selectin-mediated signaling on integrin-mediated firm adhesion required for leukocyte recruitment [32]. This process may be relevant to normal surveillance and a model has emerged in which P-selectin expression on the choroid plexus stromal vessels promotes trafficking into this compartment and the presence of small numbers of T cells in the CSF [5, 30]. During EAE, inhibition of selectins and PSGL-1 (using blocking antibodies or mice that lack PSGL-1) has yielded mixed results. Some studies showed reduced trafficking of lymphocytes into the CNS and decreased severity of disease [31, 33], while others indicated that blockade did not impact on these processes [31, 34–36]. However, it is important to distinguish between the role of selectins in mediating leukocyte rolling at the cerebrovascular endothelium versus their function in other T cell and dendritic cell (DC) activities. For example, stimulation of DCs through PSGL-1 results in an increased ability to generate Tregs, which in turn can ameliorate disease [37, 38]. Nevertheless, the combined blockade of P-selectin and α4-integrins has shown a more profound inhibition of T cell rolling along inflamed vessels and a marked decrease in the severity and onset of EAE [32, 39]. These results suggest additive effects in the functions for P-selectin and α4-integrins in T cell rolling and illustrate the complex nature of the molecular interactions involved in these processes.

Another ligand for E-selectin is the glycoprotein CD44, which is ubiquitously expressed by immune and nonimmune cells, and the blockade of CD44 during EAE reduces CNS inflammation and the development of clinical disease [40]. Additional explanations for this observation include the interaction between CD44 and its ligand hyaluronan (HA) and a report that CD44 expression on the inflamed CNS vessels can tether HA and promote recruitment of activated T cells [41]. Given the complex biology of CD44 that includes multiple, unrelated ligands and its ability to undergo conformational changes, there remains a major knowledge gap in our appreciation of how its interactions with the selectins contribute to various forms of CNS inflammation.

3.2 β7 Integrin and Its Role in CD49d and CD103

The pairing of the β7 integrin with α4 (Cd49d) or αE (CD103) chains leads to the formation of stable heterodimeric α4β7 and αEβ7 complexes, respectively. The α4β7-MadCAM-1 pairing has been implicated in leukocyte migration to several

sites of inflammation including the mucosa; however, their role in CNS inflammation is less clear. During EAE, blockade of α4β7 had no effect on disease course, but EAE in β7-integrin-deficient mice was reduced in severity and MAdCAM-1 blocking antibodies did inhibit EAE [42, 43]. Thus, the precise role of the α4β7 integrin and MadCAM-1 during EAE remains to be clarified [25], but these discrepancies may be related to the use of β7 as a component of CD103. DCs and T cells express β7 integrin, and in a model of vesicular stomatitis virus (VSV) infection, memory $CD8^+$ T cells that express CD103 have been detected in the parenchyma long after viral clearance [44]. Whether this population is a consequence of a postinfection surveillance mechanism or due to persistence of VSV-specific T cells within the parenchyma and if CD103 influences these cells are open questions.

3.3 VLA-4 (α4β1)/VCAM-1 Interactions

Numerous studies using EAE as a model have highlighted the importance of the VLA4 (α4β1)/VCAM-1 interactions as a major determinant in leukocyte recruitment to the CNS. VCAM-1, one of the major α4β1 integrin ligands, is upregulated on the vasculature in EAE and MS. Moreover, the adhesion of activated MBP-specific T lymphocytes to brain endothelial cells in tissue sections is mediated by α4-VCAM-1 interactions, and the severity of disease caused by different MBP-specific T cell clones correlated with levels of α4 expression [45]. Consistent with these findings, anti-α4 antibodies block the accumulation of T cells in the CNS and the development of EAE [45, 46]. α4β1 is also required for the adhesion of immature DCs to the CNS vasculature and their access to the brain during EAE; however, the accumulation of granulocytes and macrophages within the CNS is independent of this integrin [47]. VCAM-1 blockade also delayed the onset of EAE but had modest effects on the duration and severity of disease [34], suggesting a role for other α4 integrin ligands such as fibronectin in these events [48]. These proof-of-concept studies underscored the importance of the α4β1 integrins in leukocyte accumulation in the brain and led to the development of antibodies that interfere with the function of this integrin for treatment of MS. However, blockade of α4 integrins in other models has also been shown to compromise protective immunity to several pathogens within the brain, including *T. gondii*, SIV, and bornavirus [49–51]. These findings highlight that approaches that might interfere with natural immune surveillance can lead to unwanted consequences, which are discussed in more detail later.

3.4 LFA-1(αlβ2)/ICAM Interactions

LFA-1 (αlβ2) is one of the best-studied members of the β2 family of integrins, and its ligand ICAM-1 is constitutively expressed by endothelial cells within the CNS vasculature and is upregulated during inflammation [46, 52]. Several *in vitro* studies

have shown that LFA-1-ICAM-1 interactions are required for the adhesion of T cells to the CNS vascular endothelium and for their optimal migration across this barrier. There are also reports that entry of Th17 cells into the brain parenchyma during EAE occurs in the absence of α4 integrins but is dependent on LFA-1 [53]. However, blockade of LFA-1/ICAM-1 and the use of ICAM-1-deficient and LFA-1-deficient mice ($Cd11a^{-/-}$) during EAE have resulted in responses ranging from prevention of EAE to exacerbation of disease [54–57]. Since LFA-1 is a component of the immunological synapse and has a prominent role in T cell activation, it is possible that the broader effects of LFA-1 blockade during EAE also influence the generation of the pathological T cells. Nevertheless, LFA-1 has also been implicated in the migration of adoptively transferred dendritic cells to the brain during toxoplasmic encephalitis [58] and so appears to be part of a core adhesive program that is relevant to many immune populations. Whether this influences other facets of immune function such as migration within the brain is unknown, and some of the disparate findings in EAE indicate that the biology is more complex than currently appreciated.

3.5 Therapeutic Significance of Targeting VLA-4 and LFA-1

With the need to develop more effective treatments to manage the clinical manifestations of MS, one approach has been the development of strategies that would limit the migration of pathogenic T cells into the CNS. The identification of α4β1 as a key molecule required for immune cell access to the CNS provided the rationale for the development of a monoclonal antibody (natalizumab) directed against integrin α4, as a treatment for MS. Clinical trials with this therapy demonstrated significant benefits including fewer inflammatory CNS lesions and reduced numbers of relapses in MS patients. However, the drug was temporarily withdrawn due to reports that, in a limited number of patients, this treatment was associated with the development of progressive multifocal leukoencephalopathy (PML), a potentially fatal disease caused by reactivation of JC polyomavirus [59]. Not surprisingly the use of another antibody (efalizumab) that blocks LFA-1 also results in PML (and was withdrawn from the market in 2009) [60]. Latent JC virus can persist in multiple tissues including the kidney, bone marrow, and brain. Whether the CNS disease caused by this virus is a consequence of reduced local immunosurveillance or whether it reflects the reactivation of the virus in the peripheral compartments and spread to the CNS is uncertain. Nevertheless, natalizumab continues to be used primarily as a monotherapy for treatment of MS, with careful consideration of prior JCV antibody titers in patients and close monitoring for development of PML. The challenge in this field is to determine whether the knowledge gained from basic and clinical studies can be used to design improved or more selective approaches that allow normal surveillance while targeting pathological processes.

4 Role of Chemokines in Homeostasis and Inflammation

In humans, there are approximately 50 chemokines and 19 chemokine receptors whose expression varies among immune cells [61, 62]. Several chemokines are expressed constitutively in the steady state and control homeostatic processes such as the natural circulation and homing of different immune cells [63, 64]. For example, the chemokine, CCL25, is expressed in the gut, and T cells and dendritic cells expressing CCR9 (the receptor for CCL25) home specifically to this tissue site during the steady state. Similarly, CCR4 and CCR10 mediate normal trafficking of T cells to the skin. The impact of chemokines and their receptors on tissue-specific homing patterns has been likened to an address code for immune cells [65, 66]. Specific chemokine receptor expression has also been associated with distinct classes of T helper cell subsets: Th1 cells express CXCR3 and CCR5, whereas Th17 cells express CCR6 [65, 67, 68]. To date, no brain-specific "address code" has been identified, but numerous studies have implicated chemokines in various aspects of neuroinflammation and elements of this literature are reviewed below.

4.1 CCR7/CCL19, CCL21

Several homeostatic chemokines are expressed during the steady state and contribute to the localization and behavior of lymphocyte populations within secondary lymphoid organs [69]. Two of these chemokines, CCL19 and CCL21, are constitutively expressed in the spleen and lymph nodes where they influence the migration of CCR7-expressing naïve and memory T cells as part of normal recirculation events [70]. Within the brain, CCL19 expression has been detected in venules and it has been proposed that it promotes immune surveillance by CCR7-expressing T cells [71]. Moreover, in a model of T cell acute lymphocytic leukemia (T-ALL), CCR7 is critical for the entry of tumor cells into the CNS where they can evade chemotherapy and act as a source of relapses [72]. Thus, blocking CCR7 may prevent metastasis to the CNS and reduce the need for aggressive treatment of T-ALL.

CCR7 also appears to have a role in lymphocyte entry into the CNS during inflammation. CCR7 blockade reduced the adhesion of activated T cells to sections of inflamed brain ex vivo [71]. In the context of infectious disease, CCL21 expression increases in the brain during chronic toxoplasmosis, and $CD4^+$ T cells that infiltrate the CNS in response to infection co-localize with CCL21 [51, 73]. Indeed, in *plt*$^{-/-}$ mice, which lack CCL19 and CCL21, $CD4^+$ cells were not able to access the brain parenchyma during toxoplasmic encephalitis. However, in mice that constitutively express CCL21 in astrocytes, lymphocytes did not enter the brain in the absence of infection. Thus, CCL21 alone is not sufficient to promote the entry of lymphocytes into the uninflamed CNS, but this chemokine has a key role during infection.

4.2 CXCR4/CXCL12

In contrast to many chemokines that are expressed in response to inflammatory signals, CXCL12 is expressed constitutively by the endothelium at the BBB and in the choroid plexus. Interestingly, instead of promoting inflammation, it has been proposed that CXCL12 limits the entry of $CXCR4^{+}$ immune cells into the brain. During MS and EAE, the localization of CXCL12 changes from the basal to the luminal side of the endothelium, which may promote T cell entry into the CNS [74–77]. These studies emphasize how altered expression patterns of chemokines at the BBB or BCSFB can influence the entry of immune cells to the CNS.

4.3 CCR6/CCL20

The receptor CCR6 is expressed by multiple cell types, including IL-17 producing T cells, which are associated with pathological T cell responses in many sites, including the CNS. Several recent studies have explored the role of CCR6 during EAE. Reboldi and colleagues reported that CCR6-deficient mice were resistant to EAE, and this phenotype was ascribed to a critical role for CCR6 in the initial recruitment of activated T cells to the CNS. The observation that CCR6-expressing Th17 T cells were associated with the choroid plexus which constitutively expresses CCL20 led to the hypothesis that entry of IL-17 producing cells occurs at this portal [78]. In contrast, two other reports have found that EAE is exacerbated in CCR6-deficient mice [79, 80]. Since regulatory T cells (Tregs) also express CCR6, an increase in pathology in CCR6-deficient mice may be a result of Treg dysfunction. Indeed, Treg recruitment to the CNS is reduced in the absence of CCR6 [79, 80]. Because this receptor is expressed on multiple cell types that influence many facets of the immune responses during neuroinflammation, these apparently contradictory studies illustrate the complexities of interpreting studies that involve total chemokine receptor knockout mice.

4.4 CXCR3/CXCL9,10,11

CXCR3 and its multiple ligands have been associated with various forms of neuroinflammation and surveillance of the CNS. In the absence of inflammation, T cells present in the CSF of patients express CXCR3 [81]. During EAE, as well infections that impact the CNS, CXCR3 is highly expressed by infiltrating T cells [82–89]. In some instances, CXCR3 is required for optimal trafficking of T cells to the CNS [86, 88]. In addition, $CXCR3^{-/-}$ mice develop less severe inflammation in models of viral encephalitis and cerebral malaria [82, 83, 86, 87]. While neurons and infiltrating myeloid cells are capable of producing CXCR3 ligands, astrocytes are a predominant source of CXCL10 and microglia of CXCL9 during EAE [90–93].

Consistent with the evolving view of chemokine biology, CXCR3 and its ligands are not simply involved in the recruitment of T cells to the CNS. Recently, the influence of CXCL10 on T cell behavior in the tissue parenchyma has been visualized using multiphoton microscopy of $CD8^+$ T cells responding to infection with *T. gondii*. Surprisingly, blockade of CXCL10 decreased the velocity of $CD8^+$ T cell migration, but did not influence the directionality of movement or the walk behavior of the cells. This alteration in migration was predicted to decrease the ability of cytotoxic T cells to find infected target cells and limit parasite replication [88]. Thus, a model is emerging in which chemokines within the inflamed CNS promote the movement of T cells through tissues, which may support pathogen control but presumably would also contribute to the development of autoimmune lesions in the CNS. Interestingly, $CXCR3^{-/-}$ mice exhibit exacerbated EAE [85, 94] and one study found that the localization of Tregs in the CNS is altered in these mice [85]. In light of recent studies that Tregs acquire similar phenotypes to the effector cell populations that they regulate [95–98], this change in Treg localization may lead to a reduced ability to limit the autoimmune effector T cell responses leading to more severe disease. Taken together, studies examining the role of CXCR3 in neuroinflammation highlight the complex nature of chemokine biology, with multiple cell types expressing a common receptor, single cells expressing multiple receptors, and differential ligand expression within a tissue.

4.5 CCR2/CCL2

The receptor CCR2 and its ligand CCL2 (monocyte chemoattractant protein 1) play a major role in the mobilization of inflammatory monocytes and neutrophils. The infiltration of CCR2-expressing $Ly6C^{hi}$ monocytes has been observed in a variety of neurological conditions. In the context of EAE, blockade of CCR2 suppressed disease [99, 100] and mice lacking CCL2 developed less severe EAE associated with reduced infiltration of monocytes [27, 101]. Similarly, during certain viral infections, CCR2-dependent accumulation of monocytes is associated with development of pathology characterized by demyelination [102] or vascular injury and the onset of seizures [103]. During other infections, including *T. gondii* and MHV, the lack of CCR2 resulted in decreased leukocyte trafficking and activation of immune cells within the CNS, leading to a reduced ability to control these pathogens [104, 105]. Together, these reports on diverse experimental models highlight the contribution of CCR2 to the recruitment of myelomonocytic cells to destructive and protective immune responses.

5 Future Directions

The studies highlighted in the previous section illustrate the key role of integrins and chemokines in the orchestration of neuroinflammation. Given that chemokines utilize G-protein-coupled receptors to signal, these receptors are attractive targets for

small-molecule inhibitors that may lead to the development of selective antagonists [106–108]. However, successful treatments that target chemokines may ultimately require that specific ligands or combinations of receptors be targeted for the most efficacious result. For example, many studies that have examined the involvement of α4β1/VCAM-1 in T cell entry into the brain support a direct inhibition of T cell adhesion to CNS vessels as the possible mechanism. Similarly, while it is clear that chemokines have a central role in neuroinflammation, how initial entry of cells into the brain or subsequent events are most critically influenced by these factors is still unclear. In many instances, interfering with these pathways results in differences in the number of inflammatory cells within the CNS, which may be due to defective priming, reduced recruitment across barriers into the CNS, or an inability to retain these cells within the tissue. The development of intravital imaging of individual cells combined with the generation of fluorescent reporter mice specific for different cell types (such as DCs, neutrophils, antigen-specific $CD4^+$ and $CD8^+$ T cells, microglia, astrocytes) and cytokines has made it possible to study the interaction between infiltrating immune cells with CNS-resident cells [1, 51, 109–111]. The application of this technology has the potential to fundamentally advance our appreciation for how immune cells enter and behave in the CNS and may lead to the refinement or discovery of therapeutic strategies to better manage neuroinflammation. This information will also be relevant to the development of complex therapies that use stem cells to repair tissue damage in the brain or chimeric antigen receptor (CAR) T cells to treat tumors that affect the CNS [112–114]. In particular, for CAR T cells, an understanding of the environmental cues provided by integrins and chemokines that influence T cell functions in the brain should inform the strategies used to engineer optimal tumor-specific effector T cells that can access and operate within the CNS.

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General Physiology and Pathophysiology of Microglia

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Abstract Microglial cells are of scions foetal monocytes that migrate into and disseminate within the central nervous system in early embryogenesis and in perinatal period. After invasion, microglial progenitors undergo specific metamorphosis and acquire a ramified morphological phenotype known as "resting or surveillant microglia." These cells in the healthy brain have highly motile processes by which they scan their territorial domains. Microglial cells also acquire multiple receptors to neurotransmitters and neurohormones and retain receptors associated with their immune and defensive function. Insults to the central nervous system of diverse aetiology trigger a complex, multistage activation process that produce multiple "activated microglia" phenotypes, which have both neuroprotective and cytotoxic capabilities. These phenotypes are most likely disease/pathology context specific, and the balance between neurotoxicity and neuroprotection are critical for the resolution and outcome of neuropathological processes.

Keywords Microglia • Phagocytosis • Activated microglia • Neuropathology • Neurotransmitter receptors • Neuroprotection • Neurotoxicity

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1 Introduction: Developmental Origins of Microglia

Microglial cells, identified by Pio del Rio Hirtega in 1919 [1–3], form an innate immune and defensive system of the central nervous system (CNS) and are of the myeloid lineage [4, 5]. Invasion of microglial progenitors that derive from the extra-embryonic yolk sac (more precisely from primitive c-kit+erythromyeloid precursors [6]) occurs very early in development (about embryonic day 10 in mice [7]), thus making microglial cells the very first glia (as astrogliogenesis and oligodendrogliogenesis begin in earnest only in a perinatal period). The second wave of migration of peripheral macrophages into the brain and the spinal cord takes place in a perinatal period being complete at about perinatal day 8 when the blood–brain barrier seals. The perinatal migration mainly occurs at the corpus callosum where "fountains" of invading myeloid cells can be readily observed [8].

The myeloid cells after entering the CNS disseminate almost homogeneously throughout the parenchyma of the brain and of the spinal cord and undergo remarkable remodelling of their phenotype that converts them into the highly distinct "resting" microglial cells. The appearance of these resting microglial cells is very different from the myeloid ancestors: resting microglia are characterised by a rather small cell body (4–6 μm in diameter), from which very thin and long processes emanate in all directions [5]. Microglial processes are in constant movement, being instruments of surveying the brain tissue within their reach. These processes also define the territorial domain of an individual microglial cell; domains of neighbouring microglia cells show very little, if any, overlap. Microglial processes move with remarkable speed (up to 0.1–0.3 microns per second) so that the territorial domains of a cell is scanned every several hours or so [9, 10]. This metamorphosis is quite remarkable and may be considered as the signal example of epigenetic adaptation of the cell to a specific environment.

2 Microglial Cell Integration into CNS Cytoarchitecture

The phenotypic remodelling of microglial cells in the nervous system environment goes far beyond the changes in their morphology. Microglia physiology is also profoundly affected that allows full integration into CNS cellular networks. First and foremost, microglial cells, in contrast to macrophages, express multiple receptors to neurotransmitters and neuromodulators. Resting microglial cells are in possession of a remarkable array of these receptors including many types of ionotropic and metabotropic receptors to glutamate, ATP, adenosine, acetylcholine, GABA, adrenaline and noradrenaline, serotonin and histamine (Fig. 1). These receptors control plasmalemmal ion fluxes and numerous intracellular signalling systems, most notably Ca^{2+} signalling, which is fundamental for physiological and pathological microglial responses. At the same time, microglial cells retain their myeloid heritage by expressing numerous receptors fundamental for immune responses such as $P2X_7$ purinoceptors, receptors to chemokines and cytokines and receptors to various tissue mediators such as

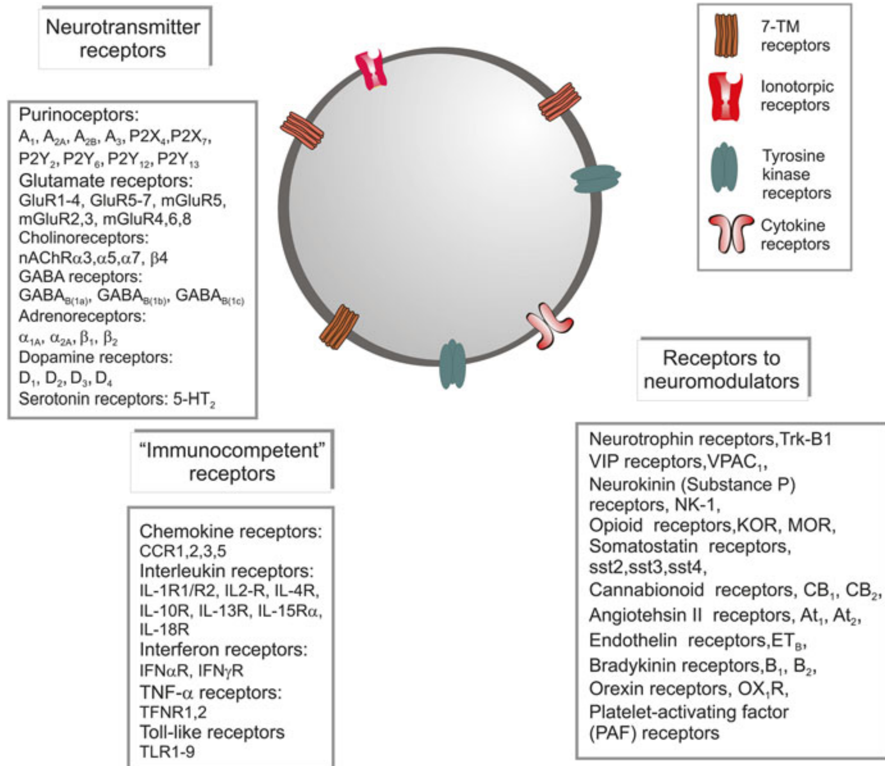

Fig. 1 A multitude of receptors expressed in microglia

platelet-activating factor, thrombin, histamine, bradykinin, etc. (for a comprehensive review of microglial receptors and exhaustive referencing, see [5, 11, 12]).

Microglia are important for developing, shaping and functional modulation of neuronal networks. At the early embryonic stages, microglial cells may assist synaptogenesis through secretion of trophic factors and possibly cholesterol. At later stages microglial cells are critical for synaptic pruning that streamlines neuronal ensembles. In the undisturbed brain, microglial processes frequently contact synaptic structures. These contacts occur on a constant basis with individual microglial process approaching presynaptic terminals and postsynaptic compartments, freezing there for about 5 min and then retracting again [13–15]. Interactions between microglial processes and synapses are regulated by neuronal activity; in the visual cortex, decrease in sensory input by light deprivation reduced the motility of microglial processes and increased the tendency of the latter to contact large dendritic spines. Re-establishing the light-induced input increased the motility of processes and the degree of their coverage of synaptic structures [13]. This specific behaviour arguably allows microglial cells to monitor synaptic activity and possibly contribute to the experience-dependent remodelling of synaptic connections. The actual mechanisms for synaptic to microglia signalling remain to be uncovered although

they are likely to involve activation of microglial neurotransmitter receptors. There is evidence, for example, for the role of ATP and purinoceptors in regulation of motility of microglial processes in vivo [9, 16, 17].

Microglial cells can signal back and modify neuronal networks by either removing synapses or secreting factors affecting neuronal and synaptic plasticity. In physiological conditions microglial cells may remove synapses by phagocytosis performed at the level of single processes without affecting the overall ramified phenotype [18]. This "physiological" phagocytosis can be visualised in acute brain slices. Arguably this process of removing synapses (known also as synaptic stripping or synaptic scaling) represents an important mechanism in controlling the number of synapses. There is evidence for the role of several specific microglial signalling systems in regulating this process. In particular, in genetically modified mice lacking the CX3R1 fractalkine receptor the transient increase in the spine density paralleled with an increase in the frequency of miniature excitatory postsynaptic currents was observed in developing hippocampus. These changes may indicate the deficit in the fractalkine-mediated microglia-dependent synaptic pruning that affects synaptic networking [19]. Similarly microglial complement receptors can contribute to the regulation of synaptic removal. In the developing brain, neurons express C1q complex that can act as a tag for synapses destined for elimination; synaptic removal subsequently is mediated through microglial C3a receptors. Removal of these receptors results in aberrant synaptic connectivity [20].

Microglia can directly influence synaptic transmission and synaptic plasticity through secretion of neuromodulatory factors. For example, microglia were reported to modulate neuronal NMDA receptors (through secreting glycine and L-serine [21]) or to affect intra-neuronal Cl^- concentration (through microglial release of BDNF), thus changing GABA-induced effects from inhibitory to excitatory [22]. The release of tumour necrosis factor-α from microglia was shown to stimulate astrocytes which in turn release glutamate acting on neighbouring synaptic structures [23]. Microglia-derived TNF-α also contributes to regulation of synaptic strength in visual cortex following monocular visual deprivation. Finally, microglial cells may affect neuronal networks through the regulation of adult neurogenesis, through controlling the integration of newly born neurons into the existing circuits and through the elimination of supranumerous neurones that undergo apoptosis [18]. All these multiple pathways for physiological interactions between microglia and neural networks are fundamental for normal development and function of the CNS; the microglia were even implicated in masculinisation of the brain and regulation of sexual behaviour [24].

The failure of microglia to perform their physiological duties may be relevant for numerous developmental brain disorders from autism to psychiatric diseases. Microglial phagocytosis, for example, is markedly impaired in mice deficient in the gene for methyl CpG-binding protein 2 (MECP2), which animals are considered a model for Rett syndrome. Clinically, Rett syndrome is manifested by an impairment of motor language and cognitive function, all these originating from defective synaptogenesis. Morphologically Rett syndrome is characterised by reduced dendritic arborisation, decreased number of spines and atrophy of neuronal somatas. It has been suggested that deficits in synaptic connectivity may result from the paralysis of

microglial phagocytosis. Specific expression of MECP2 gene in cells of myeloid lineage can partially alleviate the pathophysiological phenotype [25]. Similarly, deficient microglial function has been implicated in pathogenesis of pathological grooming behaviour observed in mice lacking Hoxb8 gene. The behavioural phenotype observed in these mice resembles a human disease trichotillomania also known as a compulsive hair-pulling disorder. Grafting of normal wild-type bone marrow cells into these mice reduced the behavioural deficit probably because of alien monocytes entering the recipient brain where they assume deficient phagocytic function [26].

3 Microglial Receptors

As has been mentioned above, acquisition of neurotransmitter receptors is the most striking part of microglial adaptation to the CNS environment. The most widespread and possibly most functionally important for microglia are purinoceptors [27].

The main type of ionotropic purinoceptors expressed in mature microglial cells are $P2X_4$ and $P2X_7$ receptors. The $P2X_7$ receptors in particular are involved in many types of neuropathology [28] and contribute to various aspects of microglial pathology. The $P2X_7$ receptors are unique among other members of P2X family in (1) exceptionally low sensitivity to ATP; the full activation of the receptors requires mM concentrations of ATP; (2) modulation of ATP sensitivity by extracellular divalent cations and (3) ability of $P2X_7$ receptors to form large transmembrane pores (permeable to molecules with m.w. up to 900 Da) upon strong stimulation. The pore formation most likely results from the dilation of the $P2X_7$ channel per se, although the role for some auxiliary proteins has been also debated [29]. $P2X_7$ receptors are abundantly present in immune cells and mediate many immune reactions, including the processing and the release of various cytokines. The $P2X_7$-mediated currents were for the first time identified in amoeboid microglial cells in situ [30]; simultaneously the $P2X_7$-mediated $[Ca^{2+}]_i$ increases were found in freshly isolated mouse microglia [31].

Microglial cells constitutively express $P2X_7$ receptors, and various brain lesions and neuropathologies (e.g. multiple sclerosis, amyotrophic lateral sclerosis and Alzheimer's disease) induce substantial up-regulation of $P2X_7$ receptor expression [27, 32]. The activation of $P2X_7$ receptors regulates multiple microglial processes from activation to apoptotic death. Stimulation of $P2X_7$ receptors was reported to be necessary for microglial activation by amyloid-β protein [33], and $P2X_7$ receptors control microglial secretion of pro-inflammatory factors (see [5] and references therein). Incidentally, direct over-expression of $P2X_7$ receptors in microglia triggers their activation in the in vitro system in complete absence of any other exogenous factors [34].

The $P2X_4$ receptors are also constitutively expressed in microglia and contribute to microglial activation in particular in the context of neuropathic pain. The role of $P2X_4$ receptors for tactile allodynia was suggested based on the pharmacological profiling and expression analysis following peripheral nerve lesion, intraperitoneal injection of lipopolysaccharide and in animals with experimental autoimmune neuritis [35]. Increased levels of $P2X_4$ receptors was found in activated (as judged by

positive staining with OX42) microglia, whereas intrathecal injection of cultured microglia bearing $P2X_4$ receptors induced allodynia in the absence of peripheral nerve damage [36].

In addition to ionotropic receptors, microglia express several metabotropic purinoceptors with predominant appearance of $P2Y_2$, $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ receptors. Stimulation of these receptors as a rule triggers Ca^{2+} signals that often involve store-operated Ca^{2+} influx; overstimulation of P2Y pathways can produce a long-lasting activation of the latter that can contribute to various aspects of microglial activation [37]. The $P2Y_6$ receptors characterised by special sensitivity to UDP regulate microglial phagocytosis [38], whereas ADP-preferring $P2Y_{12}$ receptors are fundamental for acute microglial responses to pathological insults, for morphological activation, membrane ruffling and chemotaxis [27]. In addition $P2Y_{12}$ receptors are linked to integrin-β1 signalling, which regulates extension of microglial processes [39]. In the spinal cord $P2Y_{12}$ receptors are involved in the genesis of neuropathic pain [36].

Microglial cells express ionotropic and metabotropic glutamate receptors. The ionotropic receptors are represented by all four types of AMPA receptors [40], at least three types of kainate receptors [41] and NMDA receptors which were identified only very recently and seem to be somehow linked to microglial neurotoxicity [42]. Microglia are also in possession of metabotropic receptors mGluR5 linked to intracellular Ca^{2+} signalling and mGluR2,3 (group II) and mGluR4,6,8 (group III) coupled to cAMP and involved in the regulation of TNF-α release (group II receptors) and containing microglial cytotoxicity (group III receptors) [43, 44]. Microglial cells express $GABA_B$ receptors linked to Ca^{2+} signalling and the activation of K^+ conductance [45], as well as several subunits of nicotinic acetylcholine receptors including neuronal α7 subunit that confers high Ca^{2+} permeability, and numerous metabotropic receptors including α_{1A}, α_{2A}, β_1 and β_2 adrenoreceptors, $D_{1\text{-}4}$ dopamine receptors and 5-HT_2 serotonin receptors [5].

In addition to receptors to neurotransmitters, microglia are able to sense a wide variety of neuromodulators and neurohormones. Microglial cells express all four types of adenosine receptors (which generally suppress activation process), receptors to bradykinin (with B_2 receptors being expressed in resting microglia, whereas B_1 receptors being upregulated during activation), ET_B endothelin receptors, angiotensin receptors (AT_2 receptors in resting cells and AT_1 in activated cells), somatostatin receptors, opioid receptors, neurotrophin receptors and various receptors to neuropeptides (see [5] for details). Microglial cells are further in possession of receptors to hormones, tissue and trophic factors, various blood factors, etc. In particular microglia have been found to express receptors to thrombin (PAR-1 to 4), cysteinyl leukotrienes receptors of CysLT1 and CysLT2 types, Notch-1 receptors, receptors to complement fragments C3a and C5a, macrophage colony-stimulating factor receptors, colony-stimulating factor-1 receptor, formyl peptide receptors FPR1 and FPR2, lysophosphatidic acid receptors LPA1 and LPA3 and many more [5].

Microglia also contain numerous receptors associated with defensive and immune functions. These are represented by diverse pattern-recognition receptors and by receptors to chemokines and cytokines. The pattern-recognition receptors are specifically designed to detect infectious agents. These receptors are generally classified

into (i) lectin-type, mannose and β-glucan receptors; (ii) nucleotide-binding and oligomerisation domain (NOD)-like receptors; (iii) receptors characterised by a RNA helicase domain and two caspase-recruitment domains (CARD), collectively known now as RIG-I-like receptors (RLR); and (iv) the Toll-like receptors [46–48].

These latter Toll-like receptors are particularly diversified in microglia being involved in the regulation of activation in response to multiple exogenous pathological factors. The activation of Toll-like receptors triggers several complex signalling cascades that often involve adaptor protein MyD88, transcription factors AP-1 and NF-κB [49, 50]. There are at least nine members of Toll-like receptor family, TLR1 to 9, which in the CNS are expressed mainly on glia and in particular they are present in microglial cells (see, e.g. [51–56].). The TLR1/2, 6/2, 4 and 5 are located on the cell surface, whereas TLR3, 7, 8 and 9 reside within endosomal compartments; different Toll-like receptors detect different infectious agents. The TLR1, TLR2 and TLR6, for example, are sensitive to bacterial tri- and diacyl lipopeptides, lipoteichoic acid and peptidoglycan; the TLR3 is activated by virus-specific double-stranded RNA; TKR4 is the receptor for LPS, a cell wall component of gram-negative bacteria that is an agent of choice for in vitro activation of microglia. The TLR5 detects bacterial flagellin; TLR7 and TLR8 are activated by viral RNA, whereas TLR 9 is sensitive to bacterial and viral unmethylated CpG DNA [57–60]. Stimulation of Toll-like receptors initiates the activation of microglia, and the type or receptor involved may define specific activation programmes.

Regulation of immune responses of microglia is mediated through receptors to cytokines and chemokines. Receptors to chemokines (the chemoattractive cytokines) are metabotropic 7-transmembrane domain G-protein-coupled receptors linked to multiple signalling cascades which include adenylate cyclase, phospholipases, GTPases (Rho, Rac and Cdc42) and some kinases such as mitogen-activated protein kinase (MAPK) or phosphatidylinositol-3 kinase (PI3-K) [61] are responsible for cell migration. Cytokine receptors are represented by TNF-α receptors, interleukin (IL) receptors and receptors to interferon β and γ. Microglial cells express two types of TNF-α receptors, TNFR1 and TNFR2 which positively modulate microglial activation and phagocytosis. The receptors that regulate multiple trophic and immune responses are represented by IL-1 type-I receptor (IL-1RI), IL-1 type-II receptor (IL-1RII) and IL-1 receptor accessory protein (IL-1RAcP) [5].

4 Activation of Microglia

Microglial cells form the innate defensive and immune system of the CNS. Therefore, the fundamental function of microglia is to detect pathology and to produce a defensive response. The constantly moving processes allow scanning of the brain parenchyma and multiple receptors expressed in microglial processes can detect danger signals. Detection of pathologically relevant signals triggers the activation of microglia. Microglial activation is primarily a defensive reaction which develops in many stages and results in multiple phenotypes that generally are aimed at neuroprotection although in certain conditions can contribute to cytotoxicity [4, 5, 11, 62].

4.1 Pathological Signalling of Microglia

The molecular cues for brain damage are many. It is generally believed that the activation of microglia is controlled through balancing the "ON" and "OFF" signals [4, 11, 63]. The "ON" signals are represented by molecules that are either absent in the healthy brain or are present in low concentrations. These signals appear in association with pathology and can be further classified into pathogen-associated or danger-associated molecular patterns (PAMPs or DAMPs). The PAMP signals are directly associated with pathogens and can be represented by fragments of bacterial cell walls, viral envelopes or their respective DNAs and/or RNAs. The DAMP signals are molecules normally present in the body, but either absent in the brain (e.g. blood factors entering the CNS following disruption of the blood-brain barrier, such as coagulation factors, immunoglobulins, albumin, thrombin, etc.) or localised intracellularly (enzymes and other proteins released following destruction of cells) or molecules normally utilised for intercellular signalling but appearing in unusually high concentrations (the best example being ATP, that is massively released following cellular stress or damage).

In contrast the "OFF" signals are represented by molecules associated with normal activity of neuroglial circuitry, for example, neurotransmitters, such as glutamate, GABA, ATP adenosine, etc. These are continuously signalling onto microglia to prevent their activation, and withdrawal of these molecules from the environment is indicative of stress and possible damage. These "OFF" signals are most likely responsible for linking neuronal activity with microglial status; suppression of neuronal firing triggers the activation of microglia [5]. In vagal motoneurons suppression of synaptic activity precedes and most likely initiates synaptic stripping [64]. Incidentally ATP may act as both an "ON" and an "OFF" signal; in low concentrations ATP (and its immediate metabolite adenosine) prevents microglial activation, whereas at high concentration ATP acts as a potent stimulator of microglial response. Finally microglial responses to injury are further regulated by molecules controlling microglial motility and phagocytosis. These signals are classified into "*find-me*" signals that attract microglial cells to the damaged site and "*eat-me*" signals that mark the pathological targets and induce phagocytosis [5, 65].

4.2 Multiple Phenotypes of Activated Microglia

The activation of microglia is a complex and multistage and often reversible process represented by a tightly regulated specific programme of gradual transformation of resting microglia into a multitude of activated phenotypes (Fig. 2). At the acute stage, focal lesion to a nervous tissue triggers rapid (~1.5 μm/s) movements of microglial processes converging on the site of damage in such a way that sooner or later it is completely covered by microglial processes [9]. The process motility is triggered by the activation of $P2Y_{12}$ metabotropic purinoceptors [17] and may represent the very early defensive activation response that may completely restore

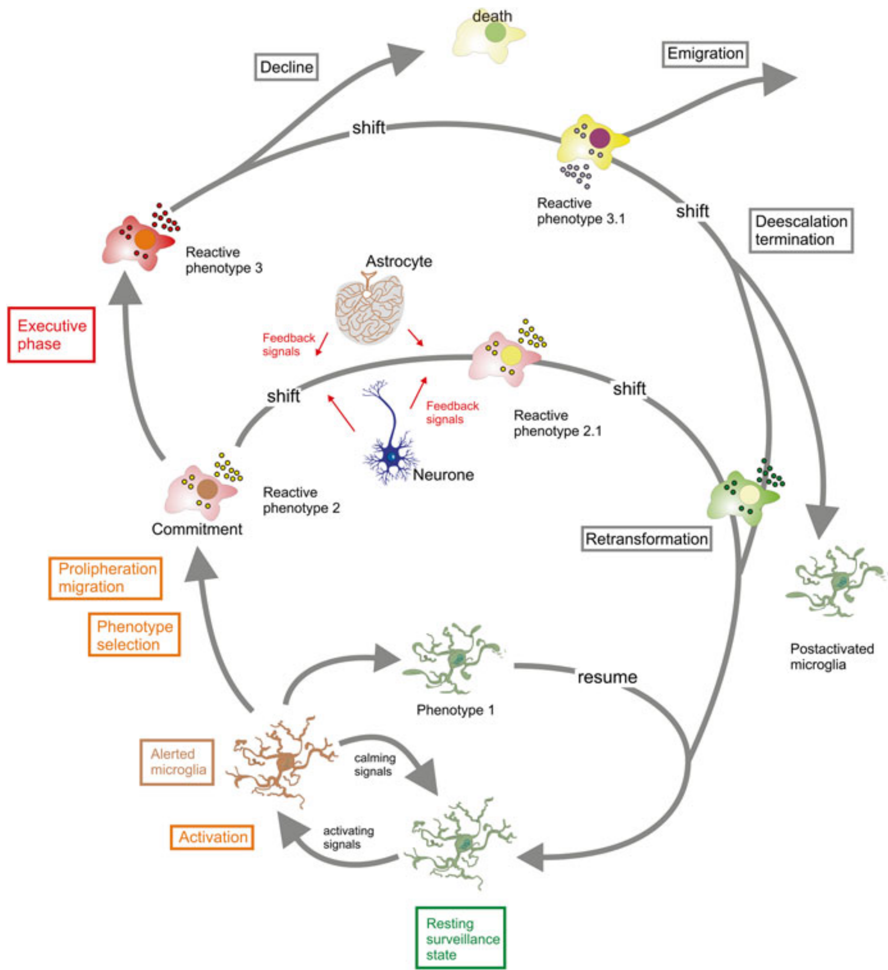

Fig. 2 Microglial activation as a continuous multistage process. The "resting" microglia constantly and actively scan their environment for exogenous or endogenous signals indicating a threat to homeostasis. Sudden appearance of "activating" signals or a loss of constitutively "calming" inputs can then trigger transitions to alerted and activated states. Cells can commit to distinct reactive phenotypes depending on the challenging stimuli and the situational context. Initial response profiles may further shift as instructed by additional influences. Not only resident CNS cells but also invading immune cells would exert such modulating influences. Initial reactive phenotypes with defence orientation may convert to repair-orientated activity profiles. Cells may eventually return to a resting state or stay "experienced." "Experienced" microglia could reveal altered responsiveness and exert distinct responses upon re-challenge. Reproduced from Kettenmann et al., 2011

limited injuries. This early "processes-executed" microglial response may also be regulated by astrocytes releasing ATP through hemichannels.

More extensive (in strength and space) lesions induce microglial transformation into reactive phenotypes. At initial stages of activation, microglia remodel their morphological appearance: the processes become fewer and thicker, some of them retract altogether and the soma size is increasing. In parallel microglial cells undergo

biochemical and biosynthetic metamorphosis, change their receptor expression and begin to secrete immune and pro-inflammatory factors [4, 66–68]. Some of the reactive microglial cells become proliferative resulting in an increase in microglial numbers around lesions. With activation proceeding, microglial cells become motile, acquire an amoeboid morphology and gather around lesioned site(s). The end point of the activation spectrum is manifested by a phagocytic phenotype that represents a final and irreversible stage of microglial activation. These main stages of microglial activation can differ between brain regions and pathological context; all in all the total number of activated phenotypes is most likely much larger that we can define currently using various (mostly derived from experiments on peripheral monocytes) markers.

4.3 Balancing Neuroprotection and Neurotoxicity

The complex programmes of microglial activation are launched very rapidly after the insult and most likely already at this stage show specificity associated with the nature of pathological damage. The activation of microglia results in profound remodelling of their physiology and biochemistry. Activated microglia alter the expression pattern of ion channels (e.g. by up-regulating inward and delayed rectifier K^+ channels [69]) and neurotransmitter receptors (e.g. purinoceptors that undergo rapid changes following activation [70, 71]). Microglia substantially increase their motility (which requires complex and coordinated changes in ion and water channels and transporters [5]). Finally, microglial cells remodel synthesis of enzymes and various inflammatory factors.

All these concerted changes produce many phenotypes of activated microglia, these phenotypes being selected for the defence of the nervous system against pathology. The defence conceptually requires balancing of two opposite arms, cytotoxic and neuroprotective. Activated microglia are in full possession of both as indeed neuroprotective as well as cytotoxic phenotypes are well characterised. Further it is possible that the very same microglial cell may simultaneously execute neuroprotection and cytotoxicity or fluctuate between these two functional extremes. This reflects a high plasticity of activated microglia which may rapidly adopt to changing pathological environments. As a result it is almost impossible to unequivocally define microglial status based on morphology or surface markers. The rather popular division of activated microglia into M1 (neurotoxic phenotype associated with activation of Toll-like or interferon γ receptors) and M2a-c (neuroprotective or regeneration-friendly or deactivating phenotypes induced following stimulation of IL 4 or IL 13, see [62, 72–75]) seems to be an oversimplification [76]. Microglial cells present many phenotypes which can execute a continuum of function which all have a global defensive meaning. Indeed to resolve a foreign invasion, for example, both infectious agent and damaged cells have to be effectively killed and ensuing debris removed, and thus cytotoxicity has been selected for its beneficial potential. Microglial cells are indeed in full possession of cytotoxic agents, such as reactive oxygen species or pro-inflammatory factors. In addition microglial cells are true phagocytes and thus indispensable for post lesion cleaning of the tissue. Neuroprotective function of

microglia is similarly assisted by numerous molecular cascades. For example, activated microglial cells are able to contain glutamate overload (by up-regulating expression of GLAST/GLT-1 glutamate transporters [77, 78]) and assist neuronal survival by secreting numerous growth factors such as, for example, NGF or BDNF [79, 80].

Finally, the activation of microglia is a reversible process that brings activated microglia to resting form after the pathological factor is attenuated [4]. How this "deactivation" of microglia proceeds remains unknown although it is possible to speculate that it may involve removal of pathology-associated signals, restoration of normal chemical environment and possibly appearance of special "terminating" factors.

5 Conclusions

Microglial cells are fundamental elements of neuropathology. In the resting conditions, these myeloid cells are important part of brain cytoarchitecture, which contribute to the development shaping and normal function of synaptically connected neuronal ensembles. Microglial cells are constantly scanning the brain tissue for any signs of damage and when these latter occurs, microglia launch a defensive response generally known as microglial activation. This microglial activation is governed by complex and multifaceted programmes that may produce many cellular phenotypes endowed with both neuroprotective and cytotoxic functions. These phenotypes are most likely disease/pathology context specific, and the balance between neurotoxicity and neuroprotection is critical for the resolution and outcome of neuropathological process.

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Inflammation and the Pathophysiology of Astrocytes in Neurodegenerative Diseases

Gordon P. Meares and Etty N. Benveniste

Abstract Astrocytes, the most abundant cell in the central nervous system, are essential for brain function and homeostasis. This chapter focuses on the immunological role of astrocytes in the pathology of major neurodegenerative diseases. Astrocyte activation, or astrogliosis, has been observed in many neurodegenerative diseases. Factors associated with neurodegeneration including extracellular oligomerized proteins such as amyloid β and α-synuclein as well as inflammatory cytokines and chemokines can influence the functionality of astrocytes. In response to such stimuli, astrocytes produce a multitude of soluble factors including cytokines, chemokines, reactive oxygen/nitrogen species, and growth factors. This astrocytic response is initially protective, limiting damage and promoting functional recovery. However, the prolonged and progressive nature of neurodegenerative diseases establishes an environment in which astrogliosis may be aberrantly sustained, and the ongoing production of astrocyte-derived molecules contributes to the non-resolving inflammatory and neurotoxic landscape associated with neurodegeneration.

Keywords Astrocyte • Glia • Microglia • Inflammation • Cytokine • Chemokine • Interleukin • Interferon

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Abbreviations

6-OHDA	6-hydroxydopamine
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
Aβ	Amyloid β
BBB	Blood–brain barrier
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
DAMP	Damage-associated molecular pattern
GDNF	Glial-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
HD	Huntington's disease
IFN	Interferon
ISG	Interferon-stimulated genes
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple sclerosis
NFT	Neurofibrillary tangles
NF-κB	Nuclear factor-kappa B
NOD	Nucleotide-binding oligomerization domain
PGD2	Prostaglandin D2
PRR	Pattern recognition receptor
STAT	Signal transducer and activator of transcription
SN	Substantia nigra
SOD1	Superoxide dismutase 1
TBI	Traumatic brain injury
Th	T helper
TLR	Toll-like receptor

1 Introduction

Astrocytes are intriguing and remarkable cells controlling virtually every facet of central nervous system (CNS) functions. Astrocytes work together with neurons, microglia, oligodendrocytes, endothelial cells, and other cells to ensure harmonious function within the unique environment of the CNS. For example, astrocytes form the tripartite synapse where they take up glutamate as well as synthesize and release glutamine for use by neurons for conversion to glutamate, together ensuring proper neurotransmission. Additionally, astrocytes release trophic factors including brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and others. Astrocyte end-feet interact with the neurovasculature and influence blood–brain barrier (BBB) function. The many diverse functions of astrocytes are too complex and

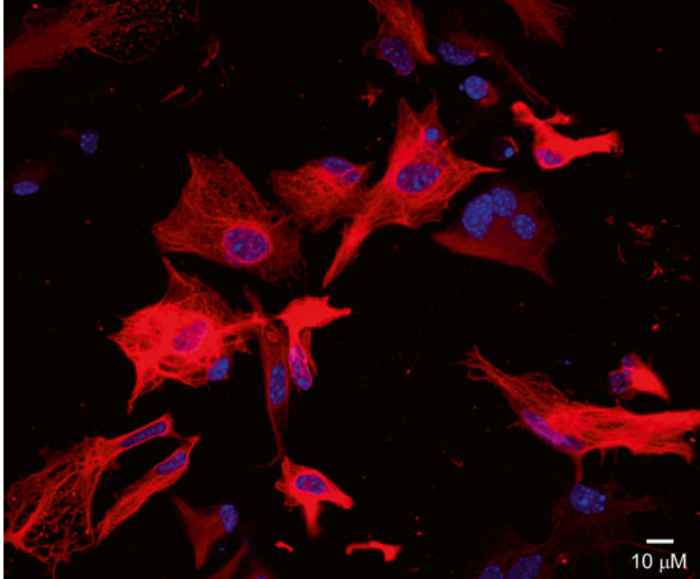

Fig. 1 GFAP expression in murine astrocytes. Astrocytes were isolated from the telencephalon of P1 pups and expanded in culture for 14 days. Cells were then stained for GFAP (*red*) and nuclei (DAPI, *blue*) and imaged by confocal microscopy. Notice the mesh-like network of GFAP

numerous to describe in detail here; however, there are a number of excellent reviews describing the phenotypic and functional characteristics of astrocytes [1–5].

The focus of this chapter is to describe the role of astrocytes in neuroinflammation in the context of neurodegenerative diseases. Microglia are typically thought of as the main innate immunity effector cell in the CNS because of their macrophage-like phenotype, robust inflammatory responses, and ability to present antigen via major histocompatibility complex (MHC) class II. It is now appreciated that astrocytes have important innate immune functions as well [6]. In response to injury, infection, disease, or any disturbance, astrocytes undergo a phenotypic change known as astrogliosis. Widely characterized as the increased expression of the intermediate filament protein glial fibrillary acidic protein (GFAP), astrogliosis involves a host of transcriptional, translational, and phenotypic changes aimed at resolving and limiting damage to the CNS [2]. GFAP is expressed at variable levels in unstimulated astrocytes and forms a fibrous network typical of cytoskeletal proteins (Fig. 1). Additionally, astrocytes express pattern recognition receptors (PRR), although their repertoire is more restricted than that of microglia. Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns and astrocytes express TLR2, TLR3, TLR4, TLR5, and TLR9. TLR3, which recognizes double-stranded RNA, appears to be the most abundant TLR expressed by astrocytes [6]. In addition, astrocytes express nucleotide-binding oligomerization domain (NOD) proteins that recognize bacterial components [7]. Astrocytes can also sense and respond to damage-associated molecular patterns (DAMPs) such as ATP through purinergic receptors and the multi-protein NLRP2

inflammasome [8, 9]. Astrocytes respond to interferons (IFN) and a wide array of cytokines and chemokines. When stimulated, astrocytes in turn produce many cytokines and chemokines including IL-1, IL-6, LIF, CNTF, IL-8, IL-10, IFN-α, IFN-β, M-CSF, GM-CSF, TNF-α, TGF-β, CCL2, CCL3, CCL4, CCL5, CCL20, CXCL10, and CXCL12 [10–12]. In addition, inflammatory stimulation of astrocytes can lead to the production of the free radical nitric oxide (NO) which is toxic to neurons and oligodendrocytes and may promote neurodegeneration [13]. While astrocytes contribute to the local inflammatory response, they are also essential to limit and resolve CNS inflammation. Following traumatic brain injury (TBI), as well as other insults, astrocytes proliferate and form a glial scar around the injury [2]. The selective ablation of proliferating astrocytes following TBI in mice results in a prolonged inflammatory response and increased neuronal degeneration [14]. In acute conditions such as injury or infection, the astrocytic response is paramount to reestablish homeostasis in the CNS. However, in chronic conditions such as neurodegenerative diseases, astrocytes may eventually contribute to pathology.

2 Astrocytes in Multiple Sclerosis

Multiple sclerosis (MS) is a debilitating T cell-mediated autoimmune disease in which leukocytes (T cells, macrophages, neutrophils, and others) invade the CNS, leading to demyelination and axonal degeneration, eventually resulting in permanent disability. The etiology of MS is complex, involving genetic, environmental, and geographic factors, and usually develops in young adults (20–40 years of age) with a bias toward females [15]. MS initially manifests as highly variable transient episodes disrupting sensory and/or motor function, followed by full or partial recovery and disease remission (relapsing-remitting MS). In conjunction with symptoms, inflammatory lesions are also observed in the brain and spinal cord. MS lesions are areas of demyelination and inflammation involving invading peripheral leukocytes as well as resident glial cells. Cytokines and chemokines are key players in this inflammatory attack. Cytokines including IFN-γ, IL-17, and IL-6 are elevated in MS lesions as are the C-C chemokines CCL2, CCL3, CCL4, CCL5, CCL7, and CXCL12 [16–18]. MS patients have multiple attacks causing incremental damage to the CNS, and many patients progress to secondary progressive MS, where remission and recovery are reduced [19, 20]. Additionally, cognitive impairment is observed in at least 50 % of MS patients, contributing to disability and reduced quality of life [21]. Treatments for MS including IFN-β, glatiramer acetate, fingolimod, and others have greatly improved the quality of life for many MS patients; however, not all patients respond to or can tolerate these treatments [22, 23]. As such, new therapeutic targets for the treatment of MS are greatly needed.

The animal model of MS, experimental autoimmune encephalomyelitis (EAE), has greatly facilitated understanding the immunological interactions with the CNS. Although EAE is by no means a perfect replica of human MS, it shares many similar pathological features. EAE can be induced in a number of animals including nonhuman primates, rabbits, guinea pigs, hamsters, rats, and mice with an array of

protocols and CNS antigens [24, 25]. Most current research utilizes the murine model. EAE, like MS, is a demyelinating disease involving perivascular infiltration of peripheral immune cells and axonal degeneration, manifesting with physical symptoms in a relapsing-remitting and/or progressive fashion. T helper (Th) cells, specifically IFN-γ-producing Th1 cells and IL-17-producing Th17 cells, are the main effector cells in the initiation of EAE [26].

In postmortem studies of MS lesions, markers of Th1 and Th17 cells have been described, among other cell types [27]. Coincident with infiltration of leukocytes, astrocyte damage and hypertrophy have been observed in MS lesions [28]. Moreover, astrogliosis is present in the CNS of MS patients [1]. These examples highlight an abundance of data that suggest astrocytes are important players in the pathogenesis of MS. This has been supported by studies in EAE. Astrocyte activation, as measured by GFAP expression, correlates with or precedes the onset of clinical symptoms [29–31]. Additionally, there is astrocyte proliferation within the white matter of the spinal cord [32]. Astrocytes in MS and EAE produce the potent leukocyte-attracting chemokines CCL2 [33, 34] and CCL20 [12, 35] among others, and disruption of either of the receptors for these chemokines, CCR2 and CCR6, respectively, results in amelioration of EAE [36, 37]. While astrocytes produce chemoattractants, they also form a barrier around perivascular lesions in EAE to block further leukocyte infiltration into the healthy parenchyma [38]. IL-6 is a multifaceted proinflammatory cytokine that is elevated in the CNS following injury or in diseases including MS [39]. Astrocytes are a major source of endogenous IL-6 in the CNS, and IL-6 drives its own expression through autocrine signaling in conjunction with the soluble IL-6 receptor (trans-signaling) [40, 41]. Transgenic mice expressing IL-6 under the control of the GFAP promoter alters EAE disease such that inflammatory leukocytes invade mainly the cerebellum rather than the spinal cord [42]. Disruption of gp130, the common signal-transducing receptor for the IL-6 family of cytokines, in astrocytes leads to exacerbated EAE, indicating that astrocytes also have a key role in limiting disease [43]. Additionally, the importance of astrocytes in EAE was further established in a recent study which demonstrated that intact IL-17 signaling in astrocytes is required for induction of disease [44]. Moreover, IL-17 enhances IL-6-induced IL-6 and CCL20 expression in astrocytes [45, 46]. This likely reflects the cooperative actions of the IL-6-induced transcription factor STAT3 and the IL-17-induced transcription factor NF-κB [47, 48]. Disruption of NF-κB activity in astrocytes ameliorates CNS inflammation and EAE disease severity [49, 50]. In MS and EAE, Th1 cells and Th17 cells contribute to the pathogenesis of disease. However, IL-4-producing Th2 cells and T regulatory cells (Tregs) are protective in EAE models [51, 52]. Thus, the repertoire of T cells interacting with the CNS is critical to the outcome of disease, and astrocytes influence this through production of chemoattractant molecules. For example, during EAE, astrocytes produce CXCL10 which recruits T cells, the monocyte chemoattractant CCL2, as well as CCL20 that can recruit both Th17 cells and Tregs [34, 35, 53, 54]. Additionally, as nonprofessional antigen-presenting cells, astrocytes, in an IFN-γ-inducible fashion, can express major histocompatibility complex (MHC class II) and present myelin-derived autoantigens to encephalitogenic T cells [55–57], potentially providing a stimulus for reactivation of T cells in the CNS. Collectively, these studies indicate that astrocytes are active participants in MS and EAE pathology and are potential therapeutic targets.

3 Astrocytes in Alzheimer's Disease

Alzheimer's disease (AD) is a progressive neurodegenerative disease that robs individuals of their memory and reduces cognitive function. Extracellular amyloid β (Aβ) deposition and tau-containing neurofibrillary tangles (NFTs) are hallmarks of AD pathology [58]. However, beginning with Alois Alzheimer's initial description of AD more than 100 years ago, alterations in glial cells have also been appreciated [59]. Upon postmortem analysis, brains from patients with AD display clear astrogliosis, and the levels of GFAP inversely correlate with cognitive function [60, 61]. Astrocytes influence several features of AD. Astrocytes are thought to phagocytosis Aβ [62], and blockade of astrocyte activation in a transgenic AD mouse model increases Aβ plaque burden [63]. The exact mechanisms leading to gliosis in AD is not well understood. Fibrillar Aβ1-42 can stimulate pattern recognition receptors, including the lipopolysaccharide (LPS) coreceptor CD14 and the NOD-like receptor NALP3, leading to microglial activation and production of IL-1β [64, 65]. Similarly, astrocytes express TLRs and NLRs which may engage Aβ1-42 and promote astrogliosis, but this has not yet been formally demonstrated. However, several molecules have been implicated in mediating various astrocytic responses to Aβ including low-density lipoprotein receptors, aquaporin-4, adenosine A2A receptor, as well as the scavenger receptors CD36 and CD47 [66–69]. More recently, Aβ was shown to interact with the α7 nicotinic acetylcholine receptor and promote astrocytic glutamate release [70, 71]. While astrogliosis is initially beneficial, the long-term production of cytokines and chemokines may be deleterious and promote AD pathology.

Glial-derived IL-1 and IL-6 are important proinflammatory cytokines elevated in the brain of patients with AD [72, 73]. From animal studies, we have learned that these cytokines may be active participants in AD pathology. Transgenic AD mice (Tg2576) that express an APP mutant associated with early onset familial AD have increased IL-6 in the brain that precedes detectable Aβ plaques [74]. Moreover, IL-6 expression, in the same transgenic mouse model, persists into the established disease state with IL-6-producing astrocytes observed near Aβ deposits [75]. Mice overexpressing IL-6 in astrocytes have learning defects, suggesting that IL-6 may exacerbate cognitive decline [76]. Moreover, IL-1β directs astrocytes to produce IL-6 [77]. As mentioned previously, astrocytes are a potent source of chemokines that likely help to recruit and direct the peripheral monocytes observed in the AD brain [78]. Direct injection of IL-1β into the rat forebrain leads to prolonged astrocyte activation with concomitant increases in GABA and glutamate [79]. Elevated glutamate may be associated with the ability of Aβ1-42 to reduce astrocyte-dependent glutamate clearance [80]. Astrocytes stimulated with IL-1β also secrete S100B [81]. Secreted S100B has cytokine-like functions and at low concentrations is neurotrophic. However, extracellular S100B is elevated in neurological disorders including AD, and at higher concentrations S100B can promote neuronal cell death [82]. Further, antibody-mediated blockade of IL-1β in 3×Tg-AD mice, which express mutants of APP, presenilin, and tau, reduces S100B expression, tau pathology, and disease pathology [83, 84]. Nitric oxide may also play an important role in AD. Mixed glial cultures respond to Aβ peptides with increased production of IL-1β and TNF-α that leads to increased

expression of iNOS and synthesis of nitric oxide [85]. In AD astrocytes appear to be the main source of nitric oxide [86]. Nitric oxide is neurotoxic and may facilitate neurodegeneration in AD [87, 88]. Moreover, stimulation with the microglial- and astrocyte-derived cytokines IL-β, TNF-α, and IFN-γ can also stimulate nitric oxide production with subsequent neurotoxicity from astrocytes [89]. Astrocytes can also modulate microglial function through the production of soluble cytokines and chemokines. Astrocyte-produced S100B can stimulate activation of microglia that includes the production of IL-1β [90], potentially reinforcing or promoting astrogliosis. Additionally, inflammatory cytokines, as well as Aβ fibrils, can also stimulate astrocyte- and neuron-dependent APP expression and Aβ production [91–93]. Ultimately, the interactions between cytokines (particularly IL-1) with neurons, microglia, and astrocytes drive a cycle of inflammation and Aβ production that culminates in neurological dysfunction and cognitive decline [94].

4 Astrocytes in Parkinson's Disease

Parkinson's disease (PD) is characterized by the selective loss of dopaminergic neurons in the substantia nigra (SN) and the associated physical manifestations. In addition to dopaminergic neurodegeneration, neuropathology includes the accumulation of α-synuclein-containing Lewy bodies, activated microglia, infiltrating $CD4^+$ and $CD8^+$ T cells, and increased numbers of astrocytes surrounding dopaminergic neurons [95–97]. Elevated levels of cytokines including TNF-α, TGF-β1, IL-1β, IL-6, IL-2, IFN-γ, and reactive oxygen/nitrogen species are also observed in brains from PD patients [98]. These findings (and many others) indicate an ongoing, nonresolving inflammatory reaction in the brain of PD patients.

Several animal models suggest that inflammation is important in the pathogenesis of PD. Mice expressing human α-synuclein driven by the thy1 promoter display activated microglia and elevated TNF-α as early as 1 month of age in the striatum [99]. Importantly, the striatum contains axon terminals emanating from the SN as part of the nigrostriatal pathway. These findings support the idea that inflammation maybe a key participant in neurodegeneration and not just a consequence of tissue damage [99]. In toxin-induced models, including MPTP and 6-OHDA, inflammatory cytokines and activated microglia are present [100]. MPTP intoxication leads to prolonged (years) glial activation, suggesting glial cells are involved in the pathological outcome. Direct injection of LPS is toxic to dopaminergic neurons [101], indicating that inflammation, even in the absence of disease, can recapitulate the cell death seen in PD. Additionally, LPS can synergize with MPTP to induce dopaminergic neuronal cell death in neuron-glia cocultures [102]. LPS-induced neuronal death is likely indirect. In support of this are in vitro studies demonstrating that microglia and astrocytes work in concert to drive neurotoxicity in response to LPS [103].

Astrocyte accumulation of α-synuclein is observed in the PD brain [104], and recent findings suggest that α-synuclein can be transmitted from neurons to surrounding cells [105]. Indeed, astrocytes can take up α-synuclein via endocytosis. Not only do astrocytes take up α-synuclein, but an inflammatory reaction is stimu-

lated that includes production of IL-6 and TNF-α as well as chemokines and matrix metalloproteinases (MMPs) [105]. Moreover, transgenic mice expressing a mutant α-synuclein associated with familial PD, A53T α-synuclein, in astrocytes display paralysis and mortality. This is associated with widespread gliosis and increased expression of TNF-α, IL-1β, and IL-6 in the brainstem. Conditioned media from the A53T α-synuclein-expressing astrocytes stimulated IL-1β and Cox1 expression in microglia [106]. These findings suggest that the effects of α-synuclein on astrocytes may contribute to the pathology of PD.

Astrocytes can have both protective and neurotoxic effects. Alpha-synuclein can enhance IL-1β-induced CXCL10 expression in astroglial cultures through mRNA stabilization [107]. CXCL10 is toxic to neurons; this has been demonstrated in the cholinergic LAN-2 cell line and in mixed human fetal neurons [108, 109]. While the dark pigment found in the SN, neuromelanin, attenuates astrocyte-derived CXCL10 [107], the direct influence of CXCL10 on dopaminergic neurons has not been examined. Astrocyte expression of the antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) in the Thy1-hSYN^{A53T} mice protects motor neurons, reduces synuclein aggregates in the brain and spinal cord, and enhances overall survival. Additionally, Nrf2 expression reduces gliosis [110].

The exact mechanisms responsible for activation of the glial reaction in PD are unknown. Gliosis is observed in PD patients and most animal models and once active a self-perpetuating inflammatory reaction may result. In adult macaques injected with MPTP, persistent astrogliosis is observed as well as elevated IFN-γ and TNF-α in the SN. Mice lacking either IFN-γ or TNF-α have attenuated gliosis following MPTP treatment [111].

Some mutations associated with familial forms of PD have been shown to alter inflammatory responses. Cortical slices from PINK1$^{-/-}$ mice have increased production of TNF-α, IL-1β, and IL-6 [112]. The cytokines produced suggest activation of microglia and astrocytes, although it should be noted that PINK$^{-/-}$ astrocytes are dysfunctional in their proliferative capacity and do not have elevated GFAP expression [113]. Astrocytes deficient for DJ-1 are more sensitive to LPS-induced inflammatory gene expression [114]. Mutations in Nurr1 are associated with a rare form of PD. Nurr1 suppresses inflammation in microglia and astrocytes through repression of NF-κB, and loss of Nurr1 enhances astrocyte-derived neurotoxic molecules [103]. This indicates that astrocytes are involved in both sporadic and familial PD.

5 Astrocytes in Huntington's Disease

In contrast to most neurodegenerative diseases in which the etiology is unknown, we know that Huntington's disease (HD) is caused by a CAG expansion (poly Q) in the huntingtin gene, leading to neuronal loss in the striatum and cortex [115, 116]. As with other neurodegenerative diseases, inflammation is likely a key player in HD. Immune activation is detectable in the periphery and CNS of HD patients. Elevated plasma levels of IL-6, IL-8, IL-4, IL-10, TNF-α, and IL-5 have been shown in HD patients compared to healthy controls. Similarly, IL-6 and IL-8 levels are elevated

in the CSF and in striatal tissue [117]. In addition to elevated cytokines, cellular alterations including microglial activation and astrogliosis are present in HD [118].

The role of astrocytes in HD is multifaceted, involving the production of inflammatory mediators as well as the potential loss of neuronal support. The expression of mutant huntingtin is not restricted to neurons; it is also expressed in astrocytes and peripherally. The targeted expression of mutant htt (160Q) in astrocytes leads to neurological and motor dysfunction [119], suggesting that astrocytes can directly influence HD pathology. A key function of astrocytes is to support neurons through the secretion of neurotrophins and buffering of extracellular glutamate. Evidence from HD mouse models suggests that astrocyte dysfunction may be an important aspect of the disease. Astrocytes produce BDNF, and this was found to be impaired in astrocytes expressing a mutant huntingtin fragment (htt552-100Q) [120]. Others have shown that astrocyte-produced BDNF provides therapeutic benefit. Astrocyte-targeted overexpression of BDNF attenuated quinolinate-induced lesions [121]. Additionally, viral delivery of BDNF driven by the GFAP promoter delayed disease progression in the R6/2 HD mouse model which expresses the 5′end of human huntingtin with 115-150 CAG repeats [122, 123]. Similarly, delivery of GDNF protects neurons and reduces disease severity [124, 125]. In vitro, astrocyte-conditioned media protect a striatal neuronal cell line expressing huntingtin Q111 from oxidative and excitotoxic cell death [126].

Evidence suggests that excitotoxic injury is an underlying mechanism of striatal neuronal loss in HD [127]. The uptake of glutamate, the main excitatory neurotransmitter, is impaired in the prefrontal cortex of HD patients [128]. Expression of mutant huntingtin in astrocytes reduces glutamate transporter expression and impairs the ability of astrocytes to take up glutamate. Moreover, in a coculture system, mutant htt-expressing astrocytes were less efficient at protecting neurons from glutamate-induced excitotoxicity [129]. In an in vivo model in which striatal astrocytes express mutant htt, reduced expression of glutamate transporters GLAST and GLT-1 as well as impaired glutamate uptake was observed. In addition, these mice displayed astrogliosis and neuronal dysfunction [130]. The ability of mutant htt to impair astrocyte-dependent glutamate handling may potentiate neuronal death in HD.

Despite evidence of microglial, astrocytic, and complement activation in the brains of HD patients, few studies have examined the contribution of glial cells to inflammation in HD [131]. In line with previous studies that mutant htt impairs astrocyte function, astrocytes from R6/2 mice express and secrete less CCL5 (RANTES). Impaired secretion results in aberrant accumulation of CCL5 in astrocytes and is observed in HD mouse models and in HD patients [132]. CCL5 has neurotrophic effects and its reduction may contribute to HD pathogenesis [132]. A recent study has examined the inflammatory responses in HD mice and astrocytes. In Hdh150Q mice, acute LPS treatment leads to enhanced TNF-α and IL-1β production in the cortex, striatum, and periphery [133]. Not only is the initial inflammatory reaction greater in the mutant htt mice, it is also prolonged. The enhanced inflammation was associated with excessive NF-κB activation in astrocytes. A single injection of LPS resulted in chronic inflammation and accelerated disease in the R6/2 mice. In addition, isolated R6/2 astrocytes stimulated with LPS produced higher levels of nitric oxide and were more toxic to isolated neurons [133].

6 Astrocytes in ALS

Amyotrophic lateral sclerosis (ALS) is caused by the selective degeneration of motor neurons resulting in progressive paralysis and premature death. In most cases, ALS is sporadic with unknown etiology. In a small number of cases, ALS is caused by mutations in the gene encoding superoxide dismutase 1 (SOD1). Through the use of SOD1 mutant mice, the mechanisms and cells involved in pathogenesis have been examined, and the non-cell autonomous processes involved in ALS have gained attention. Collectively, it appears that disease onset is determined by motor neurons, most likely through mutant SOD1-dependent damage; however, other cells including microglia and astrocytes are important in overall disease progression [134]. Consistently, ALS patients display activated microglia and astrocytes and increased expression of proinflammatory cytokines [135].

Glial activation is observed in the postmortem analysis of patients with ALS and in ALS animal models. It is likely that astrocytes and microglia work together, along with other cells types such as peripheral leukocytes, to modulate disease pathology. Using a SOD1^{G37R} mouse model in which mutant SOD1 could be deleted from astrocytes, Yamanaka and colleagues demonstrated that disease onset was unaffected, but disease progression was greatly attenuated [136]. Although astrogliosis, based on GFAP expression, was not reduced by astrocyte-selective ablation of SOD1^{G37R}, microgliosis was diminished. Concomitant with reduced microglial activation was a reduction in the expression of iNOS. These studies indicate that mutant SOD1-expressing astrocytes can influence disease progression in part through modulation of microglia [136]. Expression of SOD1^{G37R} in astrocytes elicits an inflammatory response and toxicity toward motor neurons in coculture. This includes elevated expression of iNOS and NOX2 with increased production of nitric oxide and reactive oxygen, respectively. The antioxidant apocynin attenuated astrocyte-produced ROS and motor neuron toxicity [137]. Accordingly, motor neurons are sensitive to NO-induced cell death, most likely through reaction with superoxide to form highly reactive peroxynitrite [138]. Additionally, astrocytes derived from both familial and sporadic ALS patients are toxic to motor neurons. This toxicity was associated with upregulation of a number of astrocyte-produced inflammatory molecules including several C-C and C-X-C chemokines, TNF and IL-8 [139]. Similarly, mutant SOD1-expressing mouse astrocytes are toxic to primary motor neurons in coculture [140]. Expression of SOD1^{G93A} alters inflammatory gene expression in astrocytes leading to upregulation of CCL8, CXCL7, and CCL5 [141]. In addition the prostaglandin D2 (PGD2) receptor was markedly increased. While these chemokines do not mediate the astrocyte-dependent toxicity toward motor neurons, blockade of the PGD2 receptor attenuated cell death, suggesting that prostaglandins may have role in motor neuron death [141].

IFN-γ has also been implicated in the demise of motor neurons. SOD1^{G93A}-expressing astrocytes produce IFN-γ, and antibody-mediated neutralization of IFN-γ blocks astrocyte-dependent toxicity toward motor neurons in this model [142]. The toxic effects of IFN-γ are mediated in part through stimulation of the TNF family member, LIGHT (TNFSF14), from motor neurons which binds the lymphotoxin-β

receptor (LT-βR) in an autocrine fashion, activating a pro-death signaling cascade. Consistent with a role for astrocytes in driving disease progression, deletion of LIGHT delays disease progress but not onset [142]. Type I IFNs may also have a role through stimulation of interferon-stimulated genes (ISGs) in astrocytes. ISG15 was reported to be elevated in human ALS and mouse spinal cords. Deletion of IFNAR1 delayed disease progress but not onset in SOD1G93A mice [143]. Thus, astrocyte-dependent production and responses to IFNs may have important roles in the progression of ALS.

7 Conclusions

Astrocytes have a key role in controlling inflammatory responses in the CNS (Fig. 2). Here, we have focused on astrocytes in only the most prevalent neurodegenerative diseases. It is worth noting that activated astrocytes and increased inflammatory cytokines are observed in many other neurodegenerative diseases including

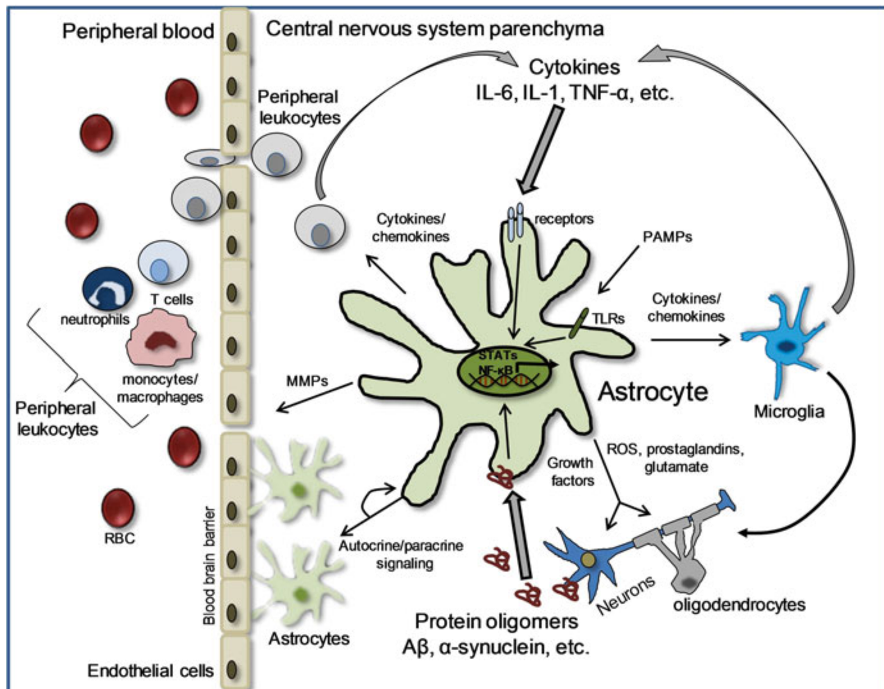

Fig. 2 Astrocytes orchestrate CNS inflammation. In neurodegenerative diseases, astrocytes respond to soluble factors including protein/peptide oligomers produced by neurons and inflammatory cytokines and chemokines produced by endogenous microglia and invading peripheral leukocytes. In response, astrocytes activate transcription factors such as NF-κB and STATs that leads to the production of a plethora of molecules which dictate the behavior and/or recruitment of the surrounding cells. The astrocyte-directed response may be beneficial through release of anti-inflammatory mediators and growth factors, or it may promote neurodegeneration through production of ROS and proinflammatory mediators

prion diseases [144] and lysosomal storage diseases [145]. While astrocytes have numerous beneficial functions [1–4, 6, 146–148], it seems that long-term perpetual stimulation, as likely occurs in neurodegenerative diseases, may exacerbate disease. Thus, we must continue to define the physiological and pathological functions of astrocytes as they may hold the key to new therapies.

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Oxidative Stress, Neuroinflammation, and Neurodegeneration

Hui-Ming Gao, Hui Zhou, and Jau-Shyong Hong

Abstract Oxidative stress and chronic neuroinflammation are two intertwined key pathologic factors in brain aging and neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases. As physiological signaling molecules, reactive oxygen species (ROS) play important roles in many biological processes. However, when excessive amounts of ROS overwhelm the antioxidant defense system, the resultant redox imbalance disrupts cellular integrity and functions. The brain is highly sensitive to oxidative stress. The failure of free radical-scavenging antioxidants in clinical trials demands new therapeutic strategies to block the major sources of oxidative stress in neurodegenerative diseases. While the mitochondrial electron transport chain is the major source of intracellular ROS, over-activated phagocytic NADPH oxidase (NOX2)—the major inflammatory oxidative enzyme—has been demonstrated as a prime mediator of chronic neurodegeneration in models of neurodegenerative diseases. Moreover, emerging evidence has suggested that dysregulated chronic neuroinflammation might be a driving force of decades-long neurodegenerative processes. Blockage of the crossroad of neuroinflammation and oxidative stress may have greater efficacy in the treatment for neurodegenerative diseases. Thus, targeting microglial NOX2 might become a disease-modifying therapeutic strategy for neurodegenerative diseases.

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1 Introduction

Neurodegenerative diseases are a group of incurable, chronic, and degenerative disorders in the central nervous system (CNS). They are characterized by the gradual loss of neurons in discrete brain areas and consequent deficits in specific brain functions (e.g., cognition, memory, or movement). More than 36 million people worldwide suffer from Alzheimer's disease (AD) and Parkinson's disease (PD), the two most common neurodegenerative diseases. Ample evidence has implicated that oxidative stress (cytotoxic consequences of redox imbalance) and neuroinflammation (the inflammatory reaction in the CNS) are two co-conspirators in the pathology of chronic neurodegeneration in various neurodegenerative diseases [1, 2]. It is important to point out that neuroinflammation and oxidative stress are two substantially different pathological events in the disease process. However, one can be a cause of the other, and they interplay in the entire disease process. Therefore, inhibition of neuroinflammation may diminish oxidative stress and vice versa. It has been well documented that excessive activation of phagocytic NADPH oxidase (NOX2, the major superoxide-producing enzyme during inflammation) is an important contributor to the pathogenesis of neurodegenerative diseases. Thus, new interventions suppressing microglia over-activation, especially NOX2-derived oxidative stress, might hold therapeutic potential for currently incurable neurodegenerative diseases.

2 Oxidative Stress

Oxidative stress refers to the redox imbalance and its cytotoxic consequences. It happens when increased generation of reactive oxygen species (ROS) overwhelms the antioxidant defense system disrupting cellular functions and integrity. At physiological levels, ROS serve as signaling molecules and play vital roles in many biological processes, such as cell growth and signaling, synthesis of biological molecules, posttranslational processing of proteins, the immune response, and metabolism [3, 4]. However, an excessive amount of ROS can oxidize proteins, nucleic acids, and lipids, thereby participating in aging and development of a diverse array of diseases, including obesity, diabetes, cancer, atherosclerosis, cardiovascular disease, and neurodegenerative diseases.

3 The Brain Is Highly Sensitive to Oxidative Stress

Although representing only 2 % of the body weight, the brain consumes 20 % of the body's oxygen. The brain derives its energy almost exclusively from oxidative metabolism of the mitochondrial respiratory chain, which produces ATP and reduces oxygen to H_2O by the sequential addition of four H^+ and four electrons. The "leakage" of high-energy electrons along the mitochondrial electron transport chain generates superoxide free radical ($O_2\bullet^-$) and hydrogen peroxide (H_2O_2, a non-radical oxidant). In the brain, nonenzymatic reaction of neurotransmitter catecholamines (e.g., epinephrine, norepinephrine, and dopamine) with oxygen forms highly cross-linked quinones and neuromelanin (a dark-colored granular pigment formed within dopamine and noradrenaline-containing neurons), when yielding reactive semiquinones, H_2O_2, and oxygen free radicals [5]. Intraneuronal metabolism of the neurotransmitter dopamine catalyzed by monoamine oxidase (MAO) yields H_2O_2. The activity of MAO increases with age [6]. Moreover, the substantia nigra (the major lesion area in PD) contains high concentrations of iron, which binds to neuromelanin and enhances hydroxyl radical (OH•) formation [7]. Thus, the high oxygen consumption, the high energy demand, and the oxidative metabolism of neurotransmitters lead to high production of ROS in the brain (Fig. 1).

The brain contains large amounts of polyunsaturated fatty acids whose double bonds within membranes allow easy removal of hydrogen atoms by ROS (e.g., OH•) and make the brain particularly vulnerable to free radical attack. Furthermore, numerous neurotransmitter systems are sensitive to ROS. Through direct interaction with neurotransmitter receptors and ion transport proteins (e.g., channels, pumps, and transporters) or alteration of ligand-receptor interactions after oxidation of lipids of cell membranes, ROS disrupt receptor activity, ionic homeostasis, and neurotransmission of adrenergic, dopaminergic, serotonergic, and GABAergic systems [8–10]. ROS impair mitochondria integrity, reduce ATP production, and increase mitochondria-derived ROS [11]. ROS-induced dysfunction of the ubiquitin-proteasome system (UPS) will reduce protein degradation, which in turn exaggerates the accumulation of abnormal proteins [11, 12]. ROS production and destructive effects of oxidative stress can be exacerbated by elevated cellular calcium, increased L-glutamine and excitotoxicity, protein aggregates, and neuroinflammation [1, 2, 13–15]. Collectively, high production of ROS, high sensitivity to ROS, moderate antioxidant defense (e.g., low level of catalase activity and only moderate amounts of superoxide dismutase and glutathione peroxidase in the brain), and a limited renewal and regenerative capacity of neurons render the brain especially susceptible to oxidative insults [1, 16] (Fig. 1). Indeed, diseased brain regions of patients with various neurodegenerative diseases reveal oxidative stress markers, such as lipid peroxidation, protein carbonyls, and reduction of glutathione levels [1, 2].

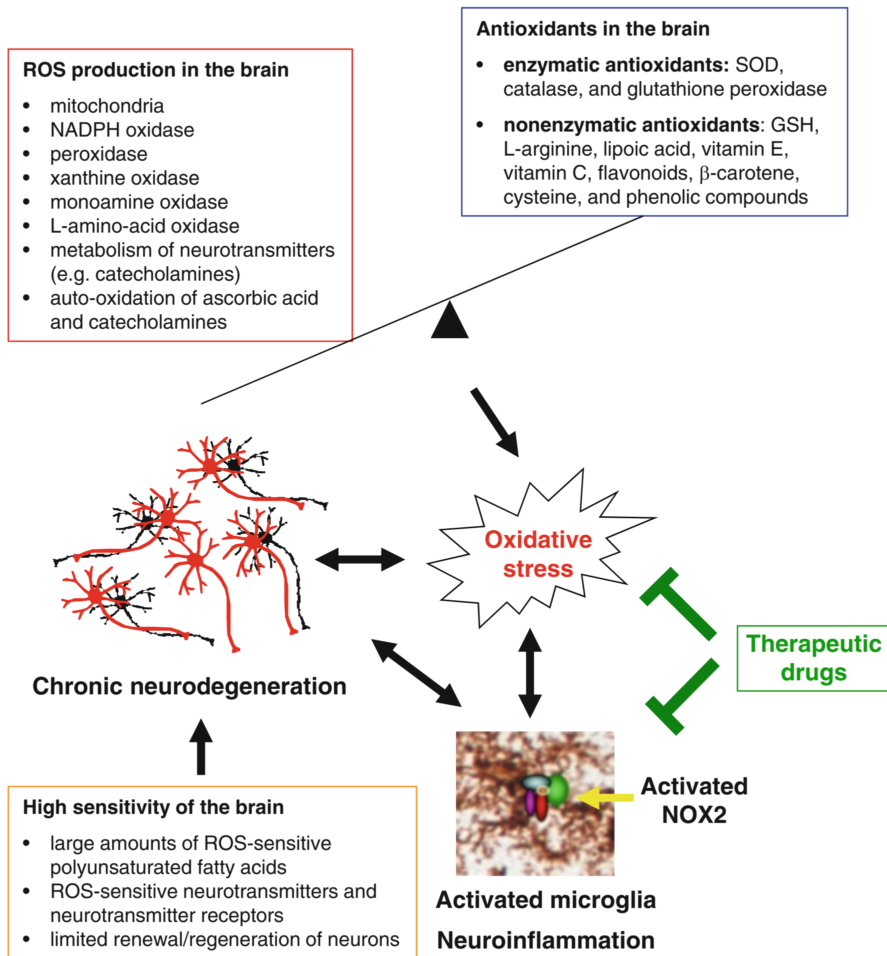

Fig. 1 A vicious cycle among neuroinflammation, oxidative stress, and neurodegeneration drives chronic neurodegeneration. Oxidative phosphorylation in mitochondria, activation of oxidase enzymes (e.g., NADPH oxidase, peroxidase, xanthine oxidase, monoamine oxidase, and L-AMINO-ACID oxidase), metabolism of neurotransmitters (e.g., catecholamines), and auto-oxidation of ascorbic acid and catecholamines in the brain generate reactive oxygen species (ROS). Mitochondrial dysfunction, increased activity of oxidase enzymes, and oxidative metabolism of neurotransmitters, combined with the high oxygen consumption and the high energy demand, lead to high production of ROS in the brain. Both enzymatic antioxidants (e.g., SOD, catalase, and glutathione peroxidase) and nonenzymatic antioxidants (e.g., GSH, L-arginine, lipoic acid, vitamin E, vitamin C, flavonoids, β-carotene, cysteine, and phenolic compounds) are essential for redox balance in the brain. Insufficient antioxidant defense also leads to excessive amounts of ROS. Thus, when excessive amount ROS overwhelms the antioxidant defense system, the resultant oxidative stress induces chronic neurodegeneration and brain dysfunction, which in turn can exaggerate oxidative stress. Dysregulated chronic neuroinflammation can mediate progressive neurodegeneration. Oxidative stress and chronic neuroinflammation are two intertwined key pathologic factors in neurodegenerative diseases; one can be a result of the other. The brain is highly sensitive to oxidative stress and neuroinflammation because of its large amounts of ROS-sensitive polyunsaturated fatty acids, neurotransmitters and neurotransmitter receptors, and the limited renewal/regeneration of neurons. A vicious cycle among neuroinflammation, oxidative stress, and neurodegeneration may drive chronic neurodegenerative process. Therefore, suppression of ROS production may dampen neuroinflammation and vice versa. Over-activated NOX2 is a major contributor to inflammation-mediated oxidative stress and chronic neurodegeneration. Blockade of the crossroad of neuroinflammation and oxidative stress, especially NOX2, may have greater efficacy in the treatment for neurodegenerative diseases. SOD, superoxide dismutase; GSH, glutathione

4 Major Sources of ROS in the Brain

Under physiological conditions, oxidative phosphorylation in mitochondria, activation of several oxidase enzymes (e.g., MAO and L-amino-acid oxidase), and metabolism of neurotransmitters (e.g., catecholamines) in the brain generate superoxide free radical or H_2O_2 as normal byproducts. Mitochondrial dysfunction, inappropriate activity of cytoplasmic or membrane oxidases (e.g., NADPH oxidase, peroxidase, and xanthine oxidase), and insufficient antioxidant defense lead to excessive amounts of ROS (Fig. 1).

4.1 Mitochondria

The mitochondrial electron transport chain is the major source of intracellular ROS [17]. During the process of oxidative phosphorylation, the partial reduction of molecular oxygen leads to the formation of superoxide free radical, which in turn can be converted into many other ROS. Within the mitochondria, antioxidant manganese-superoxide dismutase (MnSOD) catalyzes the dismutation of superoxide into oxygen and H_2O_2. Once produced, H_2O_2 can rapidly be converted to highly reactive OH• through the Fenton reaction in the presence of free iron and copper [18]. Superoxide can also be converted to hypochlorous acid (HOCl) and hydroperoxyl radical (HOO•) [19, 20].

4.2 NADPH Oxidase (NOX)

NOX, a membrane-bound, multi-subunit enzyme complex, transfers electrons across the plasma membrane from NADPH to oxygen and generates superoxide. Four of seven NOX isoforms, NOX1 to NOX4, have been detected in various brain cells (microglia, neurons, and astroglia) [4, 21]. Interestingly, many cells express several NOX isoforms, but distinct subcellular distribution and activation mechanisms may explain the non-redundancy in their functions [3, 4]. NOX2 (also named phagocytic NOX) is the major source of extracellular superoxide in phagocytes including neutrophils, monocytes, macrophages, and microglia (the resident immune cells in the CNS). Upon stimulation, the cytosolic subunits of NOX2 ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small Rho GTPase, Rac1 or Rac2) translocate to the membrane-bound $p22^{phox}$/$gp91^{phox}$ heterodimer to assemble the active NOX2 that catalyzes the reduction of oxygen to superoxide. Rac proteins, $p22^{phox}$, and the cytosolic subunits of non-phagocytic NOX, NOXO1 and NOXA1 (functional homologues of $p47^{phox}$ and $p67^{phox}$, respectively) are essential for NOX1 activation [3, 22, 23]. Both $p22^{phox}$ and NOXO1 are required for NOX3 activation [24, 25]. In summary, for the activation of NOX, the catalytic subunits and $p22^{phox}$ are indispensable; $p47^{phox}$ and NOXO1 serve as organizers or regulatory subunits; $p67^{phox}$

and NOXA1 are activators; and p40^{phox} is a nonessential, modulatory subunit [3, 26]. In the CNS, microglia generate much more NOX-derived ROS than astroglia, neurons, endothelial cells, or vascular smooth muscle cells [19, 27].

NOX-derived ROS participate in microglia proliferation and immune defense [28], glutamate release from activated microglia [29, 30], microglial induction of neuronal apoptosis during development [31], neurite outgrowth [32, 33], blood pressure regulation by angiotensin II [34, 35], astroglia signaling [36], and learning and memory formation [37, 38]. Elevated NOX activation is an important source of brain oxidative stress. Brain tissues of patients with AD reveal increased mRNA transcripts of NOX1 and NOX3 as well as elevated expression and activity of NOX2 [39–41]. An upregulation of NOX4 expression in neurons and in newly formed capillaries under ischemic conditions implies a role for NOX4 in ischemia/hypoxia [42]. Ample experimental evidence also indicates a positive association between increased expression/activation of NOX1, NOX2, and NOX4 and enhanced neuronal damage in several models of neurodegenerative diseases [43–46]. Collectively, NOX expressed in microglia and neurons is an important source of brain oxidative stress in various neurodegenerative diseases.

4.3 Peroxisomes

Another important source of intracellular ROS is peroxisomes (oxidative organelles that are present in all tissues including the brain). Within peroxisomes, enzymatic removal of hydrogen atoms from specific organic substrates (RH2) by using molecular oxygen produces H_2O_2. Through the peroxidation reaction catalyzed by peroxidase, H_2O_2 can oxidize other substrates, such as alcohol, phenols, formic acid, and formaldehyde [47].

4.4 Xanthine Oxidase

In the presence of H_2O and oxygen, xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and then xanthine to uric acid, leading to production of H_2O_2 and superoxide. Xanthine oxidase has been implicated as a source of oxygen radicals in the brain. In human neuronal cultures, oxidative stress induced by the xanthine/xanthine oxidase system activates cholesterol biosynthesis pathway and induces neuronal apoptosis, supporting a possible link between oxidative stress, cholesterol metabolism, and AD [48]. In addition, allopurinol, a xanthine oxidase inhibitor, is neuroprotective in mouse cerebellar granule cell cultures treated with kainic acid (a specific agonist for the kainate receptor) and in rodent models of cerebral ischemia [49].

4.5 Other Enzymes and Molecules

Several enzymes expressed in the brain, such as tyrosine hydroxylase, MAO, and L-amino-acid oxidase (also known as ophio-amino-acid oxidase), produce H_2O_2 as a normal byproduct during their activation [6]. The metabolism of arachidonic acid, a polyunsaturated omega-6 fatty acid that is abundant in the brain, by lipoxygenases and cyclooxygenases generates eicosanoids and superoxide [50]. In the brain, auto-oxidation of ascorbic acid and catecholamines also yields H_2O_2 [50].

4.6 Nitric Oxide Synthase (NOS) and Reactive Nitrogen Species (RNS)

In mammals, three NOS isoforms, endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS), catalyze the production of nitrogen free radical nitric oxide (NO•) from L-arginine. As an important biological signaling molecule, NO• is involved in modulation of vascular or airway tone, insulin secretion, angiogenesis, and neural development and activity [51]. In particular, iNOS-derived NO• plays an important role in immune defense, autoimmune disease, and neurodegenerative diseases [51]. Reaction of NO• with superoxide forms highly reactive and toxic peroxynitrite ($ONOO^-$), which can decompose to form OH• (the most reactive and damaging species) and $NO2^-$. S-nitrosylation, a form of posttranslational protein modification, involves the covalent incorporation of a NO• moiety into thiol groups of a subset of specific cysteine residues in proteins to form S-nitrosoproteins. Aberrant S-nitrosylation or denitrosylation (the reverse process of S-nitrosylation) has been associated with PD, AD, and amyotrophic lateral sclerosis (ALS) [52–55].

5 Antioxidant Defense System in the Brain

In order to maintain the balance between oxidants and antioxidants and to eliminate oxidative cellular destruction, cells develop numerous enzymatic and nonenzymatic antioxidant defense mechanisms. SOD, catalase, and glutathione peroxidase are enzymes responsible for coordinated degradation of superoxide and H_2O_2 (Fig. 1). Many scavengers dedicated to reducing the levels of oxidants also play an important role in modulating oxidative stress in the brain.

5.1 Enzymatic Antioxidant Defense

SOD catalyzes the dismutation of superoxide to less reactive H_2O_2. Three forms of SOD, cytosolic copper–zinc SOD (CuZnSOD; SOD1), mitochondrial MnSOD (SOD2), and extracellular CuZnSOD (SOD3), are encoded by three separate genes

in eukaryotic cells. SOD1 and SOD2 play a main role in removing superoxide in the cytosol and mitochondria, respectively. Both SOD1 and SOD2 are implicated in a variety of age-related brain disorders. Mutations in SOD1 are associated with approximately 20 % of familial cases of ALS; however, reduction in dismutase activity may not be the cause of motor neuron death in these cases, whereas a gain-of-function may be responsible for the pathogenic role of mutant SOD1 [56, 57]. Notably, multiple lines of evidence have shown an inverse correlation between SOD levels and overall susceptibility to ischemic brain injury [58]. Cerebral ischemia/reperfusion after transient focal cerebral ischemia (tFCI) downregulates the expression of SOD2 and the transcription factor STAT3 (signal transducer and activator of transcription 3) and blocks the recruitment of STAT3 into the SOD2 promoter [59]. Transgenic overexpression of SOD1 ameliorates infarction volume and neurological deficits induced by tFCI and attenuates hippocampal neuronal death elicited by transient global cerebral ischemia (tGCI) [58, 60, 61]. Similarly, SOD2 overexpression mitigates membrane lipid peroxidation, protein nitration, and infarction volume after tFCI [62]. Administration of a cell-permeable SOD mimetic attenuates hippocampal CA1 pyramidal neuronal damage and neurological deficits induced by tGCI [63]. Conversely, reduced dismutase activity in $SOD1^{-/+}$ mice and $SOD2^{-/+}$ mice aggravates tFCI-elicited superoxide production, apoptotic neuronal death, infarct volume, and neurological deficits [58, 64]. Moreover, in transgenic mouse models of Alzheimer pathology, deletion of SOD1 exacerbates β-amyloid oligomerization, cognitive impairment, and neuronal dysfunction, and deletion of one allele of SOD2 increased amyloid plaque formation [65, 66]. Thus, lost or compromised dismutase activity of SOD1 and SOD2 and consequent oxidative stress play important roles in ischemia brain damage and Alzheimer pathology.

Catalase and glutathione peroxidase mediate the decomposition of H_2O_2 to H_2O and O_2, eliminating the poisonous effect of excessively produced H_2O_2. Glutathione peroxidase also catalyzes the reduction of lipid peroxyl radicals. Catalase is mostly localized to peroxisomes in most cells of the brain, and its expression level is low in both gray and white matter. Glutathione peroxidase is present in the cytosol and the mitochondria of brain cells. In models of AD and stroke, upregulation of catalase is neuroprotective, whereas impairment in catalase increases vulnerability to neuronal injury [67, 68]. Postmortem studies reveal reduction in the activity of glutathione peroxidase and the amount of glutathione in the substantia nigra of PD patients [69]. Genetic deletion of glutathione peroxidase enhances the susceptibility of mouse brain neurons to neurotoxin [70].

Peroxiredoxins, a group of ubiquitous, nonheme peroxidases, catalyze the reduction of H_2O_2, organic hydroperoxides, and peroxynitrite, which requires an activated cysteine residue at their active site of these enzymes [71]. Transgenic mice overexpressing peroxiredoxin are more resistant to ischemic neuronal injury [72]. Lentivirus-mediated overexpression of peroxiredoxin attenuates dopamine neuron death and motor impairment in a mouse model of PD created by 6-hydroxydopamine [73].

The plasma membrane redox system (PMRS) is an electron transport chain in the plasma membrane. It transfers electrons from intracellular or extracellular donors to

extracellular acceptors, reducing membrane-associated oxidative stress. PMRS has multiple redox enzymes, such as quinine reductase, NADH-cytochrome b5 reductase, NADH-quinone oxidoreductase 1 (NQO1), NADH-ferricyanide reductase, NADH-coenzyme Q10 reductase, and NADH-cytochrome c reductase [74]. PMRS has been implicated in aging and neuronal survival, and impairment in PMRS was induced by β-amyloid and detected in the hippocampus and cerebral cortex of triple transgenic (3×Tg-AD) mice, an animal model of AD [74, 75].

5.2 Nonenzymatic Antioxidants

Nonenzymatic antioxidants are scavengers that remove ROS either by inhibiting cellular sources of oxidants or by inducing cellular antioxidant systems. They can broadly be divided into two groups. While metabolic antioxidants (e.g., glutathione, L-arginine, and α-lipoic acid) are generated during the metabolic reactions in the cell, nutrient antioxidants are taken in from diet, which include vitamin E, vitamin C, flavonoids, β-carotene, cysteine, and phenolic compounds [76] (Fig. 1).

Glutathione (GSH), a tripeptide (γ-glutamyl-cysteinyl-glycine), is synthesized intracellularly by a sequential action of two enzymes, the glutamate–cysteine ligase (γ-glutamylcysteine synthetase) and GSH synthetase. In its reduced state, glutathione nonenzymatically scavenges both singlet oxygen and OH•. Glutathione acts as a main cofactor for a family of glutathione peroxidase enzymes. The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) serves as the primary determinant and an indicator of the cellular redox state. The reduction in the ratio of GSH:GSSG is associated with aging and neurodegeneration [69]. Glutathione reductase regenerates GSH from GSSG using NADPH as an electron donor. The substantia nigra of patients with idiopathic PD or presymptomatic PD shows glutathione depletion [69]. Exogenous addition of glutathione provided neuroprotection in models of PD [15].

Vitamin E, a hydrophobic antioxidant, protects cell membrane from damage by ROS such as peroxyl radicals during lipid oxidation. α-tocopherol is the most biologically active form of vitamin E. Although α-tocopherol can react with singlet oxygen and OH•, the major antioxidant action of α-tocopherol relies on its ability to donate labile hydrogens to peroxyl and alkoxy radicals, thereby preventing lipid peroxidation. Vitamin E deficiency causes neurologic symptoms and the so-called ataxia with vitamin E deficiency, a neurodegenerative disorder [77]. Although being very effective in ameliorating neurodegeneration in animal models, vitamin E failed to show consistent neuroprotection in patients with neurodegenerative diseases [78, 79]. It seems that supplementation with vitamin E in humans is most effective when its deficiency is more evident. This phenomenon seems true in most nonenzymatic antioxidants. Ascorbic acid (vitamin C) is a hydrophilic antioxidant. It removes free radicals by electron transfer and also serves as a cofactor for enzymes of antioxidant

defense. Like vitamin E, vitamin C is neuroprotective in a variety of models of neurodegenerative diseases but has no great efficacy in the clinical setting.

Coenzyme Q10 (CoQ10), a powerful hydrophobic antioxidant enriched in the mitochondria, participates in electron and proton transport of the respiratory chain in the inner mitochondrial membrane. As an energy carrier, CoQ10 continuously goes through oxidation–reduction cycle. There are three redox states of CoQ10: fully oxidized (ubiquinone), semiquinone (ubisemiquinone), and fully reduced (ubiquinol). The reduced form of CoQ10 inhibits both the initiation and the propagation of lipid peroxidation and protein oxidation by preventing production of lipid peroxyl radicals and reducing initial perferryl radical and singlet oxygen. The reduced form of CoQ10 also effectively regenerates vitamin E from the α-tocopheroxyl radical. The ROS-scavenging role of CoQ10 is reported to be neuroprotective in preclinical studies of neurodegenerative diseases; CoQ10 also shows some clinical efficacy in a few neurodegenerative disorders, including Huntington disease (HD) and PD [80, 81]. However, in 2011, the National Institute for Neurological Disease and Stroke (NINDS) terminated a phase III clinical trial of high doses of CoQ10 (1,200 or 2,400 mg/day) because of the lack of evidence of a clinical benefit in early PD [82].

Antioxidant thioredoxin acts as an electron donor to facilitate the reduction of other proteins by cysteine thiol–disulfide exchange. Thioredoxins are present in all organisms and are essential for life in mammals. Loss-of-function mutation of either of the two human thioredoxin genes is embryonic lethal. Using NADPH as an electron donor, thioredoxin reductase converts oxidized thioredoxin back to its reduced form. Thioredoxin is reported to be neuroprotective, and its overexpression suppresses neurotoxicity in a Drosophila model of Machado–Joseph disease expressing polyglutamine expansions [83, 84].

6 Oxidative Stress and Neurodegeneration

Oxidative stress is intimately linked to aging—the best established risk factor for neurodegenerative diseases. Microarray analysis of postmortem human brain samples has revealed downregulation of genes encoding for synaptic transmission, learning, and memory after age 45, and such gene downregulation is associated with increased amounts of oxidative damage in their promoters [85]. Mitochondrial dysfunction and glutamate-mediated oxidative stress, inflammatory oxidative insults, and insufficient GSH are important mechanisms underlying age-related oxidative damage and neurodegeneration. In aged brains, iron accumulates in the basal ganglia and is believed to contribute to neurodegenerative diseases through oxidative stress in PD and AD [86]. It is commonly accepted that neurodegenerative diseases are consequence of elevated oxidative stress and compromised anti-oxidative mechanisms, regardless of initial insults or genetic defects.

6.1 Mitochondrial Dysfunction and Oxidative Neuronal Damage

Mitochondria play a key role in electron transport and oxidative phosphorylation, and they are the major cellular source of oxygen free radicals. Considerable evidence has shown that mitochondria-derived oxidative stress occurs early and acts causally in the pathogenesis of aging-related neurodegenerative diseases [11]. Oxidized nucleotides in brain mitochondrial DNA (mtDNA) are increased in aging and AD [6, 87, 88]. The accumulation of mutations in mtDNA correlates with impairment of mitochondrial function that causes imbalance of production and removal of ROS, leading to cell death. The mitochondrial uncoupler CCCP (carbonyl cyanide m-chlorophenyl hydrazone, a chemical inhibitor of oxidative phosphorylation) triggers amyloidogenic processing of amyloid precursor protein (APP) and intracellular accumulation of β-amyloid in astroglia, which are pathological features seen in Down syndrome [89]. Hemizygous deficiency of the mitochondrial MnSOD increases brain β-amyloid levels and plaque deposition in a transgenic mouse model of AD that harbor a mutant APP [90].

Both genetics and environmental evidence have revealed a prominent role for mitochondrial dysfunction and oxidative stress in the pathogenesis of PD. Immunocapture techniques have detected increased oxidative damage and reduced electron transfer rates in mitochondrial complex I subunits in PD patients [91]. Mitochondrial complex I inhibition by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1-methyl-4-phenylpyridinium (MPP^+, an active metabolite of MPTP), or rotenone replicates some of the key features of PD, such as death of dopaminergic neurons and locomotor deficits. Mechanistically, mitochondria-derived free radicals are important contributors of such pathological alterations [92, 93]. Paraquat, a free radical generator, induces loss of dopaminergic neurons, motor deficits, and increased production of free radicals by mitochondria in rodents [94]. While accidental exposure to MPTP causes Parkinsonism in human, lifetime use of pesticides rotenone and paraquat in farm workers is associated with increased risk for PD. Mutations in *parkin*, *DJ-1*, and *PINK1*, known to cause familial forms of PD, are linked to mitochondrial dysfunction and oxidative stress [95]. In addition, α-synuclein (a cytosolic protein under physiological condition) appears to interact with mitochondrial membranes [96] and to inhibit mitochondrial complex I in affected brain regions of patients and models of PD [97]. Mutant α-synuclein transgenic mice exhibit impairment in mitochondrial integrity and function [98]. Under oxidative stress or electron transport chain inhibition, which can lead to increased permeability of mitochondrial membrane or opening of the mitochondrial permeability transition pore, Bax and other pro-apoptotic molecules can result in enhanced cytochrome c release into the cytosol, caspase activation, apoptosis, and collapse of the mitochondrial membrane potential. Thus, mitochondrial dysfunction and consequent oxidative stress play a pivotal role in aging and neurodegenerative diseases.

6.2 Oxidative Stress and Glutamate-Induced Neuronal Degeneration

Glutamate-mediated oxidative stress alone or in combination with other sources of oxidants has been implicated in PD, ALS, and HD. The neurotransmitter glutamate and related excitatory amino acids account for most of the excitatory synaptic activity in the mammalian CNS. Through activation of its ionotropic receptors (NMDA receptor, AMPA receptor, and kainate receptors), glutamate activates a number of different pathways that cause oxidative stress and induces neuronal degeneration. NMDA receptor-mediated stimulation of enzyme phospholipase A2 (PLA2) by glutamate and subsequent release of arachidonic acid lead to production of superoxide and OH•. These radicals and arachidonic acid enhance the release of glutamate and inhibit its uptake and its inactivation by neurons and glia, promoting a vicious cycle [6]. Antioxidants protect cultured mouse cortical neurons against delayed NMDA receptor-mediated neuronal degeneration [99]. Transgenic mice expressing CuZnSOD gene show elevated SOD activity and are less vulnerable than wild-type mice to ischemic brain damage; neurons cultured from these transgenic mice are less vulnerable to glutamate toxicity [6]. Systemic administration of kainate acid (a specific agonist for the kainate receptor) leads to production of free radicals in the brain; concurrent neurodegeneration and lipid peroxidation accumulation induced by kainate acid are attenuated by antioxidants. Furthermore, the neurotoxic effects of intracerebral injection of kainate acid or quisqualic acid (an AMPA receptor agonist) are blocked by the centrally active antioxidant, idebenone [6].

Two recent reports reveal that superoxide production in neurons elicited by NMDA receptor activation primarily results from NOX activation [100, 101]. Deficiency in $p47^{phox}$ or NOX2 inhibitor apocynin blocks NMDA-elicited superoxide production and neurotoxicity in cultured neurons and mouse hippocampus [100]. Interestingly, the NMDA receptor antagonist ketamine also increases NOX activity with a possible involvement of neuronal interleukin-6 [102, 103]. Knockdown of NOX2 and NOX4 expression in HT22 neuronal cells reduces glutamate-induced H_2O_2 accumulation and cell death [104]. These findings indicate that NOX-derived ROS appear important for neurotoxicity induced by excessive glutamate.

7 Inflammatory Oxidative Damage Is a Major Mediator of Chronic Neurodegeneration

Neuroinflammation, a prominent and most common feature shared by all neurodegenerative diseases, has been increasingly accepted as a crucial contributor to chronic neurodegeneration in various neurodegenerative diseases [105–107]. Microglial activation is the prime component of neuroinflammation. A wide range of stimuli, such as infection, autoimmune injury, toxic insults, trauma, or ischemia, can trigger microglial activation and disrupt CNS homeostasis [105–107]. Activated microglia release a myriad of inflammatory and cytotoxic factors, such as cytokines,

chemokines, eicosanoids, proteases, excitatory amino acids, and ROS. Among these factors, NOX2-derived ROS are recognized as a crucial player in neuroinflammation-mediated oxidative stress and chronic neurodegeneration [105–107].

7.1 NOX2 Activation in AD

AD is an age-related, nonreversible brain disorder. It is characterized by a progressive decline in memory, cognitive abilities, and mental function. Three major hallmarks in the brain—extracellular amyloid plaques, intracellular neurofibrillary tangles that contain aberrant Tau, and loss of neurons responsible for memory and learning and consequent brain atrophy—are associated with the disease processes of AD. Aggregated β-amyloid (the prime pathogenic mediator of AD) induces NOX2-dependent ROS production in microglia [108–111]. NOX2 inhibition by apocynin or $p47^{phox}$ deletion promotes alternative and anti-inflammatory microglial activation during neuroinflammation evoked by an intracerebroventricular injection of LPS or β-amyloid [112]. NOX2 deletion or inhibition prevents microglia-mediated neurotoxicity triggered by β-amyloid in cell culture systems [113, 114]. Furthermore, in an AD mouse model overexpressing mutant APP, a membrane-permeable NOX2 peptide inhibitor NOX2ds-tat (gp91ds-tat) or NOX2 deletion attenuates neuronal oxidative stress, cerebrovascular dysfunction, and behavioral deficits [115]. NOX2 inhibitors diphenylene iodonium (DPI) and apocynin prevent β-amyloid-induced neuronal death through blocking ROS generation, glutathione depletion, and mitochondrial depolarization in neurons and astrocytes [12]. Thus, NOX2-mediated oxidative stress is an important contributor to AD pathogenesis.

7.2 NOX2 Activation in PD

PD is a neurodegenerative movement disorder. A gradual loss of nigral dopaminergic neurons and the formation of α-synuclein-containing Lewy body are the pathological hallmarks of the disease. The major lesion region in PD, the substantia nigra, reveals upregulation of microglial NOX2 in PD patients and a mouse model of PD [116]. Activation of microglial NOX2 aggravates dopaminergic neurodegeneration induced by aggregated α-synuclein (the key pathogenic mediator of PD); PD-producing neurotoxins MPTP and 6-hydroxydopamine; PD-associated pesticides rotenone and paraquat; inflammagens LPS and fMLP (formyl-methionyl-leucyl-phenylalanine); diesel particles; angiotensin II; the active form of matrix metalloproteinase-3; and extracellular high-mobility group box 1 (HMGB1) released from stressed/damaged neurons or activated microglia. Importantly, DPI, apocynin, or NOX2 deletion mitigates such neurodegeneration [15, 117–128]. Minocycline blocks MPTP-elicited nigral dopaminergic neurodegeneration via suppressing microglial activation and membrane translocation of $p67^{phox}$ [129]. NOX2-deficient mice are more resistant to systemic administration of MPTP or an intra-nigral

injection of LPS than wild-type mice [120, 130]. Collectively, NOX2-mediated redox imbalance exaggerates inflammation-mediated PD neurodegeneration.

7.3 NOX2 in Amyotrophic Lateral Sclerosis (ALS)

ALS (Lou Gehrig's disease), a fatal paralytic neurodegenerative disorder, is characterized by a progressive degeneration of motor neurons in the spinal cord, the brainstem, and the motor cortex leading to rapidly progressive muscle weakness and atrophy throughout the body. Microglia in the spinal cord of animal models and patients with ALS show NOX2 upregulation, increased ROS production, and oxidative damage [131]. Mutations of SOD1 are associated with autosomal dominant familial ALS. Recent studies using glial cells suggest a regulatory role of SOD1 in Rac1-dependent NOX2 activation and a pathogenic role of enhanced interaction of mutant SOD1 with Rac1 [56, 132]. In mice carrying mutant SOD1, apocynin (30–300 mg/kg) and NOX2 deficiency delays motor neurodegeneration, slows disease progression, improves neurological symptoms, and extends survival [46, 56, 131, 132]. Altogether, increased activity of microglial NOX2 is an important disease mechanism underlying the non-cell-autonomous nature of ALS neurodegeneration.

7.4 NOX2 Activation in Multiple Sclerosis (MS)

MS is a neurodegenerative disease with widespread inflammatory axonal demyelination and neuronal injury in the brain and the spinal cord. NOX2 activation in microglia has been implicated in MS pathogenesis. For instance, a low ROS-generating variant of $p47^{phox}$ in rats promotes the formation of experimental autoimmune encephalomyelitis (EAE, a widely used animal model for MS) [133]. Similarly, mice carrying a truncated and nonfunctional $p47^{phox}$ develop severer EAE than wild-type mice; however, $p47^{phox}$-knockout mice reveal decreased EAE [134, 135]. NOX2-deficient mice are resistant to EAE [136]. These seemingly conflicting findings regarding NOX2 activity and EAE development imply that the combined action and the balance among ROS, inflammatory process, neuronal injury, and neuronal repair might critically determine the outcome of inflammatory axonal demyelination and neuronal survival.

7.5 NOX1 Activation in Neurodegenerative Diseases

NOX1 deletion slows disease progression and increases lifespan of a mouse model of ALS, which carries mutant SOD1 [46]. PD-associated pesticide paraquat increased NOX1 expression, and siRNA-mediated NOX1 knockdown attenuated ROS production and cell death induced by paraquat and MPP^+ in N27 dopaminergic

neuronal cultures [43, 44]. NOX1-derived superoxide increases the release of inflammatory factors in LPS-treated microglia cultures and exaggerates presynaptic protein loss in striatal neurons after intra-striatum LPS injection [45]. Collectively, the NOX family expressed in microglia and neurons is an important contributor to brain oxidative stress in various neurodegenerative diseases.

8 The Therapeutic Potential of Targeting NOX2 in Neurodegenerative Diseases

The fact that oxidative stress is a key pathologic mediator and that several free radical-scavenging antioxidants failed in clinical trials in neurodegenerative diseases requires a better therapeutic strategy—to block major sources of oxidative stress. As described above, over-activated NOX2 has been implicated as a major contributor to inflammation-mediated chronic neuron loss in neurodegenerative diseases. In fact, recent preclinical evidence has demonstrated neuroprotective effects of pharmacological inhibition of NOX2 in models of neurodegenerative diseases [27]. Many structurally and functionally different compounds that shared common properties of dampening inflammation and NOX2 activation provide neuroprotection in models of PD. These compounds, including compound A (a potent and selective inhibitor of IKK-β in the NF-κB pathway), dextromethorphan (a widely used antitussive agent), sinomenine (a natural dextrorotatory morphinan analog), squamosamide derivative FLZ, minocycline (a broad-spectrum tetracycline antibiotic), pituitary adenylate cyclase-activating polypeptides, resveratrol (a nonflavonoid polyphenol with antioxidant and anti-inflammatory properties), TGF-β1 (transforming growth factor-β1, a known endogenous immune modulator), and verapamil (a calcium-channel blocker used to treat high blood pressure, chest pain, and certain heart rhythm disorders), lose their neuroprotective effects in the absence of NOX2 [27, 129, 130, 137–144].

By targeting estrogen receptor alpha and subsequent suppressing NOX activation, 17-beta-estradiol attenuates ischemic oxidative damage in stroke [145]. Galantamine, an alkaloid drug currently used to treat AD, suppresses activation of iNOS and NOX and offers neuroprotection in an in vitro brain ischemia–reperfusion model [146]. A 9-month treatment of aged R1.40 mice (a model of AD) with the nonsteroidal anti-inflammatory drug ibuprofen reduces oxidative damage (as shown by the reduction in lipid peroxidation, tyrosine nitration, and protein oxidation) and enhances plaque clearance at least partially through the inhibition of microglial NOX2 activation [109]. Additionally, an intraperitoneal injection of a low dose of dextromethorphan (0.1 mg/kg) suppresses NOX2 expression and infiltration of monocytes and lymphocytes into the spinal cord and thereby attenuates moderate EAE [147]. It is worth emphasizing that the long-standing clinical safety record of some “old” drugs (e.g., dextromethorphan and galantamine) makes them attractive candidates for further investigation for their clinical use in the treatment of neurodegenerative diseases. Taken together, the correlation of pharmacological

inhibition of NOX2 activity with attenuated neurodegeneration in models of various neurodegenerative diseases strongly implicates NOX2 as a promising therapeutic target for neurodegenerative diseases (Fig. 1).

The limitation of known NOX2 inhibitors, the prooxidant potential of apocynin, the insufficient specificity and high toxicity of DPI, the irreversible off-target effect on serine proteases and low efficacy with an IC50 greater than 1 mM of AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), and unfavorable administration routes of NOX2ds-tat make their clinical translation almost unfeasible [3, 148, 149]. Thus, to develop and screen novel blood–brain-barrier permeable NOX2 inhibitors with improved efficacy, specificity and pharmacokinetic profiles might discover promising clinical therapeutics for various neurodegenerative diseases. Furthermore, given the critical role of mitochondrial dysfunction in brain oxidative stress, either as a cause or a result, a combination of "mitochondrial medicine" (e.g., CoQ10) with agents targeting neuroinflammation and NOX2 will be more effective than an individual therapeutic approach for the treatment of chronic neurodegeneration.

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Peripheral Neuroimmune Interactions and Neuropathic Pain

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Abstract Neuropathic pain often results from damage to peripheral nerves, which can mobilize the immune system, as in Guillain-Barré syndrome, postherpetic neuralgia, or trauma. Although most studies focused on detrimental effects of neuroinflammation, recent experimental data provide evidence on analgesic effects of leukocytes. Pain-ameliorating actions involve anti-inflammatory cytokines and immune cell-derived opioid peptides, which activate opioid receptors on peripheral terminals of sensory neurons in injured nerves. In addition, endocannabinoids are present in leukocytes, and mechanisms involved in the resolution of inflammation are mounted, but their significance to neuropathic pain modulation is yet to be examined. Clinical evidence is less compelling, although in some conditions the occurrence of pain seems to be associated with lowered numbers of macrophages or T lymphocytes. This chapter discusses studies addressing both unfavorable and beneficial actions of neuroinflammation in the regulation of painful neuropathies.

Keywords Pain • Neuropathy • Inflammation • Immune cells • Leukocytes • Opioid peptides • Endocannabinoids • Resolvins

Abbreviations

CCI	Chronic constriction injury
CRF	Corticotropin-releasing factor
CXCL	Chemokine (C-X-C motif) ligand
DRG	Dorsal root ganglion
ICAM-1	Intercellular adhesion molecule-1

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IL	Interleukin
PSNL	Partial sciatic nerve ligation
SNL	Spinal nerve ligation
TNF	Tumor necrosis factor

1 Introduction

Neuropathic pain can result from perturbations to the peripheral nervous system, which include diseases (e.g., diabetes), trauma (e.g., amputation, nerve compression, entrapment, stretch), or cancer-related chemotherapy. Debilitating consequences are ongoing/spontaneous pain and enhanced sensitivity to normally innocuous (allodynia) or noxious stimuli (hyperalgesia) (reviewed in [1, 2]). Maladaptive alterations in the nervous system include ectopic impulse generation (at the site of axonal damage and/or in cell bodies), sensory fiber degeneration, sensory and sympathetic nerve sprouting to areas they normally do not innervate, disinhibition (i.e., decreased activity or loss of inhibitory neurons), enhanced activity of descending facilitatory, or impaired activity of descending inhibitory transmission (reviewed in [3, 4]). Furthermore, increasing evidence shows that nerve damage mobilizes the immune system, which can occur in response to infection (e.g., by varicella zoster virus in postherpetic neuralgia), autoimmune disease (Guillain-Barré syndrome), nerve compression (e.g., by tumor), or amputation [5–8]. Neuroimmune mechanisms have been predominately examined in animal models, including spinal nerve ligation (SNL), in which lumbar L5 and/or L6 nerves are tightly ligated; chronic constriction injury (CCI), in which loose ligations are placed around the sciatic or saphenous nerves; and partial sciatic or saphenous nerve ligation (PSNL), in which the dorsal third to half of the nerve is tightly ligated (reviewed in [9, 10]). Traditionally, attention has been focused on the enhancement of pain by leukocytes in neuropathy (reviewed in [10–15]). Interestingly, recent research suggests that immune cells can also ameliorate pain associated with nerve lesion (reviewed in [9, 16]). This chapter provides an overview of bimodal actions of the immune response in the modulation of neuropathic pain: detrimental, which can be mediated by proinflammatory cytokines, and beneficial, which are mediated by anti-inflammatory cytokines and opioid peptides. Other possible mediators such as endocannabinoids and resolvins are also addressed.

2 Immune Responses in the Generation of Neuropathic Pain

Injury to peripheral nerves leads to activation of resident cells, such as fibroblasts, mast cells, and macrophages as well as Schwann cells, which secrete proinflammatory cytokines (e.g., tumor necrosis factor [TNF]-α, interleukin [IL]-1β, IL-6), chemokines, nitric oxide, reactive oxygen species, prostaglandins, growth factors, or metalloproteases. Additionally, damaged nerve fibers release vasoactive and

algesic substances, including substance P and calcitonin gene-related peptide. Action of these mediators results in the blood-nerve barrier disruption, vasodilation, and enhanced blood vessel permeability and consequently in extravasation of blood-borne leukocytes (neutrophils, monocytes, and lymphocytes), which accumulate in lesioned nerves and dorsal root ganglia (DRG) (reviewed in [9–12, 14]).

Several studies have directly investigated the role of leukocytes in neuropathic pain. Systemic treatment with a cytotoxic neutrophil antibody decreased the number of neutrophils in the blood or injured nerves and diminished heat or mechanical hypersensitivity following PSNL [17, 18]. Stabilization of mast cells by injections of sodium cromoglycate increased the number of intact mast cells, reduced counts of neutrophils and macrophages at the site of nerve damage, and attenuated both forms of hypersensitivity after PSNL [19]. Treatments affecting macrophages were less consistent. Thus, mice with genetically delayed influx of macrophages had reduced heat hyperalgesia but enhanced mechanical hypersensitivity, compared to wild-type mice following CCI [20]. Treatment with liposome-encapsulated clodronate decreased the number of macrophages infiltrating injured nerves, reduced degeneration of nerve fibers, and attenuated thermal hyperalgesia following PSNL [21], but did not improve mechanical hypersensitivity after SNL [22]. Additionally, clodronate application in another study only slightly decreased sensitivity to noxious pressure and did not ameliorate sensitivity to innocuous mechanical stimulation in the PSNL model [23]. Furthermore, administration of macrophages to uninjured nerves did not induce mechanical hypersensitivity [22]. Together, these variable results suggest a limited contribution of macrophages to the generation of neuropathic pain. The role of T lymphocytes was assessed in athymic nude rats and mice, CD4 knockouts, recombination-activating gene-1 knockouts, and in mice with severe combined immunodeficiency. These animals developed less mechanical or thermal hypersensitivity compared to wild-type animals following CCI or transection of spinal nerves. However, the effects were usually moderate, often did not correlate with the temporal expression of T lymphocytes, and did not always appear to be solely attributed to their absence, but probably to the T lymphocyte genetic deficiency-related secondary alterations (e.g., decreased expression of astrocytes in the spinal cord) [24–27].

Enhancement of pain by leukocytes has been attributed to proinflammatory cytokines, of which TNF-α and IL-1β were most often examined. TNF-α mRNA or protein levels as well as TNF-α receptors were found at the site of nerve injury and in the DRG neurons following CCI [28, 29]. In animals without nerve damage, TNF-α and IL-1β applied on the sciatic nerves or into hind paws elicited discharges in peripheral sensory neurons [30] or induced mechanical and heat hypersensitivity [31]. Moreover, these electrophysiological and behavioral effects of TNF-α were enhanced following SNL [32]. Interestingly, however, the excitatory effects were produced by TNF-α and IL-1β in lower but not higher concentrations, which possibly resulted from activation of anti-inflammatory cytokines by higher doses of TNF-α and IL-1β [30–32]. Several other proinflammatory cytokines, IL-6, IL-15, IL-17, and IL-18, have also been implicated (reviewed in [14]). Consistently, strategies interfering with proinflammatory cytokine function ameliorated neuropathy-induced hypersensitivity. Thalidomide, which inhibits TNF-α synthesis, attenuated mechanical and heat hypersensitivity

following CCI. These effects were associated with decreased endoneurial levels of TNF-α and enhanced amounts of the anti-inflammatory cytokine IL-10 in the injured nerves as well as of the opioid peptide Met-enkephalin in the spinal cord [33]. Both forms of hypersensitivity were also reduced by etanercept, which prevents TNF-α binding to its receptor [34], and by antibodies to TNF-α or IL-1β, following CCI [35, 36]. Additionally, approaches indirectly affecting proinflammatory cytokine actions have been applied. Hence, blocking adenosine triphosphate signaling by purinergic 2 receptor antagonist (pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid) decreased IL-1β mRNA in injured nerves, DRG, and spinal cord (where also IL-1β protein level was reduced) as well as IL-6 mRNA in nerves and diminished heat and mechanical hypersensitivity following CCI [37]. Similar effects were also found by antagonizing estrogen receptor β with the isoflavone genistein [38]. Interestingly, systemic treatment with neural stem cells resulted in their migration to the injured nerves (but not uninjured nerves, DRG, spinal cord, or brain), diminished mRNA and protein levels of IL-1β and IL-6, slightly enhanced anti-inflammatory cytokine IL-10 mRNA (but not protein), and improved heat and mechanical hypersensitivity after CCI [39]. Likewise, analgesic effects of systemically applied human adipose-derived stem cells were associated with lower levels of IL-1β and elevated levels of IL-10 in CCI nerves [40].

In summary, most animal studies show that dampening proinflammatory cytokine responses improves neuropathic pain. Depletion of immune cells produced less coherent results, particularly regarding T lymphocytes and macrophages, which might be related to their heterogeneity. For example, while in vitro polarized T helper-1 lymphocytes (producing proinflammatory cytokines) enhanced pain, the T helper-2 lymphocytes (producing anti-inflammatory cytokines) decreased mechanical and thermal hypersensitivity following CCI [24]. Macrophages are the key phagocytic cells for removing degenerating axons' debris in a process of Wallerian degeneration, which facilitates the regrowth of injured axons (reviewed in [11]). Similar to lymphocytes, macrophages consist of M1 and M2 subpopulations [41], which might differentially modulate neuropathic pain. Additionally, immune cells are not the only source of cytokines, which are also present in Schwann, satellite glial, and neuronal cells (reviewed in [11, 13, 14]), and the relative contribution of each cell type has not been clarified. Moreover, as discussed below, immune cells produce opioid peptides, which serve as endogenous analgesics.

3 Analgesic Effects of Immune Responses in Neuropathy

3.1 Anti-inflammatory Cytokines

As described above, analgesic effects of various treatments aiming at inhibition of proinflammatory cytokines were associated with elevated levels of anti-inflammatory cytokines. For instance, amelioration of neuropathy-induced hypersensitivity by

thalidomide and stem cells correlated with increased numbers of macrophages expressing IL-10 or elevated levels of IL-10 in CCI nerves [33, 39, 40], and splenocytes driven in vitro to produce IL-4, IL-10, and IL-13 attenuated CCI-induced hypersensitivity following in vivo transfer [24]. Additionally, an injection of IL-10 at the site of nerve damage diminished the number of endoneurial TNF-α-expressing cells and attenuated thermal hypersensitivity after CCI [42]. Additionally, a viral vector encoding IL-4 led to the expression of IL-4 protein in DRG neurons and reduced mechanical and heat hypersensitivity following SNL. These effects were associated with decreased levels of IL-1β, prostaglandin E2, and phosphorylated p38 mitogen-activated protein kinase in the spinal cord [43]. The beneficial effects of anti-inflammatory cytokines are predominantly mediated through suppression of proinflammatory cytokines. Additionally, IL-4 upregulates opioid receptors [44].

3.2 Opioid Peptides

Opioid peptides belong to three main groups represented by endorphins, enkephalins, and dynorphins, which derive from the respective precursors, pro-opiomelanocortin, proenkephalin, and prodynorphin. Two additional opioids, endomorphin-1 and endomorphin-2, are known, but their precursors have not been identified. Opioid peptides possess different selectivity for the three opioid receptors, μ (endomorphins, endorphins, enkephalins), δ (enkephalins, endorphins), and κ (dynorphins), which belong to the family of seven transmembrane domain Gαi/o protein-coupled receptors. In addition to the nervous system, opioid peptides are synthesized by leukocytes. The full-length pro-opiomelanocortin transcripts as well as enzymes required for processing of pro-opiomelanocortin and proenkephalin have been detected in rodent or human immune cells. Importantly, β-endorphin, Met-enkephalin, Leu-enkephalin, dynorphin A, and endomorphins were found in T lymphocytes, neutrophils, and monocytes/macrophages infiltrating injured tissues. Opioid receptors have also been found in immune cells; however, their significance in pain transmission has not been directly examined. Thus, in peripheral injured tissue, leukocyte-derived opioid peptides activate opioid receptors on peripheral sensory neurons to locally inhibit pain. Mechanisms of such peripheral opioid analgesia have been extensively examined in animal models of inflammatory pain and are addressed in recent review articles [16, 45, 46]. Briefly, opioid peptide-producing immune cells use adhesion molecules (selectins, intercellular adhesion molecule-1, integrins α_4 and β_2) and chemokine (C-X-C motif) ligands (CXCL1, CXCL2/3) to accumulate in peripheral inflamed tissues [47–49]. Upon stressful stimulation (e.g., experimental cold water swim) or in response to releasing agents, such as corticotropin-releasing factor (CRF), cytokines (TNF-α, IL-1β), chemokines (CXCL1, CXCL2/3), and formyl peptides, immune cells secrete opioid peptides. The release of opioids from leukocytes is CRF-, IL-1-, and formyl peptide-receptor specific. Depending on the cell type, opioid peptide secretion is mediated by extracellular Ca^{2+} or by inositol trisphosphate receptor-triggered release of Ca^{2+} from

endoplasmic reticulum and is mimicked by potassium, consistent with vesicular secretion [50–52]. Moreover, blocking aminopeptidase N and neutral endopeptidase on leukocytes and peripheral terminals of sensory neurons prevented degradation of enkephalins and dynorphin A and locally ameliorated inflammatory pain [53].

The role of neuroimmune interactions involving opioids in the regulation of neuropathic pain has been recently investigated. β-Endorphin, Met-enkephalin, and dynorphin A proteins and their precursors' mRNA were found in neutrophils, macrophages, and T lymphocytes accumulating at the injured nerves following CCI or PSNL [27, 54–56]. All three opioid receptors were expressed in sensory fibers, and μ- and δ-receptors were upregulated in injured nerves [54, 57, 58]. Consistent with the expression of CRF receptors on opioid peptide-containing leukocytes, application of CRF at the site of nerve damage reversed mechanical hypersensitivity following CCI. These analgesic effects were blocked by locally applied CRF receptor antagonist, antibodies to β-endorphin, Met-enkephalin or dynorphin A, and selective antagonists of μ-, δ-, and κ-opioid receptors, as well as by systemic injections of antibody to intercellular adhesion molecule-1 [54]. While opioid peptide-containing neutrophils and macrophages are involved at early (2–3 days) and later (14–15 days) neuropathy stages, β-endorphin-containing T lymphocytes mediated analgesia in advanced neuritis, as demonstrated in mice with severe combined immunodeficiency. Hence, attenuated CRF-induced analgesia in these mice was restored by transfer of wild-type mice-derived T lymphocytes in the CCI model [27]. Additionally, peripherally restricted opioid receptor antagonist (naloxone methiodide) applied at the nerve injury site enhanced heat hyperalgesia following PSNL, suggesting a tonic control of neuropathic pain by endogenous opioids [55]. Furthermore, systemic treatment with granulocyte colony-stimulating factor enhanced the number of granulocytes containing β-endorphin in injured nerves and attenuated heat and mechanical hypersensitivity in the naloxone methiodide-sensitive manner. Concomitantly, TNF-α and IL-6 were downregulated in the DRG, whereas microglial activation was attenuated in the spinal cord [56]. Clearly, immune cells can be protective against neuropathic pain by utilizing opioid peptides.

3.3 Other Mediators

Endocannabinoids *N*-arachidonoylethanolamine (anandamide) and 2-arachidonoyl-glycerol are synthesized from polyunsaturated fatty acid, and their levels are regulated by metabolizing enzymes. Anandamide is primarily inactivated by the fatty acid amide hydrolase, while 2-arachidonoyl-glycerol is metabolized by monoacylglycerol lipase. Endocannabinoids exert their actions via cannabinoid receptors 1 and 2, which belong to the family of seven transmembrane domain Gαi/o protein-coupled receptors. Both cannabinoid receptors and their ligands are expressed in the pain-modulating pathways of the central and peripheral nervous system. Additionally, anandamide and 2-arachidonoyl-glycerol were found to be produced

and secreted by macrophage cell lines or native cultured macrophages. Similarly, cannabinoid receptor 2 and to a lesser extent cannabinoid receptor 1 are expressed on splenocytes, lymphocytes, natural killer cells, mast cells, monocytes, macrophages, and neutrophils in cell cultures and in vivo. Macrophage-derived anandamide and 2-arachidonoyl-glycerol interacting with vascular cannabinoid receptors were implied in endotoxin-induced hypotension, whereas activation of leukocytic cannabinoid receptors modulated leukocyte function (e.g., cytokine production and/or release, cell proliferation, migration, and apoptosis) (reviewed in [16, 59]). However, the significance of such immunomodulatory effects of cannabinoid receptors and of immune cell-derived endocannabinoids in pain transmission is yet to be established. For example, a peripherally restricted inhibitor of fatty acid amide hydrolase elevated levels of anandamide in peripheral tissue and suppressed hypersensitivity in inflammatory and neuropathic pain in a cannabinoid receptor 1-dependent manner, but the cellular source of anandamide was not identified [60].

Resolvins D and E are lipid mediators synthesized from polyunsaturated fatty acid by several enzymes, including cyclooxygenase-2, cytochrome P450, and 5- and 15-lipoxygenases, and are involved in the resolution of inflammation. Application of synthetic resolvin E1 into inflamed tissue reduced local neutrophil infiltration and expression of TNF-α, IL-1β, IL-6, and chemokine CCL2 and diminished heat hypersensitivity in an inflammatory pain model. Analgesic effects were also observed following injection of resolvin E1 on the spinal cord, in inflammatory and SNL pain models. These actions were mediated by Gαi-associated ChemR23 receptor. The receptor has been found on spinal microglia and central and peripheral terminals of DRG neurons as well as in macrophages accumulating in inflamed tissue (reviewed in [61]). Nevertheless, the relative contribution of each cell type as sources of ChemR23 receptors and resolvins to the modulation of neuropathic pain awaits clarification.

4 Clinical Evidence

Several clinical conditions associated with peripheral nerve damage involve immune reactions. In patients with neuropathies of various etiologies (including vasculitis, Guillain-Barré syndrome, alcohol abuse, and AIDS), the immunoreactivity of TNF-α in Schwann cells as well as of TNF-α, IL-1β, and IL-6 in macrophages and T lymphocytes in sural nerve biopsies was higher in patients with painful compared to those with nonpainful neuropathies. In addition, serum levels of TNF-α and IL-2 mRNA were higher in patients with painful neuropathy, while in those with painless neuropathy anti-inflammatory IL-4 and IL-10 mRNA levels were elevated. Proinflammatory IL-8 concentration in the cerebrospinal fluid was higher in postherpetic neuralgia patients compared to healthy controls, but there were no differences in serum levels of various other pro- and anti-inflammatory cytokines (reviewed in [62]). Interestingly, however, there were significantly fewer $CD3^+$ and $CD8^+$

T lymphocytes in biopsies of zoster skin lesions in these patients [5]. Furthermore, lowered plasma $CD4^+$ T lymphocyte counts paralleled increased incidences of sensory neuropathies in HIV patients [63]. Additionally, patients who developed phantom pain had significantly lower number of macrophages in nerve biopsies compared to patients without phantom pain after leg amputation; there were no differences in the number of T and B lymphocytes, cells expressing TNF-α or TNF-α receptor 1 in nerves, as well as in the serum levels of TNF-α and IL-6 [8]. Together, it appears that in some conditions, decreased counts of macrophages or T lymphocytes were associated with the presence of pain, suggesting their beneficial role in neuropathy. It is more difficult to find clear relationships between neuropathic pain and the expression of cytokines in patients, which might be related to the variety of neuropathies, stages of the disease, and/or examined tissue.

The investigation of the role of opioid peptide-containing immune cells in neuropathic pain has just begun in preclinical studies. So far, the clinical relevance of peripheral endogenous opioid analgesia has been shown for somatic inflammatory pain. β-Endorphin and Met-enkephalin were detected in synovial granulocytes, monocytes/macrophages, lymphocytes, and plasma cells, while opioid receptors were found in synovial tissue sensory neurons in patients with acute knee trauma and chronic arthritis. Blockade of opioid receptors by the antagonist naloxone injected into such tissue exacerbated pain after knee surgery. Furthermore, in these patients, CRF receptors and β-endorphin were co-expressed in synovial leukocytes, and the injection of CRF into the knee joint resulted in a transient but significant reduction of postoperative pain. This strongly indicates that immune cells continuously release and can be stimulated to secret opioid peptides to counteract inflammatory pain (reviewed in [16]). It remains to be examined whether immune mechanisms involving opioids, cannabinoids, and resolvins contribute to the regulation of neuropathic pain in patients.

5 Conclusions

There is a compelling body of evidence on the association of neuropathy with activation of the immune system. Although a majority of studies concentrated on pain-generating properties of immune responses, the analgesic actions of opioid peptide-containing leukocytes in experimental neuropathy were recently reported, and the presence of pain in some clinical neuropathic conditions correlated with lowered numbers of macrophages or T lymphocytes. Thus, it will be interesting to investigate the opioid production/release in leukocytes in such patients. These findings suggest that immunosuppressive strategies for the treatment of inflammatory diseases carry a risk to exacerbate pain. Clinical therapy of neuropathic pain with immunomodulatory agents such as steroids, nonsteroidal anti-inflammatory drugs, or anti-TNF-α drugs showed limited efficacy and can be associated with serious side effects, such as gastrointestinal ulcers and bleeding, kidney and liver toxicity,

infection, cardiovascular complications, and risk for tumor induction as well as neurological disorders, including demyelinating neuropathies (reviewed in [10, 64, 65]). Clearly, immune responses accompanying nerve injury are not exclusively maladaptive, and their favorable actions are not restricted to the removal of tissue debris and improvement of nerve regeneration. It appears that immune cells need to be stimulated to secrete opioids to produce adequate pain relief. Technology-oriented research [66] is needed to find novel ways to target opioid-containing cells, anti-inflammatory cytokines, and mediators involved in the resolution of inflammation in the relevant damaged tissues. This represents an attractive opportunity to use intrinsic beneficial effects of neuroinflammation as possible therapies of painful neuropathies. Importantly, since chronic pain is a complex biopsychosocial phenomenon, an interdisciplinary management, including psychological, physical, and occupational therapy, needs to be combined with pharmacological treatments [67].

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Part II
Neurological Diseases and Inflammation

Innate and Adaptive Immune-Mediated Neuroinflammation and Neurodegeneration in Parkinson's Disease

Rebecca A. Wilshusen and R. Lee Mosley

Abstract Innate and adaptive immunity affect the pathogenesis of Parkinson's disease (PD). In particular, the activation of microglia that feed neuroinflammation and oxidative stress influences the degeneration of dopaminergic neurons along the nigrostriatal axis during disease. Activated microglia that proximate degenerating neurons within the substantia nigra are a hallmark of PD. Other PD hallmarks include neuronal Lewy body inclusions composed primarily of aggregated ubiquitin and neuronal proteins. Under inflammatory conditions, oxidative or nitrative modifications of neuronal proteins, such as ?-synuclein, lead to misfolding and formation of neurotoxic species that accumulate within the neuron. Release of misfolded proteins from injured or dead neurons intensifies neuroinflammation and neuronal injury within the surrounding area. Moreover, once in the peripheral immune compartments, processing and presentation of modified proteins, such as nitrated ?-synuclein by dendritic cells, induce effector T cells to those nitrated epitopes that act as neoantigens. Robust effector T cell immune responses against nitrated or modified self-proteins and migration of those effector T cells to inflammatory sites exacerbate neuroinflammation and dopaminergic neurodegeneration, which lead to accelerated disease progression. The links between T cell immunity and nigrostriatal neurodegeneration are supported by laboratory and animal models as well as human investigations of immune-associated biomarkers in the spinal fluid, peripheral blood, and brain tissue of patients with idiopathic or familial forms of PD. Regulatory T cells modulate both innate and effector T cell-mediated immunity to attenuate neuroinflammation and alleviate neurodegeneration along the nigrostriatal axis. Thus, harnessing proinflammatory and neurotoxic effector immune responses with drugs, vaccination, or immunomodulation affords promising therapeutic strategies

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either alone as an interdictory therapy or in combination as adjunctive therapy in the context of neuronal replacement. Herein immune-mediated inflammation, oxidative stress, and neurodegeneration as linked to PD pathogenesis are examined as well as the potential benefits of efficacious immune regulatory control over those neurotoxic processes.

Keywords Parkinson's disease • Neuroinflammation • Neurodegeneration • Dopaminergic • Adaptive immunity • Innate immunity • Microglia • Effector T cells • Teffs • Regulatory T cells • Tregs

Abbreviations

PD	Parkinson's disease
SNpc	Substantia nigra pars compacta
LB	Lewy body
α-syn	α-Synuclein
TNF	Tumor necrosis factor
LPS	Lipopolysaccharide
IFN-γ	Interferon gamma
CD40L	CD40 ligand
tPA	Tissue plasminogen activator
MMP-3	Matrix metalloproteinase-3
β-Amyloid	Amyloid beta
MHC	Major histocompatibility complex
ADCC	Antibody-dependent cell cytotoxicity
LFA-1	Lymphocyte function-associated antigen-1
ICAM-1	Intercellular adhesion molecule-1
VCAM-1	Vascular cell adhesion molecule-1
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
iNOS	Inducible nitric oxide synthase
COX1	Cyclooxygenase-1
COX 2	Cyclooxygenase-2
NADPH	Nicotinamide adenine dinucleotide phosphate
HLA-DR	Human lymphocyte antigen-DR
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
6-OHDA	6-Hydroxydopamine
NO	Nitric oxide
PGE2	Prostaglandin E2
PRRs	Pattern recognition receptors
PAMPs	Pathogen-associated molecular patterns
DAMPs	Damage-associated molecular patterns
TCR	T cell receptor
APCs	Antigen-presenting cells

Th1	Type 1 T helper
Th2	Type 2 T helper
Th17	Type 17 T helper
HSP	Heat shock protein
GFAP	Glial fibrillar acidic protein
NGF	Nerve growth factor
BBB	Blood-brain barrier
RANTES	Regulated on activation normal T cell expressed and secreted
Tregs	Regulatory T cells
N-α-syn	Nitrated α-syn
Teffs	Effector T cells
GDNF	Glial cell-derived neurotrophic factor
nTregs	Natural Tregs
IPEX	Immune-dysregulation polyendocrinopathy, enteropathy X-linked
iTregs	Induced or inducible Tregs
TGF-β	Transforming growth factor-β
NF-κB	Nuclear factor-kappa B
NSAIDs	Nonsteroidal anti-inflammatory drugs
MAP	Mitogen-activated protein
MCP-1	Monocyte chemotactic protein-1
BDNF	Brain-derived neurotrophic factor
VIP	Vasoactive intestinal peptide
GM-CSF	Granulocyte/macrophage colony-stimulating factor

1 Introduction

Parkinson's disease (PD) is a relatively common neurodegenerative disorder, second in prevalence only to Alzheimer's disease, but the most common neurodegenerative movement disorder. Rare before 50 years of age, PD affects approximately 1–2 % of the population older than 60 years, with the incidence increasing to 3–5 % for those over 85 years of age [1]. Most studies in the USA and Europe estimate the annual incidence rate at 16–19 per 100,000 [2]. An estimated five million people worldwide are afflicted with PD, with 1–1.5 million people each in the USA and Europe. The average age of onset for PD is approximately 60–65 years with the peak incidence between 70 and 80 years of age. Thus, age represents a primary risk factor for PD with an increased prevalence among the population of increased age. This is particularly noteworthy given the increasing levels of neuroinflammation and oxidative stress associated with progressive age [3].

Clinical signs and severity of disease associated with PD result from the progressive loss and neuropathology of neurons that synthesize the neurotransmitter dopamine and are responsible for initiation of movement. The dopaminergic neurons that are primarily lost in PD are those that originate in the substantia nigra pars compacta (SNpc) and innervate the dorsal striatum. This nigrostriatal pathway represents one

of the four major dopamine signaling pathways in the brain and is prominently involved in controlling movement [4]. Thus, degeneration of these neurons and loss of dopamine along this axis result in the presentation of the pathophysiological clinical signs of PD: tremors, gait disturbance, muscle rigidity, and cognitive dysfunction. Dynamic neuronal dysfunction and loss that progress without intervention eventually lead to total loss of physical movement and ultimately to death [5].

Other pathological hallmarks in PD include neuronal cytoplasmic inclusions known as Lewy bodies (LBs). LBs are eosinophilic inclusions composed primarily of aggregated proteins including α-synuclein and ubiquitin as two major components [6–10]. Although, typically only observed among postmortem brain samples from PD patients, LBs are considered diagnostic for PD. In the brain, α-synuclein (α-syn) exists as a natively unfolded and soluble protein localized in the presynaptic terminals of neurons and is postulated to be involved in chaperoning vesicles to synaptic membranes. Posttranslational modifications of α-syn through nitration, oxidation, or phosphorylation enhance misfolding, oligomerization, fibril formation, and aggregation [11–14]. These processes are responsible for converting nontoxic molecules of α-syn into toxic fibrillar species that induce annular pore formation in neuronal membranes and eventually lead to neurodegeneration [15, 16]. Misfolding of α-syn is also caused by genetic mutation or overexpression. While most cases of PD have no family history of disease, point mutations in the gene encoding α-syn (SNCA; OMIM 163890) are linked to autosomal dominant parkinsonism (PARK1, OMIM 168601) [17–20] as are duplications and triplications of the SNCA gene (PARK4, OMIM 605543) [21, 22], all of which increase the aberrant misfolding of α-syn [23–25]. Taken together, these observations led to the α-syn burden hypothesis of PD which posits that misfolding of excess wild-type or mutated α-syn and the inability of the cell to clear the misfolded toxic species lead to the etiology or progression of familial and sporadic disease [26].

The reason(s) for increased susceptibility of dopaminergic neurons along the nigrostriatal axis in PD remains enigmatic. Neuronal death can be induced along several different, but cross-signaling pathways. One parameter thought to be responsible for increased susceptibility was the oxidative synthesis and metabolism of dopamine, which would increase oxidative stress within neurons [27, 28]. Additionally, dopaminergic neurons in the substantia nigra possess low intracellular glutathione which results in reduced antioxidant capacity and therefore are rendered more susceptible to oxidative stress than other types of cells located in the same area [29]. However, dopaminergic neurons in close proximity to the SNpc, which exist under similar conditions, appear undisturbed. Reactive oxygen species produced by activated microglia also have the potential to cause dopaminergic cell death by directly crossing the membrane to enter the dopaminergic neuron and overwhel the endogenous antioxidant systems to elevate oxidative stress that eventually leads to increased apoptosis and degeneration of the neuron [30]. In that same vein, since dopaminergic neurons in the substantia nigra widely express the death signaling tumor necrosis factor (TNF) receptor, chronic inflammation and delivery of proinflammatory cytokines such as TNF-α can induce and augment apoptotic cell death processes [30].

2 Neuroinflammation and Neurodegeneration in Parkinson's Disease

While the etiology of PD remains enigmatic, strong evidence now supports a non-cell autonomous theory, which promotes the notion that neuroinflammation is strongly implicated in etiology and/or disease progression [31]. Indeed, other hallmarks of PD include the abundant signs of neuroinflammation as evidenced by activated glia in ante- and post-mortem analyses of patient brains [32–34]. Interestingly, the predominant inflammatory glial cell population in PD is the microglia, whereas reactive astrocytes and myelinating oligodendrocytes are largely absent [32, 35, 36].

Microglia are a population of cells derived from the myeloid lineage of the innate immune system. They constitute approximately 20 % of the glia in the brain and represent the brain's resident phagocytic cells, thereby making them the first line of defense against pathogens and foreign material [37, 38]. Microglial progenitor cells enter the brain during early embryogenesis and develop throughout the brain within the parenchyma, choroid plexus, leptomeninges, and among the microvasculature [38, 39]. Resident microglial cells in a healthy brain typically are in a resting or dormant state with a ramified morphology, and not until activation are structural changes observed that include motile branches or the migration of somata [40]. Resting microglia have small rod-shaped somata that remain fixed with few signs of migration capacity. Numerous and highly ramified processes extend from the somata and continuously undergo cycles of de novo formation and withdrawal, participating in immune surveillance by sampling the extracellular space in a seemingly random fashion with a high turnover rate [30, 40]. These processes enable the microglia to efficiently sample and control the microenvironment of the brain and clear the surrounding tissue of increased metabolic products and deteriorated tissue components. The extensive processes and protrusions of the microglia enable direct contact with other cells of the brain, particularly astrocytes, neuronal cell bodies, and blood vessels, suggesting that microglia dynamically interact with other cellular elements to generate a finely tuned communications network that quickly allows microglia to become activated in response to insult and injury [37, 40]. In contrast, some interactions seem necessary for homeostasis and regulation, such as the interaction of CD200 on neurons and endothelial cells with CD200R on microglia, whereby deletion of CD200 or blockade with CD200R antagonists results in increased levels of microglia demonstrating an activated phenotype [5].

Microglia have a multitude of receptors on the cell surface, including toll-like receptors, cytokine and chemokine receptors, and ion channels that allow the microglia to remain sensitive to small changes in the extracellular environment. These changes result from a plethora of stimuli and danger signals including pathogens, viral DNA, lipopolysaccharide (LPS), interferon gamma (IFN-γ), CD40 ligand (CD40L), chemokines, and proteases such as thrombin and tissue plasminogen activator (tPA) as well as neurotransmitters, gangliosides such as GM1, matrix metalloproteinase-3 (MMP-3), and even simple alterations in ion homeostasis [37, 41–47]. More important to neurodegenerative disorders such as Alzheimer's disease and PD are microglial responses to misfolded proteins such as amyloid beta (β-amyloid) and

α-syn, respectively. Under homeostatic conditions damaged and misfolded proteins in the brain are cleared by the ubiquitin-proteasome pathway [48]. The immune surveillance function of myeloid cells in the brain, such as microglia, enables the cells to contact and control the neuronal microenvironment, thereby maintaining the homeostatic conditions of the brain. However, encounters with misfolded proteins, such as aggregated or modified α-synuclein, that cannot be cleared adequately from the microenvironment can lead to increased microglial activation [49].

Detection of disturbances within the neuronal microenvironment induce microglia to become activated with graded responses relative to the extent of perturbation [50]. Upon activation, resting microglia undergo several physical and metabolic changes including increases in proliferative capability, morphogenesis, cell volume, and extension of their processes into an amoeboid appearance, which is morphologically distinct from resting microglia that present small cell bodies with ramified processes [5, 37, 51]. Morphological changes of microglia become more macrophage-like in appearance, express surface proteins such as CD11b or major histocompatibility complex (MHC) antigens, and change the expression pattern of cytokines to resemble a more proinflammatory profile [38, 52, 53]. During activation, maximal densities of reactive microglia are found close to the lesion or epicenter of injured cells [52].

Among their many innate immune functions, activated microglia are well adapted for the induction of inflammation as well as cytokine-mediated cytotoxicity and antibody-dependent cell cytotoxicity (ADCC). Moreover, microglia with antigen presentation and co-stimulatory capabilities are able to induce and regulate T cell responses [54]. In the activated state, microglia show increased expression of Fc receptors leading to increased IgG reactivity necessary for ADCC, upregulation of complement receptors, and increased expression of cell adhesion molecules such as lymphocyte function-associated antigen-1 (LFA-1), intercellular adhesion molecule-1 (ICAM-1) (CD54), vascular cell adhesion molecule-1 (VCAM-1) (CD106), and CD1 [50], thus amplifying the abilities of microglia to traffic and migrate. Of interest, deletion of the FcγR by genetic ablation inhibits microglial activation and dopaminergic cell death in animal models of PD [55]. Activated microglia are known to produce a variety of toxic substances that, in addition to killing infectious agents, also can accelerate neuronal injury and death. These toxic substances include reactive oxygen species (ROS), reactive nitrogen species (RNS), and proinflammatory mediators such as cytokines, chemokines, and prostaglandins [37, 40, 52, 56]. As expected, generation of reactive species is potentiated by upregulation of enzymes such as inducible nitric oxide synthase (iNOS), cyclooxygenase 1 (COX1), COX 2, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase resulting in increased levels of oxidative stress. These inflammatory conditions alter most cellular functions including proliferation, differentiation, and cell death. High levels of ROS and RNS can inactivate or damage proteins, which then result in cellular death and degeneration [5]. iNOS produces high levels of nitric oxide (NO) and superoxide radicals which increase the nitration and modification of neuronal proteins such as α-syn and in turn preferentially amplify microglial activation and cell-surface expression of MHC class II (MHC II) molecules [8, 9, 56]. Large numbers of activated microglia that express increased human lymphocyte antigen-DR (HLA-DR) (i.e., a human MHC II) have been shown in the substantia nigra of postmortem samples from brains of patients with PD or

parkinsonism with dementia [32, 33] and in antemortem analyses by PET imaging with PK11195, a ligand for the peripheral benzodiazepine receptor (PBR) that is preferentially expressed by activated microglia and correlates with disease severity in PD [34]. Additionally, activated microglia are present in the SN and striatum of animals used in models of PD such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)- and 6-hydroxydopamine (6-OHDA)-induced parkinsonism [49, 57–65].

Unregulated microglial activation has proven toxic to neurons due in part to release of proinflammatory factors including, but not limited to, interleukin (IL)-1β, TNF-α, IL-6, IFN-γ, NO, prostaglandin E2 (PGE2), and superoxide radical [8, 9, 52, 62, 66]. IL-1β activates microglial iNOS and NADPH oxidase that generate NO and superoxide radicals, respectively [38, 39], both of which directly and indirectly produce oxidative damage to neurons that is sufficiently severe to result in imminent death [56]. In addition, IL-1β secretion from microglia activates the cells in an autocrine fashion and upregulates expression of cell-surface receptor CD23 (FcεRII), an early biomarker of microglial activation. Subsequent activation after CD23 expression induces microglia to express greater amounts of iNOS which subsequently leads to the production and buildup of NO [56]. This cascade of inflammation set up by acutely activated microglial cells can eventually progress to chronicity and lead to the activation and recruitment of the adaptive immune system in PD [52] (see Sect. 3 below).

Interestingly, the SN contains the highest concentration of microglia in the brain, especially in the ventral tier of the pars compacta [67, 68], thus making this region especially susceptible to perpetuated microglial activation. Abnormally high levels of IL-1β and TNF-α found in the plasma and cerebral spinal fluid in patients with PD support this contention [69, 70]. Reactive microglia are highly localized, found close to cell bodies of dead or injured nigral dopaminergic neurons, but not around striatal degenerating termini [36], which suggests a retrograde mechanism for neuronal death. Cell death can also result from loss of trophic support stemming from microglia-induced neuritic beading or synaptic stripping along dendrites [71, 72]. These findings suggest a direct link between dopaminergic neuronal death in PD and microglial activation. Indeed, postmortem examinations demonstrate that neuronal degeneration in PD is associated with a substantive gliosis linked to activated microglia that has been shown in MPTP-induced parkinsonism in primates, rodents, and humans [33, 38, 73–75]. Moreover, activated microglia have been shown phagocytizing dying dopaminergic cells and are well correlated with α-syn deposition in neuronal inclusions [12]. Importantly, in vivo and in vitro studies demonstrated that microglia become activated in response to overexpression of α-syn or nitrated and aggregated forms of α-syn, a major component of LBs found in the brains of PD patients [49, 76]. Release of modified and misfolded proteins such as α-syn from injured or degenerating neurons increases proximate microglial activation and subsequent degeneration of dopaminergic neurons in the SNpc. Moreover, oxidative stress is upregulated by a multitude of mediators including increased levels of reactive oxygen, nitrogen, and carbonyl species, which in turn raise high oxygen demand in the absence of low antioxidant defense mechanisms [37]. The increased oxidative stress induces more modified α-syn to become misfolded and aggregated with formation of more fibrillar species and LBs associated with degenerating nigral neurons that feed this cyclic cascade of progressive neuroinflammation and neurotoxicity in PD [48, 49, 52, 62, 77].

3 Adaptive Immune-Mediated Exacerbation of Neuroinflammation and Neurodegeneration

In addition to the involvement of the immune system's innate arm, adaptive immunity plays a profound role in neurodegeneration of the nigrostriatal pathway and disease progression. A controlled immune response to infection and injury supports homeostasis of the central nervous system (CNS) microenvironment by protecting the host via killing pathogens and clearance of pathogenic debris to promote healing. A chronically activated immune response without regulation amplifies inflammatory and immune responses that lead to further tissue and cellular damage [78]. Adaptive immunity utilizes immunological memory to recognize specific pathogens and mount a stronger, amplified response than that afforded by innate immunity alone. Adaptive immunity is typically initiated after innate immune cells, such as dendritic cells, macrophages, or microglia via their pattern recognition receptors (PRRs), recognize broad specificities of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), which are associated with microbial pathogens, cellular stress, or cell components of damaged tissues [79]. By activating antigen processing programs, innate immune cells digest and process these substances, insert the processed peptides into the groove of MHC molecules, and present the peptide epitopes in the context of the MHC I or II molecules. Upon recognition of antigen by T cells and their T cell receptor (TCR) for antigen, antigen-presenting cells (APCs) become activated to express co-stimulatory molecules and second signals such as IL-12 and IL-4 that aid in the maturation of type 1 T helper (Th1) or Th2 effector T cells that influence cell- or antibody-mediated immunity, respectively.

While autoantibodies against dopamine neuron antigens are present in the sera and CSF of PD patients [80–82], the role of antibody-mediated or humoral immunity in PD has only recently begun to be investigated in depth. In addition to a variety of antibodies directed against globally expressed tissue antigens such as heat shock protein (HSP)-65 and HSP-70 [83], PD patients also exhibit autoantibodies to brain-associated molecules including GM1, S100B, glial fibrillar acidic protein (GFAP), nerve growth factor (NGF), neurofilament, myelin basic protein, tau, Aβ, and neuronal calcium channels as well as α-syn and its modified and fibrillar forms [84–92]. Immunohistochemical staining of tissues from idiopathic and familial PD patients show dopaminergic neurons within the SN that have bound IgG, but not IgM, while tissues from age-matched controls and non-nigral control tissues show no detectable bound immunoglobulin [93]. The IgG was reacted with approximately 30 % of the dopaminergic neurons within the nigra and directly correlated with numbers of MHC II$^+$ and CD64$^+$ (FcγRI) reactive microglia, but not with disease duration. In PD patients, about 4 % of the pigmented neurons contain LBs, and all pigmented, LB-containing neurons presented detectable amounts of bound IgG as well as α-syn within the inclusions. These patterns of antibody reactivity were consistent with numbers of activated microglia around dopaminergic neurons that are at risk of dying in PD. Together these data suggest that endogenous antibodies

of unknown specificity have the capacity to cross the blood-brain barrier (BBB) and bind cognate antigens expressed by dopaminergic neurons. Moreover, levels of antibodies to α-syn and catecholamine-derived melanin (i.e., neuromelanin) are increased in PD patients, while anti-neuromelanin immunoglobulin binding to neurons is more prevalent in early disease [94]. Opsonization or autoantibody binding of neurons targets those cells for phagocytosis and degradation by mononuclear phagocytes such as macrophages and microglia and also can activate the complement system, a major mediator of cell lysis and inflammatory reactions. Interestingly, activation of the complement system may also be involved in microglia-mediated neuronal death since microglia are the only cells within the SN that express the initial recognition component of complement, C1q [95]. Moreover, compared to controls, PD patients exhibit increased areas of C1q-opsonized extracellular depositions of neuromelanin within the parenchyma, and C1q-expressing phagocytic microglia surround those areas as well as cells around the luminal surfaces of the vasculature which express neuromelanin and C1q, suggesting a role for anti-self antibodies and C1q-mediated clearing pathways in PD.

In addition to activated microglia and astrocytes, T cells also comprise an integral component of the PD lesion, although their function or even the mechanisms by which they infiltrate remain enigmatic. Early autopsy evidence within the SN of PD patients showed increased numbers of $CD8^+$ T cells in close proximity to activated microglia and degenerating neurons [33]. More recently, both $CD4^+$ and $CD8^+$ T cells have been discovered within the SN of PD patients as well as MPTP-treated mice [65, 96–100]. As PD patients exhibit some leakiness across the BBB, the lymphocytic infiltration was thought to reflect a dysfunctional BBB [101]; however, CD4:CD8 lymphocyte ratios of 1:5 in the SN of PD patients [96] and 1:4 in the SN of MPTP mice [100] contrast the typical 2:1 ratio expected for peripheral T cells performing surveillance functions and strongly suggest the involvement of active processes other than dysfunctional BBB. Thus, the mechanisms by which these T cells gain access to the SN, their activation state, and their actual function are questions that remain to be answered.

Peripheral immune aberrations, particularly in T lymphocyte subsets are abundant in PD patients. Total numbers of lymphocytes have been shown to be diminished by 17 %, while $CD19^+$ B cells are diminished as much as 35 % and $CD3^+$ T cells diminished by 22 % [102]. Among $CD3^+$ T cells, numbers of $CD4^+$ T cells have been shown to be diminished by 31 %, whereas numbers of $CD8^+$ T cells are not significantly changed. Naïve helper $CD4^+$ T cells ($CD45RA^+$) are diminished and levels of effector/memory helper T cell subsets ($CD29^+$ or $CD45R0^+$) are either unchanged or increased. Selective loss of $CD4^+CD45RA^+$ naïve cells are also detected in other neuropathological-associated disorders such as MS and Down's syndrome, suggesting a common immunological abnormality in those neurological disorders [103, 104]. Studies of PD patients' peripheral blood showed increased frequencies of activated $CD4^+$ T cells expressing Fas [105], increased IFN-γ-producing Th1 cells, diminished frequencies of IL-4-producing Th2 cells, and $CD4^+CD25^+$ T cells [106]. Circulating levels of IL-15, RANTES (regulated on activation, normal T cell expressed and secreted), IL-10, and IL-12 are significantly

elevated in PD patients compared to controls suggesting that immune effector cells are activated in PD [107, 108]. Evidence for the increased mutual co-expression of CD4 and CD8 by CD45RO$^+$ T cells with increased expression of CD25 (α-chain of the high-affinity IL-2 receptor), TNF-α receptors, and diminished expression of IFN-γ receptors also suggest the presence of activated T cell subsets in PD patients.

In addition to T cells that express α- and β-chains of the T cell receptor (TCRαβ$^+$ T cells), elevated frequencies of T cell populations expressing γ- and δ-chains of the TCR (TCRγδ$^+$T cells) also have been found in the CSF of PD patients [109] and are thought to play a regulatory role in CNS inflammation [110–112]. Moreover, a larger proportion of the TCRγδ$^+$ T cells also express CD25 compared to controls, suggesting these CSF-obtained T cells are preferentially activated in PD patients [104]. More recently, T cell frequencies were compared to disease severity as measured by the Unified Parkinson's Disease Rating Scale III scores. Those studies showed that in peripheral blood from PD patients compared to age- and environment-matched caregivers, frequencies of CD45RO$^+$ and FAS$^+$ effector/memory CD4$^+$ T cells were increased and were directly correlated with disease, while those of CD31$^+$ and α4β7$^+$ CD4$^+$ T cells were diminished and inversely correlated with disease progression [113]. No correlations could be established between frequencies of CD4$^+$ cells within any T cell subsets and age or disease duration. Additionally, the ability of CD4$^+$ regulatory T cells (Tregs) from PD patients to suppress anti-CD3-stimulated effector T cell function from healthy donors was impaired. Overall, these data support the notion that chronic immune stimulation, notably effector/memory T cell activation and Treg dysfunction, is linked to PD pathobiology and disease severity, but not disease duration. The association of T cell phenotypes with motor symptoms provides fresh avenues for novel biomarkers and therapeutic designs.

One mechanism by which T cells may be mobilized to infiltrate the CNS during PD is through the drainage of aberrant forms of α-syn into the lymphatic system, which activates APCs to induce T cells that recognize aggregated or nitrated α-syn (N-α-syn) species (Fig. 1). Indeed, in MPTP-intoxicated mice, α-syn and most likely other inflammatory mediators drain to cervical lymph nodes where APCs are activated to induce effector T cells [97]. An influx of α-syn-specific Th1 or Th17 effector T cells into the brain during PD could increase the inflammatory phenotype and neurotoxic response of microglia near dopaminergic neurons by increasing the concentration of proinflammatory molecules in the SN [63]. Taken together, increased frequencies of memory and activated peripheral T cell subsets, as well as those cells within the nigra of PD patients, suggest putative roles of T cells in disease progression and possibly PD etiology. Although those roles have yet to be delineated, activated effector T cells (Teffs) or Tregs both exhibit effector/memory T cell phenotypes and may migrate to foci of inflammation in PD patients and lead to Teff-mediated exacerbation or Treg-mediated attenuation of PD-associated neuroinflammation and neurodegeneration. Thus, Tregs have the capacity to keep the disorder in check during the early or asymptomatic phase, while Teffs can accelerate disease progression (Fig. 1). Whether T cell aberrations in PD patients reflect specifically activated effector or regulatory T cell subsets and to which specific antigen(s) those T cells are activated require answers to develop more precise immune-based therapeutic strategies.

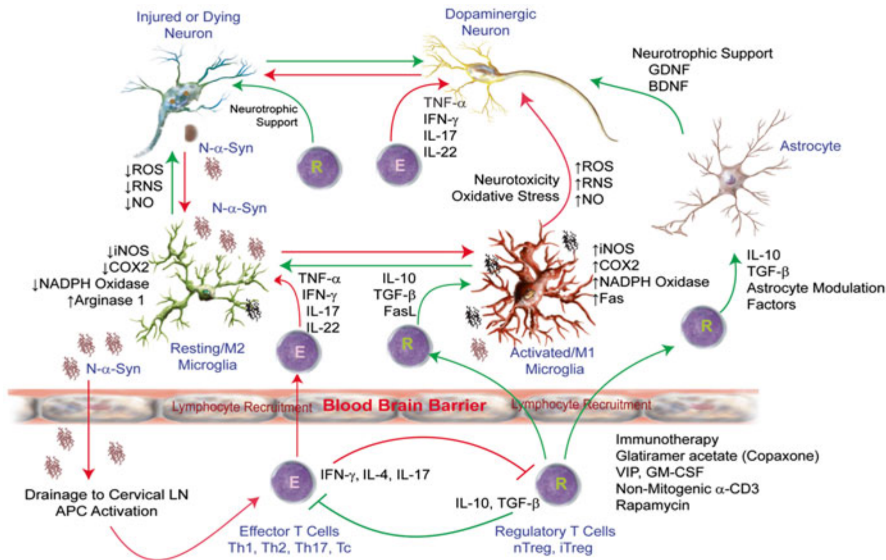

Fig. 1 Innate and adaptive immune contributions to PD pathogenesis. Reactive microglia are a prominent feature of PD. Microglia are sensitive to changes in their microenvironment, which include factors released from damaged/dying neurons such as DAMPs and self-proteins such as α-synuclein (α-syn) that have been modified, misfolded, or aggregated. These factors drive resting microglial to an activated M1 phenotype characterized by secretion of proinflammatory and neurotoxic factors and upregulation of proinflammatory mediator enzymes such as iNOS, COX-2, and NADPH oxidase. Induction of these enzymes increases ROS/RNS production and oxidative stress, which perpetuate neuronal injury and death. Microglia-derived proinflammatory cytokine/chemokine gradients recruit other lymphocytes to migrate across the BBB to sites of inflammation. One such T lymphocyte population is the regulatory T cell subset (Tregs) (R in figure). Tregs produce anti-inflammatory cytokines, such as IL-10 and TGF-β, which induce microglia to switch from a proinflammatory (M1) phenotype to an anti-inflammatory (M2) phenotype. The M2 phenotype is characterized by downregulation of iNOS, COX-2, and NADPH oxidase and upregulation of arginase-1, an enzyme with capabilities to suppress activated microglia or T cells. Changes in these enzyme functions diminish ROS/RNS formation and maintain oxidative stress at homeostatic levels. Moreover, in the presence of activated microglia which upregulate Fas, Tregs upregulate Fas ligand (FasL) that leads to subsequent killing of activated microglia via Fas-FasL interactions. Additionally, Tregs induce astrocytes to increase expression of neurotrophic factors GDNF and BDNF, which afford another neuroprotective mechanism. However, with age PD progresses, Treg functions decline, and regulatory control over proinflammatory microglia and neurotrophic support is not maintained. Thus, inflammation and dopaminergic neurodegeneration increase with augmented misfolding of modified self-proteins such as nitrated α-synuclein (N-α-syn). These proteins and the ensuring inflammatory milieu may drain to periphery immune compartments, where, as neoantigens, adaptive immune responses such as effector T cells (Teffs) (E in figure) are mounted against the modified epitopes. With increased inflammation, Th1 and Th17 Teffs are recruited and migrate across the BBB to the sites of inflammation and encounter cognate antigen presented by activated microglia. Th1 and Th17 cells express proinflammatory cytokines such as TNF-α, IFN-γ, IL-17, and IL-22, which drive microglia to higher levels of inflammation, thus indirectly enhancing neurotoxicity. A mechanism by which Teffs may directly kill neurons is suggested since both Th1 and Th17 cells produce TNF-α, and nigral neurons express TNF receptors capable of triggering apoptotic pathways. Of importance, proinflammatory cytokines from Teffs inhibit Treg functions, which further restrict regulatory control and exacerbate Teff- and microglia-mediated inflammatory responses. Treg numbers or function can be augmented with exogenous agents such as VIP, GM-CSF, anti-CD3, or rampamycin. Thus, strategies aimed at inducing, boosting, or reprogramming Treg responses by increasing Treg numbers and function, transforming Teffs to Tregs, or activating Tregs in an antigen-specific manner show promise as possible disease-modifying therapies that slow or halt progression of PD

Modified α-syn exposed to the immune system in sufficient quantity and under the appropriate conditions, such as a proinflammatory milieu, can function as a foreign antigen to engage adaptive immunity. We previously demonstrated that nitrated α-synuclein (N-α-syn) acts as a neoantigen that breaks immunological tolerance, exacerbates MPTP-induced neuroinflammation, and enhances subsequent dopaminergic neurodegeneration along the nigrostriatal axis [8, 48, 97, 114]. In MPTP-treated mice, the inflammatory milieu, which includes N-α-syn, drains to the cervical lymph nodes within 24 h and activates APCs. Conceivably, N-α-syn that is chronically released from degenerating neurons would reach the circulation and afferent lymphatics of PD patients, be sequestered in the secondary lymphoid tissues to be processed and presented by MHC II of activated APCs and induce effector T cells [48]. Indeed, after several weeks, MPTP-treated mice produce antibodies to N-α-syn, but not to unmodified α-syn [97]. Moreover, immunization with N-α-syn induces effector T cells that recognize nitrated epitopes, but not non-nitrated α-syn moieties. In contrast, immunization with unmodified α-syn yields no detectable effector T cells that recognize either unmodified or N-α-syn. The induced N-α-syn-specific Teffs are thought to be recruited to areas of inflammation surrounding the dopaminergic neurons in the SN. Since the Teffs are specific for the disease-associated protein modifications in α-syn, presentation of N-α-syn epitopes by MHC II of already activated APCs (microglia, macrophages, or dendritic cells) within the inflamed SN would evoke a helper Teff-mediated immune response and promote expansion and/or cytokine secretion appropriate for the type of induced helper Teff subtype that includes Th1, Th2, or Th17 Teffs. Th1 cells express various proinflammatory cytokines such as IL-2, IFN-γ, and TNF-α that act to induce activated microglial release of reactive oxygen species, NO, and proinflammatory factors, all of which are neurotoxic [30, 48] (Fig. 1). Th17 cells also release proinflammatory cytokines such as TNF-α, IL-17A, IL-17F, IL-21, and IL-22 which (a) induce non-hematopoietic tissue reactions due to the distribution of IL-17 and IL-22 receptors among both epithelial and endothelial cells; (b) stimulate the production of inflammatory cytokines such as TNF-α, IL-1β, and IL-6; and (c) induce IL-21/IL-21 receptor-mediated signaling by T cells, B cells, myeloid-derived cells, and natural killer cells [48, 115–119].

Secretion of proinflammatory cytokines such as TNF-α, IL-1β, and IL-12 disrupts the BBB integrity by increasing the expression of cellular adhesion molecules, such as E-selectin, while inducing chemokine gradients to allow the extravasation of Teff subtypes through the endothelial cells of the microvasculature and into the brain at the site of inflammation [8, 9, 37, 38, 63]. Once within inflammatory sites of the SN, Th1 and Th17 effectors can secrete proinflammatory and neurotoxic cytokines (Fig. 1). Alternatively, Teffs could drive microglia to a hyperactivated or neurotoxic phenotype that produces greater levels of neurotoxic mediators within the microenvironment, killing surrounding dopaminergic neurons, and leading to accelerated disease progression. In the MPTP model, N-α-syn-specific Teffs exacerbate the extent of the neuroinflammatory response with increased numbers of activated microglia, prolong the intensity of the inflammatory response, accelerate the rate of dopaminergic cell death, and worsen the nigrostriatal lesion by 50 % [63, 97].

Moreover, N-α-syn Teffs polarized to yield Th17 effectors afford significantly greater capacity to kill dopaminergic neurons compared to those Teffs polarized as Th1 effectors [8, 63]. Thus, the omnipresent inflammatory cytokine gradients produced not only from Teffs but also from hyperactivated microglia, combined with injured and dying neurons that release modified and misfolded proteins come together to form the perfect storm. This storm is sufficient for a chronically inflamed CNS environment and persistent oxidative reactive state along the nigrostriatal axis that ultimately is responsible for disease progression in PD [8, 48, 119, 120].

4 Control of Immune-Mediated Neuroinflammation and Neurodegeneration in PD

While proinflammatory effector T cells upregulate neuroinflammatory responses of microglia, Th2 effector cells secrete anti-inflammatory cytokines such as IL-4, IL-5, and IL-13 which work to enhance the neuroprotective activity of the microglial cells. Induction of Th2 effectors has been successfully utilized in strategies of immunomodulation therapy in multiple sclerosis using glatiramer acetate [121, 122]. In that same vein, $CD4^+$ Th2 effectors from glatiramer acetate-immunized mice attenuate MPTP-induced neuroinflammation and dopaminergic neurodegeneration presumably by diminishing levels of activated microglia resulting from anti-inflammatory cytokine production as well as providing neurotrophic support via increased glial cell-derived neurotrophic factor (GDNF) production by astrocytes [39, 98, 123]. Interestingly, cytokines from one particular Teff subtype tend to regulate the other types [124]. For instance, Th2-produced IL-4 suppresses Th1 and Th17 development, and Th1-specific INF-γ inhibits Th2 and Th17 cell maturation, while IL-17 produced by Th17 effectors inhibits Th1 and Th2 function.

Another T cell population with neuroprotective capabilities is the Tregs. Tregs play an indispensable role in maintaining immune homeostasis and controlling immune-mediated inflammation, and are thought, at least in part, responsible for the establishment and maintenance of immunological tolerance to self and protection from autoimmunity [125]. Moreover, after foreign pathogens and antigens are removed by immune responses, suppression of active effectors is necessary to minimize cytotoxic effects of inflammation on self-tissues. Natural Tregs (nTregs) are generated in the thymus and express both CD4 and CD25 cell-surface markers in addition to the transcription factor FOXP3, which is required not only for development and maintenance in the periphery but also for function [78]. Indeed, some mutations in FOXP3 lead to Treg dysfunction and a systemic autoimmune disorder designated as immune-dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome [126], which, without hematopoietic reconstitution of the Treg component, is a fatal condition. While nTregs are derived directly from the thymus, generation of induced or inducible Tregs (iTregs) is possible with $CD4^+$ T cells and the appropriate polarizing conditions, such as coculture in the presence of transforming growth factor-β (TGF-β), IL-2, IL-10, and/or all-trans retinoic acid [8]. Tregs

regulate immune responses by different mechanisms at several different levels. Mechanisms of Treg control include secretion of anti-inflammatory cytokines such as IL-10 and TGF-β [127, 128], cytolysis of Teffs [129], metabolic disruption by removing IL-2 ("IL-2 sponge") [130], and modulation of dendritic cell maturation or function through CTLA4 ligation [131, 132]. Effector cells such as T cells, B cells, or myeloid and APCs including microglia are inactivated or neutralized via cell-cell interaction of receptors with Tregs, albeit the requirement is determined through in vitro measurements, thus the necessity of proximal contact in vivo has not been adequately addressed [133–135]. We have reported that Tregs induce significant reductions in the number of activated microglia in the SN by decreasing mRNA expression for TNF-α and iNOS in the midbrain, indicating the reduction of microglia-mediated inflammation and oxidative stress [8, 30, 37, 49, 61, 62, 64]. Tregs also reduce nuclear factor-kappa B (NF-κB) gene expression and nuclear translocation, thereby reducing production and release of proinflammatory factors from activated microglia. Interestingly, coculture with Tregs prior to stimulation of microglia with N-α-syn results in the suppression of microglial production of ROS and proinflammatory cytokines/chemokines; however, coculture after microglial stimulation results in the upregulation of FAS by microglia and FAS ligand by Tregs with subsequent killing of the stimulated microglia by FAS-FAS ligand interactions [64].

5 Immune-Targeted Therapeutic Strategies in PD

The lack of efficacious treatment for PD warrants new strategies for disease progression and regeneration. One such strategy targets neuroinflammation, which should have profound implications at two therapeutic levels. Efficacious anti-inflammatory therapy could serve to inhibit further inflammation-mediated neurotoxicity and interdict dopaminergic neuronal loss. This alone could mitigate further disease progression. At another level, since dopaminergic neurogenesis along the nigrostriatal axis is limited, if occurring at all, attenuation of inflammatory and oxidative stress will be important to successful neuron replacement modalities. The importance of this is underscored by three independent studies wherein fetal nigrostriatal cells were transplanted to PD patients [136–139]. Postmortem analysis from several transplanted patients revealed evidence of α-syn accumulation and LB formation in older engrafted neurons from 14-year survivors, but not within those from survivors of 4 years or less [136–138]. Taken together these data suggest that dopaminergic cell loss may result from nonneuronal autonomous processes and that neuroinflammation plays an integral role in those processes [8, 31, 140, 141]. Therefore, to better facilitate neuronal engraftment, regeneration, and survival along the nigrostriatal axis, adjunctive therapies to control neuroinflammation will be an integral component of neuronal replacement therapies. Interestingly, stem cell transplants into the CNS have been shown to attenuate inflammatory responses and promote neuroprotection during early transplantation [142, 143]; however, whether these hypo-inflammatory conditions are retained throughout the life of the graft remains to be determined.

Several immunotherapeutic and pharmacological strategies that target inflammatory components of PD are actively being pursued. One potential target is COX-2, which is upregulated in nigral dopaminergic neurons of both PD patients and animal models. Among large cohorts, those who use nonsteroidal anti-inflammatory drugs (NSAIDs), particularly ibuprofen at least twice weekly, exhibit a lower risk of developing PD [144, 145]. Also pretreatment with NSAIDs and deletion of COX-2 is protective in MPTP- or 6-OHDA-induced neurodegeneration [75, 146, 147]. However, for PD patients the mechanism(s), best drug formulation, and dosage regimen that yield the most efficacious results remain to be determined.

The proinflammatory enzyme iNOS is thought to play a major role in dopaminergic neurodegeneration. Ablation by genetic manipulation or inhibition with specific pharmaceutical agents protects nigral neurodegeneration induced by MPTP or 6-OHDA but is less active at protecting striatal termini [148–150]. Interestingly, not all microglia express iNOS and inhibition of iNOS does not attenuate all reactive microglia suggesting that only a subpopulation of reactive microglia may participate in neurodegeneration [148, 150].

Minocycline is a long-acting second-generation tetracycline that exhibits a high capability to penetrate the brain parenchyma and CSF and act on activated microglia [151]. The mechanisms of action include inhibiting the phosphorylation of p38 mitogen-activated protein (MAP) kinase and the upregulation of iNOS as well as reducing production of IL-1β-converting enzyme (ICE) and IL-1β [146, 151–153]. In the MPTP and 6-OHDA models, minocycline reduces numbers of reactive microglia and inhibits neurodegeneration of nigral dopaminergic neurons as well as striatal termini in a dose-dependent fashion [146, 154, 155]. PET analyses of PD patients suggest that minocycline interferes with microglial activation; however, assessment of motor function has failed to detect clinical benefits [156–158]. In part, issues of becoming refractory to chronic anti-inflammatory pharmaceutical agents for neuroinflammation have more recently evoked strategies that afford long-term regulation via immunomodulation.

As detailed above, proinflammatory innate and adaptive immune responses are upregulated in PD and are thought to play a major role in disease exacerbation and hence progression. Thus, therapeutic strategies aimed at modulating the immune response during disease may be applicable. One strategy involves the use of Tregs to target neuroinflammation. This strategy is being investigated not only for PD but also for other neurological and autoimmune diseases for which inflammation is a major component [159–165]. In the MPTP mouse model, we showed Tregs attenuate microglia-mediated neuroinflammation, completely protect nigral neurons, and protect up to 90 % of the striatal termini [61, 63–65]. Moreover, protection is transferable in a fashion that is dependent on Treg dose. Attenuation is accomplished by Treg-mediated phenotypic switch from an M1 neurotoxic microglial phenotype to a more neurotrophic M2 phenotype with downregulation of proinflammatory mediators such as iNOS, IL-12, TNF-α, IL-1α, IL-1β, INF-γ, and monocyte chemotactic protein-1 (MCP-1) as well as concomitant upregulation of arginase-1. Moreover, Tregs control microglia function by suppressing ROS production and NF-κB activation via processes that modulate redox enzymes, cell migration, and phagocytosis.

An additional mechanism by which Tregs protect neurons is through increasing astrocytic production of brain-derived neurotrophic factor (BDNF) and GDNF [61]. Furthermore, whereas α-syn-specific Th17 and Th1 Teffs exacerbate neuroinflammation and dopaminergic neurodegeneration, interactions of those Teffs with Tregs lead to Teff augmentation of Treg function and enhanced ability of those Tregs to protect dopaminergic neurons within the nigrostriatal system. This Treg-mediated neuroprotection can be enhanced further by increasing the number or function of Tregs via immune modulatory agents such as vasoactive intestinal peptide (VIP), granulocyte/macrophage colony-stimulating factor (GM-CSF), non-mitogenic anti-CD3 antibody, rampamycin, and 1,25-dihydroxyvitamin D3 [8, 37, 48, 63]. Together, these data suggest that Tregs may be used to suppress the activity of innate and adaptive immune responses operative in PD pathogenesis by inhibiting Th1 and Th17 Teff function and transforming the neurotoxic phenotype of activated microglia. Finally, for PD patients whose innate CNS immunity is chronically activated, adaptive T cell-mediated immunity is skewed toward an effector/memory phenotype, and Treg-mediated function, which is tasked with controlling innate and Teff responses, is diminished [113, 158]. Therapeutic strategies are warranted that repair Treg deficits and harness those proinflammatory conditions in the CNS and periphery which support neurodegeneration.

6 Conclusion

In PD, activated microglia become unregulated and chronically activated for reasons that remain unknown. Neurotoxic and proinflammatory mediators produced from activated microglia fuel a cycle of inflammation, oxidative/nitrative stress, protein modification, and protein misfolding that lead to dopaminergic neurodegeneration along the nigrostriatal axis. The cyclic production of modified and aggregated self-proteins, such as nitrated α-synuclein, in the chronic presence of proinflammatory mediators is sufficient to overwhelm Treg-mediated immunological tolerance and induce peripheral immune responses to those modified epitopes. In the face of continuing neuroinflammation, effector T cells with migratory capabilities are able to cross the BBB to the inflammatory foci and again encounter their cognate antigen presented by proinflammatory microglia. Responding effector T cells exacerbate nigral inflammation with subsequent increase in neurodegeneration, which in turn accelerate disease progression. Thus, key therapeutic strategies for PD should include approaches that interdict the cycle of microglia- and effector T cell-mediated inflammation. Indeed immune modulatory regimens and vaccine approaches that upregulate regulatory T cell activities attenuate CNS inflammation and promote dopaminergic neuroprotection in laboratory and animal models. In PD patients, this approach would be expected to diminish oxidative stress, reduce protein modifications and misfolding, and lead to decreased neuronal loss with improved clinical outcomes.

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Multiple Sclerosis: Impact on Functioning of the Blood–Brain Barrier

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Abstract Proper function of the neurovasculature is required for optimal brain function and preventing neuroinflammation and neurodegeneration. During a large number of neurological disorders, dysfunction of the blood–brain barrier (BBB) is an apparent feature and may significantly contribute to disease progression. In particular, during the neuroinflammatory disorder multiple sclerosis (MS), the function of the BBB is severely hampered and immune cells gain access into the brain, causing neurological deficits. Consequently, transport of compounds, including drugs, may be altered under disease condition.

Within this chapter, we will discuss alterations of the function of the BBB and its consequences in the neuroinflammatory disorder multiple sclerosis.

Keywords Multiple sclerosis • Blood–brain barrier • Astrocytes • Immune cell trafficking

1 Multiple Sclerosis

1.1 Clinical Features and Diagnosis

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS), affecting over 2.5 million individuals worldwide. The onset of disease generally occurs between the age of 20 and 40 and is considered to be one of the most disabling neurodegenerative diseases in young adults [1]. MS incidence and prevalence is highest in Western countries (Northern Europe, North America)

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where the lifetime risk of MS development is approximately one per 1,000, affecting more women than men in a ratio of 3:1.

During MS, immune cells infiltrate the CNS causing damage to the protective myelin sheaths that surround the axons, which gradually leads to motor and sensory deficits. Clinical features of MS depend on lesion number, size, and location in the CNS and may be heterogeneous. A common sensory disturbance is impaired vision due to optic neuritis, and motor disturbances include muscle weakness, tremor, paralysis, and spasms. Depending on clinical features, four main subtypes of MS can be distinguished [2, 3].

The majority (~70 %) of MS cases progress in a relapsing-remitting course (RRMS) characterized by clearly defined alternating episodes of neurologic impairment and recovery. About half of all RRMS cases develop a secondary progressive course of MS (SPMS) within 10 years, characterized by increasing permanent neurologic impairment. Two other disease courses that comprise a small part (~15 %) of the total MS population are primary-progressive MS (PPMS), characterized by increasing neurologic impairment from the point of MS onset without recovery, and progressive-relapsing MS (PRMS; ~5 %), characterized by a steadily increasing neurologic impairment combined with acute attacks of disability [4, 5]. The diagnosis of MS is primarily based on clinical history and neurological examination and is supported by cerebrospinal fluid analysis and magnetic resonance imaging (MRI) of the brain and spinal cord, to determine the number and size of MS lesions and blood–brain barrier (BBB) leakage [4, 5].

1.2 Etiology

MS is conventionally viewed as an autoimmune disorder that has a multifactorial background, but its precise etiology is largely unknown. Both environmental and genetic factors may contribute to disease susceptibility and disease outcome. Sibling and twin studies have demonstrated that the incidence of MS is higher in monozygotic twins (25–30 %) compared to dizygotic twins (2–5 %) [6]. Moreover, genome-wide studies have revealed that susceptibility of MS is linked to genes in the major histocompatibility complex (MHC) on chromosome 6. Alleles for certain class II genes, human leukocyte antigen (HLA), confer the strongest risk of contracting MS [6, 7]. Family studies have revealed that first-degree relatives of MS patients are more likely to develop MS compared to non-related persons [6–8]. Furthermore, supporting a genetic component in MS susceptibility, twin studies showed a higher concordance rate of MS in monozygotic twins compared to dizygotic twins [8–11]. Concerning genetic associations, certain HLA alleles are associated with susceptibility to MS. The allele with the strongest association with MS is HLA-DRB1*15 (HLA-DR2) showing consistency of effect across several western European and Scandinavian countries and the Unites States. In addition, various genes coding for cytokines (interleukin (IL)7, IL12A, IL12B), cytokine/chemokine receptors (CXCR5, IL2 receptor A, IL7 receptor, tumor necrosis factor (TNF) receptor, IL12 receptor), adhesion molecules (vascular cell adhesion molecule (VCAM)-1), and

co-stimulatory molecules (CD37, CD40, CD80, CD86) are associated with pathogenesis of MS [12].

Environmental factors also contribute to the risk of developing MS as the prevalence of MS generally increases with distance from the equator and is particularly high in Northern Europe and North America. This may be related to exposure to sunlight (vitamin D), diet, or viral or other infections. Numerous viruses have been associated with MS pathology, including Epstein-Barr virus, human herpes simplex virus, measles virus, or Chlamydia pneumoniae [13–22], although no single infectious agent has been directly related to MS.

Epidemiological data further suggest that associated with living around the equator during the first 15 years of life environmental factors induce an MS resistant state that is maintained even when persons move toward high-risk areas for MS [7, 23–25]. Many explanations have been sought for this interesting phenomenon, one of them being the occurrence of helminth infections [24, 25]. Areas with high frequency of such infections show an extremely low frequency of MS [24, 25]. In addition, helminth-infected MS patients show significant lower number of relapses, reduced disability scores, and lower MRI activity compared to uninfected MS subjects [25]. These initial studies indicate that helminth infections possibly may reset the innate immune system which results in diminished clinical symptoms of MS.

1.3 Pathogenesis

Although the exact cause of MS remains unknown, MS is characterized neuropathologically by the presence of multiple focal lesions throughout the CNS [2]. MS lesions occur in all brain areas, although there are several predilection sites, like the optic nerves, periventricular white matter, brain stem, cerebellum, and spinal cord. MS white matter lesions are histopathologically characterized by the destruction of myelin sheaths, oligodendrocyte cell death, axonal damage, and glial scar formation and the presence of inflammatory cell infiltrates. Based on the degree of myelin loss and the presence of inflammatory cells or microglial cell activation, MS lesions in the white matter can be classified as preactive, active, chronic active, and chronic inactive lesions [26].

Active white matter MS lesions contain high numbers of immune cells, mainly T cells and monocyte-derived macrophages, of which the latter are mainly responsive for causing damage to the myelin sheaths surrounding axons, resulting in neuronal dysfunction. The mechanisms of CNS inflammation involve activation of autoreactive, myelin specific T helper (TH) cells in the peripheral lymphoid organs, possibly by molecular mimicry, which gain entry to the CNS and form perivascular infiltrates, a process that is accompanied by enhanced permeability of the BBB [27–30]. Local antigen-presenting cells (APCs), like microglia or perivascular macrophages, subsequently reactivate transmigrated T cells by presenting their specific target antigens. Consequently, increased amounts of proinflammatory cytokines and chemokines are locally produced, which in turn attracts more monocyte-derived macrophages and lymphocytes to the site of inflammation. This inflammatory cascade

finally leads to destruction of myelin sheaths and axonal loss [31–34]. Characteristic subsets of myeloid cells present in demyelinating lesions are foamy macrophages, which obtain their distinctive morphology by ingestion and accumulation of vast amounts of myelin-derived lipids. Glial fibrillary acidic protein (GFAP)-positive reactive astrocytes with long processes are evenly distributed throughout the demyelinated areas where they inhibit remyelination and axonal sprouting and regeneration by glial scar formation [35].

MS has long been considered an autoimmune disease primarily affecting the white matter (WM). However, it has become increasingly clear that grey matter (GM) pathology is an important aspect of the disease. To date, evidence that extensive demyelination occurs in the cerebral cortex of patients with chronic MS is increasing [36, 37]. In the cortex and deep GM brain regions, areas of demyelination can be detected in the presence of infiltrating inflammatory cells [37]. GM lesions are differently classified compared to WM lesions, based on their location within the cortex. So-called type I lesions are mixed GM/WM lesions, which are also visible on conventional histochemistry; type II lesions are mostly located around cortical blood vessels; type III lesions are subpial lesions and represent the most common type of cortical GM lesions; type III lesions reach from the pial surface downward into the cortex and may cover multiple gyri. Finally, type IV lesions are large, cortex-spanning lesions covering all six layers of the cortex while never reaching into the WM [38, 39].

However, the exact GM lesion pathology remains largely unknown [39], as WM lesion hallmarks like BBB dysfunction and leukocyte infiltration are not apparent in GM lesions [40].

1.4 Disease-Modifying Drugs

Despite many advances in both molecular and clinical MS research, MS has not been curable and current therapies consist of lifelong disease and symptom management. They are based on the hypothesis that MS is an autoimmune disease and is therefore anti-inflammatory, immunosuppressive, or immunomodulating agents. The most widely used drugs during relapses are corticosteroids like prednisone and methylprednisolone, which have both immunosuppressive and anti-inflammatory properties and reduce the duration of a relapse and accelerate recovery. RRMS patients are often treated with interferon beta (IFN-β) or glatiramer acetate, which have both been shown to reduce exacerbation frequency and severity and to improve neurological disability [41, 42].

One of the recent drugs that seems promising is natalizumab (Tysabri), which is a recombinant humanized monoclonal antibody directed against the α_4 chain of the $\alpha_4\beta_1$ integrin (or very late antigen-4 (VLA-4)) that is expressed on activated lymphocytes and monocytes and is involved in transendothelial migration [43–45]. Natalizumab treatment significantly reduced the number of new lesions and clinical relapses by blocking leukocyte migration into the brain [46].

A novel immunosuppressant drug recently on the market for treatment of RRMS is FTY720, which can reduce both relapse rate and the number of new lesions [47, 48].

FTY720 targets lymphocytes that result in sequestering of these cells in secondary lymphoid organs. Furthermore, it actively blocks lymphocyte passage over the BBB thereby reducing disease progression, indicating that limitation of the inflammation process during MS pathology is an attractive therapeutic strategy, although such drugs have limited efficacy when patients have already entered the progressive phase.

1.5 Animal Model for MS, Experimental Allergic Encephalomyelitis

Most of our current knowledge about the pathogenesis of MS is extrapolated from an animal model called experimental allergic encephalomyelitis (EAE). EAE is a widely accepted animal model for MS, sharing its clinical, immunological, and pathological characteristics [49, 50]. Rodents or nonhuman primates display MS-like symptoms after active immunization of these animals with myelin components or total myelin in combination with a strong adjuvant like (in)complete Freunds' adjuvant. Immunization leads to the development of autoreactive T cells in the peripheral lymphoid organs that recognize myelin proteins. These T cells finally enter the CNS where they find their antigens, which results in CNS inflammation, loss of neurological function, and subsequently paralysis. Depending on the immunization protocol, disease pattern may vary from monophasic type of disease with only minor myelin damage (acute EAE or transfer EAE) to the demyelinating and relapsing-remitting form (chronic EAE). Generally, clinical signs manifest themselves in an ascending manner, beginning with loss of tail tonus followed by paralysis of the hind limbs, and the disease may progress to the front limbs and occasionally even to death of the animals. However, EAE is far from being a perfect approximation of MS because MS is more heterogeneous and the target antigen(s) is (are) not known. Further advances in MS therapy will depend on our growing understanding of the pathogenesis of this still incurable disease [50].

2 The Blood–Brain Barrier

The vasculature of the brain is specialized to function as a barrier to protect the CNS by restricting entry of unwanted molecules and immune cells into the brain, by active removal of cytotoxic compounds from the brain, and by supplying the brain with essential nutrients and oxygen through specific transport mechanisms. Therefore, the BBB is not static, but reacts dynamically to the local demands of neurons for their need of oxygen, glucose, and other nutrients.

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

2.1 Features of the Blood–Brain Barrier

A key structure that protects the CNS microenvironment from the systemic circulation is the BBB. It plays a crucial role in maintaining brain homeostasis by restricting transport of immune cells and molecules into the brain parenchyma. The BBB is composed of highly specialized brain endothelial cells, surrounded by two basement membranes, pericytes, perivascular macrophages, and astrocytes, of which the latter project their end feet to the BBB, thereby inducing specific barrier properties [43–45]. Several characteristics of brain endothelium support the barrier function and exhibit functional and morphological properties that distinguish them from peripheral endothelium. The paracellular cleft between adjacent endothelial cells (ECs) is tightly sealed due to the presence of well-developed tight junctions (TJ) and adherens junctions (AJ), thereby impeding the entrance of circulating hydrophilic molecules and immune cells into the CNS. However, small gaseous molecules and a number of lipophilic agents may diffuse freely through the lipid membranes of the ECs. Other prominent features of brain endothelium are the absence of fenestrations, low pinocytotic vesicular activity, and the presence of high densities of mitochondria in the cytosol.

To maintain brain homeostasis and provide the brain with essential nutrients, specific transporters and carrier molecules strictly regulate the uptake of nutrients and metabolites into the CNS. Furthermore, potential harmful compounds like drugs and toxins are excluded from the CNS by a large family of efflux pumps, which contribute to the multidrug-resistant (MDR) phenotype of the CNS. Due to their specific features like TJs and efflux pumps, brain endothelial cells are crucial gatekeepers of the CNS and understanding the regulation of these structures and molecules will open avenues for the treatment of brain disorders complicated by BBB dysfunction.

2.1.1 Tight Junctions

The main structures responsible for endothelial sealing are TJs and AJs [43–45, 51–60]. These intercellular structures are located between adjacent brain endothelial cells, consisting of transmembrane and cytoplasmic proteins that are associated with the actin cytoskeleton. The transmembrane proteins occludin and various claudins mediate cellular interaction between brain ECs and play a major role in TJ functioning. Occludin is a phosphoprotein that spans the plasma membrane four times with intracellular location of both the amino and the carboxy termini and is associated with increased electrical resistance. Claudins comprise a multigene family consisting of more than 20 members and contain two extracellular loops and four transmembrane domains and interact in both a homophilic and heterophilic way with claudins of adjacent cells. At the BBB, the presence of claudin-1, claudin-3, claudin-5, and recently claudin-12 has been reported. Claudin-5 is a critical component of the BBB as it closes the BBB for small molecules up to 800 Da [43–45, 51–53].

The carboxyterminal parts of both occludin and claudins interact with membrane-associated recruiting proteins of the zona occludens (ZO) protein family. ZO proteins are reported to link transmembrane proteins to the actin cytoskeleton and have signaling potential. Through its interaction with TJ molecules, the actin cytoskeleton plays an active role in maintaining TJ integrity and BBB function. AJ are composed of cadherins, catenins, vinculin, and actinin. Although both AJ and TJ act to restrict endothelial permeability, TJ are primarily responsible for the low transendothelial permeability and high transendothelial electrical resistance (TEER) due to the limitation of ion transfer. Several cytoplasmic signaling molecules, such as Rho, PI3 kinase, protein kinase C (PKC), $Ca2^{+}$, heterotrimeric G proteins, cyclic adenosine monophosphate (cAMP), and phospholipase C, have been localized to TJ and AJ complexes and may regulate their assembly and disassembly. These studies strengthen our knowledge about molecular mechanisms underlying BBB regulation at the level of TJs and may therefore provide novel therapeutic opportunities to prevent TJ disassembly and subsequent BBB dysfunction [43–45, 51–53].

2.1.2 BBB Transporters

To maintain brain homeostasis, the BBB strictly regulates the influx and efflux of a variety of proteins and molecules by different transporters and carrier molecules, which makes the BBB a selective transport barrier. The brain endothelial transporters that supply the brain with nutrients include the glucose transporter 1 (GLUT-1), several amino acid carriers like excitatory amino acid transporters (EAAT) and transporters for nucleosides, nucleobases, and many other substances [43–45].

In contrast, potential harmful compounds are excluded from the CNS by the large family of ATP-binding cassette (ABC) efflux transporters, enabling multidrug resistance of the brain to xenobiotics and toxic compounds. ABC transporters consist of a variety of drug efflux pumps, including P-glycoprotein (P-gp), breast cancer resistant protein (BCRP), and the multidrug resistance-associated proteins (MRPs). These efflux pumps are expressed in the luminal and abluminal membrane of brain capillary endothelial cells and can drive cellular exclusion of a variety of exogenous compounds and drugs through the endothelial membrane against a concentration gradient at the cost of ATP hydrolysis [54–57].

The best-known and most widely studied representative of the ABC transporter family is P-gp (MDR1; ABCB1), a phosphorylated glycoprotein that was first identified in tumor cells, where overexpression conferred multidrug resistance. Moreover, it was the first drug efflux transporter to be detected on BBB endothelial cells where it locates at the luminal membrane. P-gp actively effluxes a wide variety of substrates and drugs, although the physiological substrates of P-gp have not been identified yet. At the transcriptional level these ABC transporters are under control of the orphan nuclear receptors such as steroid and xenobiotic receptor (SXR in human or pregnane X receptor (PXR) in rodent. Expression of these ABC transporters is under the influence of a number of environmental factors like neurotransmitters, endothelial factors, and inflammatory cytokines [54–57].

2.2 Astrocytes and the BBB

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

Astrocytes are able to influence a number of features of the brain EC, leading to increased integrity of the BBB. TJ expression and tightening, expression and localization of EC transporters, and specialized enzyme systems have been shown to be upregulated under astrocyte influence [43]. The notion that astrocytes can induce and maintain BBB properties in brain EC through physical interaction and secreted agents has been widely accepted. Astrocyte processes extending toward CNS microvessels terminate in specialized (perivascular) endfeet structures onto the basal lamina surrounding the BEC. Astrocyte endfeet associated with BEC shows a high density of orthogonal arrays of particles (OAPs) and organized arrays of ion- and volume-regulating membrane particles identified by freeze fracture [58], containing channels like the water channel aquaporin-4 (AQP4) and the potassium ion channel Kir 4.1 [59]. Membrane proteins in OAPs represent a strong polarization of perivascular astrocyte function and correlate with the expression of the basement membrane molecule agrin, an important proteoglycan for BBB integrity, which is responsible for the correct localization of AQP4. The distribution of these channels in OAPs is most likely important in the regulation of BBB homeostasis, as disruption of this distribution is associated with microvascular damage in, among other pathologies, Alzheimer's disease (AD) [60].

The observation of astrocyte-conditioned medium inducing junction formation in EC in vitro [61] gave rise to the idea that astrocyte-derived secreted factors were able to influence their BBB properties. Numerous astrocyte-derived agents have since then been described, mainly by in vitro studies, as modulators of EC barrier function. Transforming growth factor-β (TGFβ) secreted by astrocytes has been shown to mediate the regulation of tissue plasminogen activator and the anticoagulant thrombomodulin [62]. Glial-derived neurotrophic factor (GDNF) has been found to enhance barrier function in BEC through the TJ [63]. Fibroblast growth factor (FGF) was found to decrease BBB permeability [64], consistent with the observation that FGF knockout mice show decreased levels of TJ proteins and BBB integrity loss [65].

More recent data indicate that factors involved in CNS development such as sonic hedgehog, angiotensin, and retinoic acid have a critical role in the induction of barrier characteristics in the brain endothelium [66–68].

3 The BBB in MS

In MS pathology, numerous changes in BBB structure and functioning have been described. These observations, derived from in vitro, in vivo animal model, and patient tissue studies, show a high involvement of the disruption of BBB integrity

and function in MS pathology. The combined outcome of these studies has led to the notion that BBB disruption represents an early event in MS lesion formation, preceding the massive infiltration of leukocytes (mainly T lymphocytes and monocyte-derived macrophages) leading to myelin degradation and nervous tissue destruction. Even before clinical symptoms arise, MRI scans of animals with EAE, a validated animal model for the inflammatory phase of MS, show leakage of the BBB before leukocytes infiltrate [69].

3.1 Inflammation at the BBB in MS

Pathological events that may occur at the BBB include structural and spatial alterations of the TJs, enhanced permeability for blood-derived components, and infiltration of inflammatory cells into the CNS [70]. In these processes, proinflammatory mediators like chemokines [71, 72], cytokines [73, 74], matrix metalloproteinases [75, 76], and reactive oxygen species (ROS) play an important role [77–79].

Not only structural changes of the BBB occur during neuroinflammation; also the expression of P-gp, one of the ABC transporters, was severely decreased in its function. Work from our group showed a significant reduction of microvessel P-gp expression in various MS lesions in patients, compared to normal-appearing white matter [80]. These results suggest that a loss of P-gp expression might be involved in lesion formation or aggravation. In the same study P-gp expression was seen to be upregulated in astrocytes in MS lesions, suggesting a possible role for astrocytes as a complementary drug resistance barrier in areas of BBB disruption. Importantly, P-gp was found to mediate the release of the chemokines CCL2 (or MCP-1) and the proinflammatory lipid platelet activating factor [81, 82] which may contribute to the neuroinflammatory process.

During MS pathogenesis, reactive astrocytes participate in various mechanisms that contribute to neuroinflammation. Reactive astrocytes aggravate inflammation by increasing vascular activation and leukocyte accumulation in the CNS and are involved in loss of BBB integrity, mediated by local release of proinflammatory molecules like IL-1β, IL6, and CCL2 [83–85]. In addition, once inflammation has abated, astrocytes are the major cell type involved in glial scar formation and are thereby directly associated with inhibition of axonal regeneration [86]. In contrast, during pathophysiology, astrocytes may also exert protective properties and promote cellular regeneration. Astrocytes are able to produce antioxidant enzymes and glutamate metabolizing enzymes and transporters suggesting an important role in scavenging ROS and extracellular glutamate [87, 88]. Furthermore, reactive astrocytes maintain the capacity to secrete T-cell suppressive factors, anti-inflammatory cytokines, and neurotrophic factors and possibly rearrange their contact sites with the endothelium [60, 89, 90]. Through secretion of proinflammatory molecules, astrocytes not only contribute to direct disruption of the BBB but also facilitate upregulation of endothelial cell adhesion molecules (CAMs) thereby promoting recruitment and adhesion of leukocytes to ECs.

Recently, we demonstrated an increase in the production of the proinflammatory lipid ceramide in reactive astrocytes in active MS lesions, which contributed to the neuroinflammatory process. During MS pathogenesis, stress signals such as ROS, TNF-α, and interferon are present in the inflamed brain parenchyma and may be responsible for the observed increase in astrocytic ceramide. In turn, ceramide was found to impair the function of the BBB in vitro [91], illustrating the impact of the reactive astrocyte phenotype on the barrier properties in MS. Strikingly, reactive astrocytes were found to have an induced expression of the sphingosine-1-phosphate (S1P) receptors which after triggering with the S1P analogue Fingolimod (FTY-720P) resulted in a diminished production of proinflammatory mediators [91, 92]. Together, these data indicate that the dampening of the reactive astrocyte phenotype is an attractive new therapeutic strategy [93], although this may also result in the loss of important protective aspects of reactive gliosis.

3.1.1 Immune Cell Trafficking Across the Brain Endothelium

Before leukocytes adhere and transmigrate through the BBB, the cerebral endothelium must be activated by inflammatory mediators to express cell adhesion molecules (CAM) with which leukocytes interact. TNF-α and the chemokine MCP-1 are two examples of numerous proinflammatory molecules which can cause upregulation of endothelial CAMs such as E-selectin, P-selectin, VCAM-1, and intracellular adhesion molecule-1 (ICAM-1) [94–96].

The presence of various immune cells in the CNS is an important hallmark of MS pathology. It is unknown which factor initiates the infiltration of immune cells into the CNS, but the current hypothesis is that myelin-specific CD4+ T cells are primed in the peripheral lymphoid organs, which encounter their target antigens during immune surveillance of the CNS, thereby triggering an immune response. The general principles governing leukocyte extravasation have been thoroughly documented. It occurs according to the multistep model of leukocyte extravasation, which consists of rolling, tethering, firm adhesion, and finally transmigration of immune cells across brain EC. Endothelial cells play an active role during the extravasation process. Upon interaction with leukocytes, various signaling pathways are triggered in brain ECs that lead to rearrangement of the cytoskeleton and TJs, thus facilitating transendothelial migration [94–96].

In addition to the family of cell adhesion molecules, members of another class of cell surface molecules are involved in the transendothelial migration process. The transmembrane 4 superfamily, or tetraspanins, are small membrane proteins differentially expressed by all mammalian cells. The size of tetraspanins ranges from 204 to 355 amino acids and they contain four transmembrane domains; the first of the two resulting extracellular loops is short, while the second loop is long [97, 98]. This long, second loop in combination with the four transmembrane domains are important in promoting associations of the tetraspanin with additional proteins, such as other tetraspanins, integrins, CAMs, and intracellular signaling molecules. Resulting structures are referred to as tetraspanin-enriched microdomains (TEMs)

and they operate as molecular organizers for other transmembrane proteins [99, 100]. The biological function of tetraspanins depends on the ability of the tetraspanin to organize TEMs Moreover, these tetraspanins, CD9, CD81, and CD151, also localize to docking structures on endothelial cells which are formed at sites of leukocyte adhesion [101, 102]. More specifically microdomains containing tetraspanins and adhesion receptors were present on activated endothelial cells even before leukocytes adhered and studies demonstrated that CD81 and CD9 play a role in the transendothelial migration of immune cells [101, 102].

Besides the involvement of CAMs in the migration process, the release of inflammatory mediators severely contributes to the migration process. Monocytes, the effector cells within MS since they induce demyelination and axonal damage, are attracted to the MS lesions in high numbers. Within the process of monocyte trafficking across the BBB, it has been demonstrated that ROS play a dominant role. ROS are produced by monocytes upon firm adhesion to ECs and subsequently enhance migration and adhesion of monocytes. Treatment of EAE animals with antioxidants such as flavonoids and lipoic acid suppressed the development of EAE by lowering the entry of inflammatory cells into the CNS. Histological examination demonstrated a reduced number of infiltrated T cells and macrophages, suggesting a role for ROS in BBB permeability. Moreover, it was shown that super oxide is the predominant ROS treatment which induces BBB disruption by inducing TJ rearrangements and cytoskeletal changes, allowing cell migration [77–79]. Pathological changes at the neurovascular unit are summarized in Fig. 1.

4 Conclusions and Future Directions: Impact on Drug Delivery

The BBB is specialized to function as a barrier to protect the CNS by restricting entry of unwanted molecules and immune cells into the brain. An important feature of MS pathology is a dysfunctional BBB and consequent loss of the imperative CNS homeostasis. The unrestrained access of immune cells and harmful compounds into the CNS play a central role in demyelination and axonal damage, two hallmarks of MS pathology strongly contributing to the clinical symptoms of MS. Strategies that restore the impaired function of the BBB are therefore a promising new strategy together with the dampening of the proinflammatory phenotype of the reactive astrocytes.

Consequentially, drug delivery to the brain will be altered and disposition of drugs in the brain during different disease states may limit efficient treatment. Altered BBB permeability and function during CNS diseases such as MS may be mediated via changes in various transport pathways and receptor systems. Conversely, disease state of the CNS also provide opportunities for drug targeting to sites of affected brain regions, since drugs that normally are unable to traverse the BBB (either due to ABC transporter activity or presence of TJs) may now reach their target in the diseased brain. To accurately treat complex CNS disorders, future

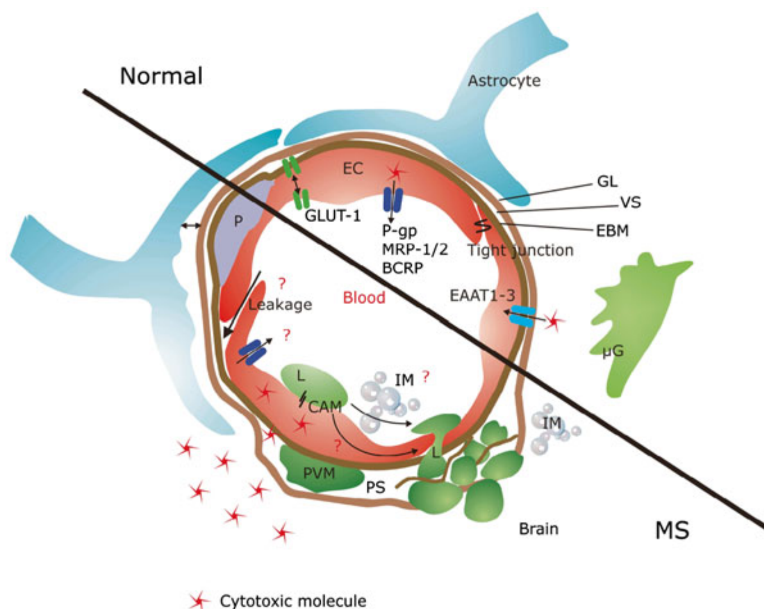

Fig. 1 The BBB during health and disease. Under healthy conditions, brain endothelial cells (EC) are interconnected by tight junction structures and have several transport systems and efflux pumps on their luminal or abluminal membranes. ECs are surrounded by a basement membrane (EBM), pericytes (P), perivascular macrophages (PVM), and astrocytes, which project their endfeet to brain EC, thereby forming a glia limitans (GL). During MS pathology several processes occur including BBB leakage, retraction of astrocyte endfeet, leukocyte (L) adhesion via cell adhesion molecules (CAM) and diapedesis, accumulation of these cells in the perivascular space (PS), and finally transmigration into the brain parenchyma. Inflammatory mediators (IM) are involved in several events, but it is not known which mediators affect TJ integrity, endothelial signaling, or BBB leakage. Moreover, cytotoxic molecules accumulate in the brain, which may be due to a decreased efflux capacity of the BBB

research should therefore aim to gain more and detailed insight into the effects of neuroinflammation on different BBB properties.

Whether the described dysregulation of transport and receptor systems are favorable or unfavorable for drug delivery across the BBB largely depends on the type of transport route or receptor involved, the regulation of its expression, and specific localization of transporters or receptors in the brain. Thus, when relying on such mechanisms in delivery strategies, targeting efficiency may be changed during CNS disease states, potentially increasing or decreasing the therapeutic efficacy of drugs. In the past and also more recently, novel and specific drug delivery approaches have been developed to overcome the BBB.

Development of new protein vectors for the physiologic delivery of large therapeutic compounds to the CNS is still ongoing [103]. Since most of these targeting approaches are dependent on specific or nonspecific targeting and trafficking mechanisms at the BBB, understanding the regulation of these mechanisms is of

crucial importance. Recent data from our group indicated that during EAE, the enhanced delivery of methylprednisolone to the brain by the means of glutathione pegylated conjugated liposomes is beneficial for the treatment of neuroinflammation associated with EAE and possibly MS [104].

For proper selection of a suitable carrier or targeting moiety, the described dysregulations of transport routes and potential target receptors during neuroinflammatory conditions must be taken into account. For instance, paracellular transport is often increased in MS due to decreased TJ expression, potentially allowing for more efficient drug targeting to affected brain regions. Likewise, the downregulation of ABC transporters during CNS diseases may lead to reduced efflux of exogenous compounds, thereby increasing brain retention of therapeutics and rendering the application of drugs that are ABC transporter substrates more effective. In contrast, application of drugs may themselves cause an increase in ABC transporter expression and function, thus hampering drug delivery to the brain.

Drug delivery strategies that depend upon the expression of a specific internalizing receptor are at risk of missing their target if its expression is markedly reduced during disease status. On the other hand, enhanced or de novo expression of specific BBB ligands under inflammatory conditions holds promise for the development of new targeting strategies. Finally, it is of importance to realize that transport mechanisms and receptor expression may change during the course of the disease, complicating the selection of an efficient strategy even more.

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Neuroinflammation in Alzheimer's Disease

Veronika M. Reinisch, Daniela L. Krause, and Norbert Müller

Abstract The neuropathology of Alzheimer's disease (AD) is still only partly understood. Beyond doubt neuroinflammation plays a key role in pathophysiology of the disease. Still it has not been fully understood when and how inflammation arises in the course of AD. Whether inflammation is an underlying cause or a resulting condition in AD remains unresolved. Mounting evidence indicates that microglia activation contributes to neuronal damage in neurodegenerative diseases. However, also beneficial aspects of microglia activation have been identified. The purpose of this review is to highlight new insights into the detrimental and beneficial role of neuroinflammation in AD. In regard to this, we discuss the limitations and the advantages of anti-inflammatory treatment options and identify what future implications might result from this underlying neuroinflammation for AD therapy. Here we put a special focus on the therapy with COX-1 and COX-2 Inhibitors as well as anti-Aß antibodies.

Keywords Inflammation • Alzheimer • Microglia • COX-inhibitors • Abeta DNA vaccination

1 Introduction

Alzheimer's disease (AD) is the most common form of dementia, amongst others, that humans are at risk of as they age. The most achievable long-term aim is to diagnose AD in an earlier stage, thus starting treatment before most clinical symptoms are present [1]. This is possible with new diagnostic imaging, e.g. amyloid-PET imaging, which can show the amyloid burden in the brain [2]. Anyway the

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pathophysiology of AD is not yet clearly identified. Still a lot of issues related to what causes AD are still unclear, limiting the identification of effective disease-modifying therapies. The main neuropathologic hallmarks of AD are peptide deposition (senile plaques), extracellular ß-amyloid (Ae) and intracellular neurofibrillary tangles containing hyperphosphorylated tau protein [3]. Today it is possible to demonstrate neuroinflammation induced by Aß burden in the brain in vivo. Apart from the disease's distinct pathological markers, the neurodegenerative features are characterized by chronic neuroinflammatory processes. Yet, those inflammatory markers are not exclusively associated with AD. Also, brains of 'healthy aged' individuals show concentrations of serum markers related to inflammation, such as elevated homocysteine and altered cholesterol homeostasis associated with cognitive functioning in the nondemented healthy ageing population [4]. In AD pathology, these ageing-related inflammatory processes are increased.

The suggestion that inflammation may participate in AD first appeared more than two decades ago. As several clinical trials have shown a beneficial effect for nonsteroidal anti-inflammatory drugs for the occurrence and course of AD, the inflammatory hypothesis in AD gained a lot of attention. In regard to treatment and prevention of AD, several classes of medications have emerged on the market, which improve the cognitive symptoms of this disorder (e.g. the cholinesterase inhibitors). But the relief that these drugs provide remains symptomatic—so it is a major goal for the future to develop effective disease-modifying therapy.

Different substantial efforts have been made to identify potential strategies to ameliorate or prevent AD pathology, with data stemming from basic research as well as from animal and epidemiological studies. Because many investigators have concluded that neuroinflammation contributes to neuronal damage in the brain during AD [5, 6], the use of anti-inflammatory drugs as a possible treatment option has been widely investigated [7–9]. Anti-inflammatory therapy has therefore been credited as a strategy for reducing the risk or slowing the progression of AD. However, the results of these studies remain inconsistent [10]. Until now, many questions regarding the inflammatory response are still unresolved. Discussion continues whether neuroinflammation is an underlying cause or a resulting condition in AD [11]. There are several studies showing that an intact immune response including intact T cell immunity is a prerequisite for cognitive function. T-cell-deficient mice show impaired learning abilities, which can be reversed with T cell substitution [12, 13].

Regarding the fact that the T cellular immune response declines with age, starting from about the age of 55 years—'immunosenescence'—such immunodeficiency in aged patients may also explain cognitive deficits. The "Maastricht Aging Study" followed this approach. Nearly 100 healthy people, mean age 57 years, were followed up in a longitudinal analysis with regard to inflammatory markers and cognitive tests. It could be shown that high levels of haptoglobin (an acute phase protein) correlated significantly negatively with cognitive abilities, measured by the Stroop test and the auditory verbal learning test. High levels of the inflammatory marker C-reactive protein (CRP) also correlated negatively with the auditory verbal learning test after 3 and 6 years follow-up. Lower cognitive abilities were associated

with higher concentrations of CRP and haptoglobin [14]. In a similar design, a prospective cohort study in 4,200 healthy persons examined CRP and interleukin (IL)-6 as inflammatory markers combined with cognitive tests at 7 years and 12 years of follow-up. It could be shown that CRP and IL-6 were significantly associated with cognitive performance, in particular in men. Higher levels of pro-inflammatory markers during midlife correlated significantly with lower cognitive abilities and weakly with the decline of cognitive abilities. Interestingly, in animal experiments it was shown that an increased secretion of IL-6 leads to deficits in learning and memory, while IL-6 knockout (KO) mice were less prone to forget learned skills and exhibited a better cognitive performance compared to wild-type mice. Accordingly, the intraventricular administration of anti-IL-6 antibodies resulted in improved memory function.

Inflammation in the brain is characterized by activation of glial cells (mainly microglia and astrocytes) and expression of key inflammatory mediators as well as neurotoxic free radicals. It has been suggested that neuroinflammation is associated with neurodegenerative disorders—both acute (e.g. stroke, injury) and chronic (e.g. multiple sclerosis, AD). In this context, microglial cells play a crucial role, and therefore, microglia and cytokines have been extensively studied in these conditions. In the central nervous system, microglia are the resident phagocytes of the innate immune system. Microglia are found in a highly activated state in close anatomical proximity to senile plaques within the AD brain. In this activated state, microglia produce various pro-inflammatory cytokines and other immune mediators that create a neurotoxic milieu leading to disease progression [6, 15].

It is our intention in this chapter to focus on newer controversies in the field of microglia activation and its role in AD pathology. For this purpose, we asked ourselves several questions: Are neuroinflammatory alterations neuroprotective—or are they rather an underlying cause of AD? And what strategies result from this underlying neuroinflammation for future treatment options?

2 Characteristics of Neuroinflammation in AD

The relevance of neuroinflammation to AD pathology has been established by multiple lines of direct and indirect evidence. One argument is that increased microglia activation has been shown in regions associated with Aβ deposition [16]. Upregulated inflammatory mechanisms co-localize in the AD brain with those regions that exhibit high levels of AD pathology (e.g. frontal and limbic cortex) and are minimal in brain regions with low AD pathologic susceptibility (e.g. cerebellum) [17].

As a second point, many of the inflammatory mechanisms that have been uncovered in the AD brain are established to be cytotoxic in the periphery of the body. Therefore, it seems likely that they are also cytotoxic in the brain, an organ that is sensitive to inflammation (e.g. in meningitis and encephalitis). However, inflammation in the brain is different from inflammation in the peripheral body. AD brains lack the classical hallmarks of inflammation such as neutrophil infiltration and

perivascular mononuclear cuffing. As for other neurodegenerative diseases, a local inflammatory reaction is sustained by activated microglia and reactive astrocytes [11]. This is indicated by the presence of antigens associated with microglia activation and inflammatory mediators, such as factors of the complement system, cytokines and free radicals [18].

Only modest elevations of inflammatory markers are found in the autopsy of patients lacking a clinical presentation of dementia but who exhibit sufficient Aβ and neurofibrillary tangles to otherwise qualify for the diagnosis of AD. Their level of inflammatory markers is significantly greater than levels of nondemented patients, but dramatically less than AD patients [19]. These findings further strengthen that inflammation is a necessity for clinical symptoms of AD.

For AD a huge variety of pro-inflammatory markers have been identified, whereas this was not the case for other forms of dementia. A relevant reduction of monocyte chemotactic protein-1 levels in the grey matter in dementia patients has been shown. For IL-6 and related markers of this pro-inflammatory cytokine system, decreases were observed in the brain and cerebrospinal fluid of demented patients [20, 21]. It is unclear, however, whether this decrease is related to further psychopathological symptoms such as depression [21]. On the other hand, IL-6 has also neuroprotective properties and decreased IL-6 might be associated with decreased neuroprotection [22].

There also is direct evidence of inflammatory toxicity in the AD brain. For instance, complement fixation and lysis of neurites could be demonstrated ultrastructurally in AD cortex, but in contrast it was only very weakly detected in nondemented elderly cortex under the same conditions [23].

Finally, many clinical and animal studies have strongly suggested that especially nonsteroidal anti-inflammatory drugs (NSAIDs) could be used as preventive or treatment strategies in AD. This aspect is further discussed in a later section of this chapter, where we focus on anti-inflammatory treatment.

Even though there are many indicators that neuroinflammation plays a key role in AD pathology, this does not answer which of these inflammatory activities are causing disease progression. The question remains: do some of these processes help to fight against the disease? In order to address this question, the role of microglia seems important, because these cells are known for neuroprotective and—degenerative functions.

2.1 Are Activated Microglia Neuroprotective or Neurodegenerative in Brain of AD Patients?

There are three glial cell types in the central nervous system (CNS), one of which is microglia. Since the 1970s there has been wide recognition that microglia are immune effectors in the CNS that respond to pathological conditions and participate in initiation and progression of neurological disorders (including AD) by releasing potentially cytotoxic molecules such as pro-inflammatory cytokines, reactive

oxygen intermediates, proteinases and complement proteins [24]. This means that their phagocytic function can be beneficial while their inflammation-related functions might be detrimental.

Several studies give evidence for an increased number of morphologically reactive microglia in AD brains compared to nondemented individuals [25, 26]. The location of these reactive microglia has been indentified directly around plaques [27]. This finding has been verified in a recent imaging study using a specific ligand for positron emission tomography (PET), which showed increased microglia activation in regions associated with amyloid deposition [16]. Up to now, the exact timing of this association could not be identified. Microgliosis might be an early component of the disease process and not necessarily dependent upon Aβ plaque interaction as a stimulus. What is known so far is that activation of microglia by Aβ fibrils is associated with a chemotactic response and extensive clustering of microglia around Aβ plaques in the AD brain [28]. These findings indicate the prominent role of microglia cells in AD. Nonetheless, it remains unclear, whether their functions are beneficial or detrimental.

The following section explains the checkered role of activated microglia in AD pathology.

2.1.1 Neuroprotective Properties of Microglia in AD

Perhaps activated microglial cells are beneficial in neurodegenerative diseases. For the useful role of microglia is that neuroprotection results from the microglia glutamate removal. Glutamate has been identified as a relevant neurotoxic substance that acts through N-methyl-D-aspartic acid (NMDA) receptors on neurons and can lead to increased neuronal cell death. Microglial cells can increase their capacity to take up glutamate upon stimulation with lipopolysaccharide (LPS) via a mechanism that is tumour necrosis factor (TNF)α dependent [11, 29] . In AD this microglia function could be relevant because memantine (the NMDA receptor antagonist) has been shown to improve cognition, function (activities of daily living), agitation and delusions in AD patients [30]. Taken together, microglial cells are important for the control of glutamate levels and might therefore contribute to neuronal survival. There is also evidence that microglia are capable of secreting neurotrophic or neuron survival factors (e.g. nerve growth factor and neurotrophin 3) upon activation via inflammation or injury [31].

Furthermore, it has been suggested that newly recruited microglia have different phagocytotic properties than intrinsic microglia, which is important for Aβ elimination. Lysosomes from the macrophage cell line are more acidic than those of microglia lysosomes [32]. This indicates that microglia derived from the periphery might be more efficient in eliminating Aβ than brain microglia. Furthermore, phagocytic activity of microglia is dampened by pro-inflammatory cytokines like TNF-α [33]. These findings show that microglia that are committed to an inflammatory response may have a lower phagocytotic capacity than newly recruited microglia. In mouse models of AD, it could be demonstrated drugs with anti-inflammatory properties like minocycline improve cognitive function and reduce the activation of

microglial cells but do not alter Aβ plaque deposition and distribution [34]. Seabrook et al. showed in amyloid precursor protein transgenic mice an age-dependent effect of minocycline: in young animals the drug increased the amyloid load indicating a beneficial effect of microglia in clearing amyloid [35]. Minocycline has been investigated not only as a potential treatment for AD but also in schizophrenia as an adjunctive therapy where it appeared to be effective in cognitive performance and reducing a broad range of psychotic symptoms [36]. Another mechanism that might help microglial cells with elimination of Aβ involves transforming growth factor-β 1 which has been demonstrated to promote microglia Aβ clearance and reduce plaque burden [37]. This supports the idea that microglia activation is useful in the clearance of Aβ.

A recent review explains that microglia—when they are challenged—may adapt to different stimulatory contexts and pass through a sequence of reactive profiles. This is in line with the finding that microglia are not just ‘resting’ but have active sensor and versatile functions [11, 38].

Are most microglia cells functions beneficial in AD? Several studies suggest an overbalance of the detrimental microglia properties.

2.1.2 Neurodegenerative Aspects of Microglia

In order to address this question, it is important to focus on timing: One must investigate when microglia activity begins during the time course of the disease. An increase in microglia activation has been observed in very early stages of AD. This increase surprisingly disappeared over time [39]. The suggestion of Vehmas et al. strengthens the assumption that microglia activation begins early in disease progression [39]. Microglia initially try to eliminate Aβ, but over time of the disease microglia fail and therefore decrease their activity. Alternatively, the microglia role in AD could be detrimental and they initiate the underlying AD pathology.

In order to further evaluate this issue, a closer look needs to be taken on what causes the microglia activation in AD, and it seems important to distinguish between acute and chronic stimulation of microglial cells. While an acute insult may trigger oxidative and nitrosative stress, it is typically short-lived and unlikely to be harmful to long-term neuronal survival. Therefore, it is believed that an acute neuroinflammatory response is generally beneficial to the CNS, since it tends to minimize further injury and contributes to repair of damaged tissue. The opposite is the case for a chronic stimulation. Chronic neuroinflammation is most often detrimental and damaging to nervous tissue. Thus, whether neuroinflammation has beneficial or harmful outcomes in the brain may depend critically on the duration of the inflammatory response. The progressive deposition of Aβ in AD disease might provide a chronic stimulus to microglial cells. Also, the chemotactic functions of Aβ to attract microglia contribute further to the ongoing inflammatory process [28]. The ratio of the pro-inflammatory cytokine IL-1β to the anti-inflammatory cytokine IL-10 is drastically elevated in the serum of AD patients, giving these patients a definite long-term pro-inflammatory profile [40], indicating a chronic neuroinflammatory

state of the CNS. In addition, the accumulating loss of neurons that characterizes AD further contributes to generation of debris and keeps microglia activated indefinitely maintaining microglia in an activated state long term. These data indicate that in AD the inflammation might be chronic, therefore contributing to disease progression [11].

There is also the emerging idea that an inflamed CNS environment may influence the ability of microglia to contribute to plaque deposition rather than plaque removal [33]. This strongly suggests that the microenvironment of the brain can influence whether microglia perform beneficial or deleterious functions in pathophysiological states. This means that microglial cells functionally adapt to their environment [38]. Recent studies show that in response to certain environmental toxins and endogenous proteins, microglia can enter an overactivated state and release reactive oxygen species (ROS) that cause neurotoxicity [41]. Overactivated microglia can be detected using imaging techniques and therefore this knowledge offers an opportunity not only for early diagnosis but eventually also for the development of targeted anti-inflammatory therapies that might diminish the progression of the disease [24].

In addition, activated microglia release the excitotoxin quinolinic acid [42] and microglia activated by AD plaques produce an apparently novel amine that evokes fulminant excitotoxicity [43]. One interesting implication of an excitotoxic contribution to inflammatory mechanisms is the potential for limited damage to functional cellular compartments. Because excitatory amino acid receptors are restricted to synapses and dendrites, these subcellular compartments are preferentially vulnerable.

As a result, microglia-produced excitotoxins may lead to cognitive impairment that is not necessarily correlated with neuronal cell loss [5]. However, activated microglia not only produce neurotoxic metabolites: Some of their products like 3-hydroxyanthralinic acid (which is—like quinolinic acid—one of the downstream products of the tryptophan metabolism) exert antioxidant and anti-inflammatory functions [44, 45].

Since tryptophan/kynurenine metabolism—i.e. the degradation of tryptophan to the partly neuroprotective, partly neurotoxic metabolites of the degradation to quinolinic acid—is driven by the enzyme indoleamine 2,3 dioxygenase (IDO), immune mechanisms are key players in this system. IDO is activated by pro-inflammatory cytokines such as interferon-gamma or IL-2. Immune activation is associated with an increased degradation of tryptophan and kynurenine. In an interesting study it was investigated whether an imbalance between neurotoxic and neuroprotective kynurenine metabolites could be detected in patients with AD. Serum levels of tryptophan, kynurenic acid, 3-hydroxykynurenine (HK), picolinic acid and quinolinic acid were measured in patients with AD, and it was found that serum levels of 3-HK were markedly increased in AD patients compared to the comparison groups ($p < .0001$), while serum levels of the other KP metabolites were not significantly different between groups. In contrast to its downstream metabolites, quinolinic acid and picolinic acid, 3-HK can cross the blood-brain barrier via an active transport process. These data therefore indicate an enhanced availability of

3-hydroxykynurenine in the brain of AD patients, which may be related to the previously reported higher production of quinolinic acid in AD brains [46].

Therefore, the balance of these products that result from activated microglia is important for the inflammatory process.

Finally up the results from microglia studies, clear evidence that exists for an important role of neuroinflammation contributing to disease progression in AD was found. However, some aspects of microglia activation might also be beneficial during the course of AD. As explained above, neuroinflammation is a critical event in AD. It has been suggested that anti-inflammatory therapy could be beneficial in delaying the onset or slowing the progression of AD. Cyclooxygenase (COX) is a unique enzyme. First, it exhibits two catalytic activities, a bis-oxygenase activity, which catalyses prostaglandin G_2 (PG) formation from arachidonic acid, and a peroxidase activity, which reduces PG G_2 to PG H_2. The peroxidase activity also results in the production of free radicals, which are in part utilized by COX itself [47]. Although nonsteroidal inflammatory drugs (NSAIDs) may have other effects as well, it is generally assumed that their primary mechanism of action is by competitive inhibition of COX activity, thereby reducing the production of inflammatory prostaglandins from membrane-derived arachidonate. COX not only helps mediate production of prostaglandins and other inflammatory factors; it is itself upregulated by pro-inflammatory mediators [11, 47].

In AD, Aβ neurotoxicity may result from several mechanisms, most likely in combination. These mechanisms include oxidative damage, direct cytotoxicity and induction of destructive inflammatory mechanisms; efforts have been directed at the control of each of these processes [48].

3 Possible Mechanisms of Action of NSAIDs in AD

The treatment of AD with NSAIDs is one of the most promising approaches. If NSAIDs are beneficial in AD, the presumed mechanism would be inhibition of COX expressed in the brain. Both COX-1 and COX-2 are expressed there and COX-2 plays a unique role in the brain compared to the periphery: Only in the brain is COX-2 expressed constitutively, whereas elsewhere the expression is activation dependent. Although in vivo the majority of COX-2 appears to be made in neurons, COX-2 was also seen in rat astrocytes and microglia [49]. It has been demonstrated that COX-inhibiting NSAIDs reduce microglia activation following infusion of Aβ in rats [50]. Neuronal stress, such as ischaemia and excitotoxicity, is associated with strong upregulation of neuronal COX-2 expression. This suggests that COX-2 is involved in neurotoxic mechanisms and may therefore represent a target for drug therapy in the treatment of AD [51, 52].

Several epidemiological studies provide the background for possible mechanisms of action of NSAIDs in AD. In most studies COX-2 inhibitors are used, because neuronal COX-2 is upregulated in response to exposure to Aβ [53], and focal increases in COX-2 have been shown in the region of amyloid plaques in

double transgenic mice carrying genes that encode both mutant APP and mutant presenilin 1 [54]. Many studies seem to show that COX-2 inhibition confers neuroprotection [55–58]. Some studies have revealed an upregulation of neuronal COX-2 in the brains of patients with AD [59, 60], though this has not been a universal finding [61, 62]. One explanation for the variation of COX expression is the short half-life of COX-2 transcripts or individual variability of inflammatory-related processes.

COX-1 is also localized in microglia and is actively involved in brain injury induced by pro-inflammatory stimuli including Aß, LPS and interleukins. A study with 20-month-old triple transgenic AD mice showed that their memory function increases, when treated with COX-1 inhibitors. In addition, amyloid deposits and tau hyperphosphorylation in hippocampus decrease [63]. Triflusal, a platelet anti-aggregant and irreversible COX-1 inhibitor, could protect against cognitive deficits by reducing the dense-core amyloid plaque load, associated glial cell activation and pro-inflammatory cytokine levels in a transgenic mouse model [64]. Unfortunately, this could only be clearly demonstrated in animal experiments.

Another principle of how NSAIDs could act comes from the finding that prostaglandin E2 levels are elevated in patients with AD, especially in early stages of the disease [65]. Therefore, NSAIDs blocking prostaglandin E2 synthesis might be beneficial. This issue is further strengthened by glial culture studies indicating that prostaglandins, particularly prostaglandin E, alter the production of several inflammation-related molecules, including IL-6, chemokines and APP [66–68].

In addition to the more traditional inflammatory mechanisms associated with COX, unique functions of COX-mediated damage may also occur in the AD brain. For example, several of the prostanoid products of arachidonate metabolism potentiate glutamate excitotoxicity, and COX-2 overexpressing transgenic mice exhibit increased neuronal susceptibility to excitotoxic insult [69].

Some of the previously mentioned studies of COX in ischaemia also suggest that intraneuronal COX-2 levels may contribute to neuronal death by production of free radicals [70]. In addition, increased COX-2 levels in AD neurons may directly damage neurons or increase their vulnerability to other detrimental processes occurring in AD brain [70]. Resulting, the inhibitory action of NSAIDs on COX-mediated production of apoptotic factors by neurons could be one of the mechanisms by which these anti-inflammatory drugs cause beneficial effects in AD.

Another non-COX-dependent mechanism of NSAIDs is to attenuate inflammatory processes in a manner by directly activating the peroxisome proliferator-activated receptor gamma (PPARγ), a receptor and nuclear transcription factor [71–73]. PPARγ is a member of the orphan nuclear receptor family. In cells of the monocytic lineage, including microglia, PPARγ acts to suppress the expression of a broad range of proinflammatory genes [71, 73]. Some NSAIDs act as PPARγ agonists, directly binding to it and initiating its transcriptional activity. Activation of PPARγ inhibits the Aβ-stimulated activation of microglia and monocytes and their secretion of proinflammatory and neurotoxic products. For example, PPARγ agonists act to inhibit the Aβ-stimulated expression of IL-6 and TNF-alpha [74], by

microglia and monocytes, and to prevent Aβ-mediated conversion of microglia into an activated phenotype [11, 75].

A further underlying mechanism of AD pathology is oxidative stress [76, 77]. Activated microglial cells are known to release ROS, which might possibly cause this oxidative stress. However, glial cells can also exhibit antioxidative functions by releasing hemeoxygenase-1 (HO-1) triggered by accumulation of 3-hydroxyanthrallinic acid (3-HAA), a downstream product of the tryptophan metabolism. The association of neuronal injury in AD and oxidative stress has been demonstrated by overexpression of immunoreactive HO-1 protein in neurons and astrocytes of the cerebral cortex and hippocampus. HO-1 was found to be co-localized to senile plaques, neurofibrillary tangles and corpora amylacea [78]. It is widely accepted that a moderate activation of heme catabolism is neuroprotective and contributes to degradation of neurotoxic protein aggregates. Regulatory interactions between HO-1 and COX pathways have also been reported [79]. However, experimental observations indicate that the extent of HO-1 induction may be critical because excessive heme degradation may result in toxic levels of carbon monoxide, bilirubin and iron. Pharmacological modulation of HO-1 levels in the brain shows promising results in models of AD and Parkinson's disease [80].

Referring to the oxidative stress underlying AD pathology, one further aspect of these ROS includes activation of COX-1/2, which is blocked by NSAIDs. It has been shown that daily doses of NSAIDs increase circulating levels of antioxidants [81]. In a rat model of AD, it was suggested that treatment with a COX-2 inhibitor reduces oxidative stress and might therefore be beneficial for the course of AD [82].

Another neuroprotective mechanism has been suggested for NSAIDs whereby these drugs directly affect amyloid pathology in the brain by reducing Aβ-42 peptide levels over the gamma-secretase activity independently of COX activity [83]. Weggen et al. reported that the NSAIDs ibuprofen, indomethacin and sulindac sulphide preferentially decrease the highly amyloidogenic Aβ-42 peptide produced from a variety of cultured cells by as much as 80 % [84]. However, for some NSAIDs the lowering effect of Aβ-42 could not be shown; instead, an increase in Aβ-42 levels was observed [85]. The underlying mechanism of how NSAIDs decrease Aβ-42 was clarified by Lleo et al., who demonstrated that Aβ-42 by lowering NSAIDs specifically affects the proximity between APP and presenilin 1 and alters a novel allosteric mechanism of action [86].

4 Anti-inflammatory Treatment Studies in AD

In recent years it has become widely accepted that inflammatory processes are an underlying condition of AD. Therefore, a number of clinical trials investigating different anti-inflammatory treatment regimens have been performed. In the following paragraph, we summarize the most import findings in regard to first mainly COX-2 dominant and second COX-1 inhibitors.

4.1 COX-1 and COX-2 Inhibitors

A prospective cohort study with 6,989 subjects showed that long-term use of NSAIDs protects against AD but not against vascular dementia [7]. More recently, Szekely et al. provided very similar findings. They concluded that NSAID use reduced the risk of preferentially AD versus vascular dementia but mainly in those individuals having an apolipoprotein E (APO) epsilon 4 allele. This study was done with over 3,000 subjects aged 65 years and older [8]. Not only selective COX-2 inhibitors were shown to be associated with decreased risk of AD; a reduced occurrence of AD could also be demonstrated for the use of the mixed COX-1/COX-2 inhibitor aspirin [9]. A meta-analysis of 17 epidemiological studies yielded strong, generally consistent, statistical evidence that NSAID and steroid use is associated with reduced risk of AD [87]. Vlad et al. investigated 49,349 patients with AD and 196,850 controls: long-term (>5 years) nonsteroidal anti-inflammatory drug use was shown to be protective against AD. These findings were clearest for ibuprofen, but did not appear for other NSAIDs [88].

Naproxen, which is slightly more selective for COX-1 than COX-2, cuts the risk of developing AD in 117 patients with MCI from whom CSF was collected 21–41 months after treatment was terminated. The tau to Aß42 ratio was reduced by more than 40 % in the group treated with naproxen [89]. Also, another NSAID with preferential COX-1 selectivity such as indomethacin reduced amyloid burden in transgenic mice [90].

To conclude there are at least ten studies showing beneficial effects of NSAIDs on amyloid burden and inflammation in mice.

In humans however, not all studies showed a positive outcome for COX-inhibitors. The failure of selective COX-2 inhibition (rofecoxib) over placebo was found in a 1-year randomized controlled study. The authors argued that their results could indicate that the disease process was too advanced to be modified, as the goal of the study was slowing the progression of dementia in patients with already established AD [10]. For another COX-2 inhibitor, celecoxib, no beneficial effect on the occurrence of AD could be demonstrated in an age group over 70 years [91]. Also, Wolfson et al. looked retrospectively at a case-control population and found no support for a beneficial effect for NSAIDs in the AD subjects [92]. However, this negative result may have been caused by an insufficient period of data collection before disease onset.

4.2 Passive and Active Anti-Aß Immunotherapies

In the last few years, most of the efforts of the pharmaceutical industry were directed against the production and accumulation of Aß. The most revolutionary development in the last several years consists in the removal of brain ß-amyloid via anti-Aß antibodies. Both passive and active anti-Aß immunotherapies can clear Aß deposits

from the brain of the AD patients. AN1792, which was used in AD patients, showed some clues of clinical efficacy but was associated with aseptic meningoencephalitis in about 6 % of patients. So this medication has been abandoned. The next generation of active and passive vaccines has been developed in the past few years and is currently under clinical investigation. The aim of these vaccines is to clear the brain from Aß deposits and to stop the progression of AD.

Bapineuzumab, composed of humanized anti-Aß monoclonal antibodies, is the most advanced product. It has been tested in two phase II trials and Aß burden was reduced in the brain of AD patients. Some patients experienced vasogenic edema especially apolipoprotein E4 carriers. This limits its clinical use, especially in higher doses (2 mg/kg). The proposed remedy is to treat AD patients with lower doses, particularly in APOE4 carriers. A large phase III trial with bapineuzumab is ongoing. This study will tell us if passive anti-Aß immunization is able to reduce progression of the disease [93]. Of course, improvements in vaccine design are needed to improve the safety and the efficacy of anti-Aß immunotherapy. Unfortunately, at this point we cannot definitely identify individuals in the preclinical stages of AD; therefore, passive immunotherapy is indicated in patients that are diagnosed with AD, i.e. have clinical symptoms. At that point patients have already accumulated substantial neuropathology in affected regions of the brain [94].

Perhaps amyloid-PET imaging combined with genetic markers and last but not least with clinical symptoms such as mild cognitive impairment will potentially detect individuals with a higher risk to develop an AD [95]. Development of valid biomarkers for AD should be a high priority aim of research on AD in the next few years.

5 Conclusion

Neuroinflammation plays a key role in the pathophysiology of the AD. Mechanisms that parallel those encountered in inflammatory diseases involving other organ systems are readily identified, along with detailed pathways for how the mechanisms interact. Although still controversial, on balance, it is likely that AD neuroinflammation exacerbates AD pathogenesis.

A general treatment principle in neurology and psychiatry that an intervention as early as possible leads to the best outcome seems to be especially true for AD. However, lack of appropriate biomarkers such as genetic risk factors or neuroimaging techniques is a problem that needs to be solved in the next few years. Until then, the treatment cannot start early enough.

Many lines of evidence show that Aβ-induced neuroinflammation is an early event in neurodegeneration of AD [96], as increases in microglia activation have been observed in very early stages of AD and disappeared over time [39]. The fact that neuroinflammation occurs very early in AD could explain why anti-inflammatory treatment seems to be most efficient as preventive or early treatment.

There are several reasons why early use of NSAIDs is superior to a late treatment. COX expression in the brain decreases over time in AD brains [97]. And the CSF PG E_2 levels in patients with AD are high when their short-term memory scores were just below those of controls, but were low in later stages of the disease. These findings further support that inflammatory processes predominate early in AD [98] and therefore require early intervention with anti-inflammatory agents.

This might also explain that the failure of some prospective clinical trials of selective COX-2 inhibitors delayed the onset of treatment. Lack of clinical efficacy could also be due to drug selection (regarding different effects of COX-1 and COX-2) or to dose and duration of treatment. The drug selection seems essential as some NSAIDs have recently been shown to increase Aβ-42 levels. It also has to be noted that the protective effects of NSAIDs may be via non-COX-inhibitory mechanisms, such as lowering of Aβ levels and activation of the peroxisome proliferator-activated receptor [gamma] [99]. These non-COX-dependent mechanisms might be differentially distributed amongst different COX-inhibitors.

While the harmful inflammatory processes seem to dominate in AD pathology, there are also some beneficial functions for inflammatory subsets. If AD neuroinflammation is approached with realistic expectations and rational drug design, AD patients could significantly benefit from anti-inflammatory treatment, especially with NSAIDs.

Another aspect could be to not only utilize the efficient treatment properties of NSAIDs in early AD but also make use of the neuroprotective aspects of neuroinflammation with combination therapy that maximizes the potential of glial activation. This would include treatment with NSAIDs and drugs that enforce anti-inflammatory and antioxidative properties (e.g. with 3-HAA and HO-1 enhancement).

Finally, a promising therapeutic approach using passive and active immunization against amyloid-beta has emerged. If in the next few years we are able to detect AD much earlier, starting treatment with immunization before the clinical symptoms appear could prove effective.

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Neuroinflammation in Huntington's Disease

Roland G.W. Staal and Thomas Möller

Abstract Huntington's disease (HD) is a progressive, eventually terminal, neurodegenerative disease caused by autosomal-dominant mutations in the huntingtin gene (*HTT*). The early symptoms of HD typically include subtle changes in mood and/or cognition, as well as poor coordination and unsteady gait. These symptoms progressively worsen until coordinated movement is virtually impossible and mental abilities have declined to a state of dementia. There is no cure and patients generally succumb to comorbid complications within 20 years of onset. The mutation is an expansion of the CAG triplet repeat stretch in the *HTT* gene, resulting in an expanded poly-glutamine (polyQ) stretch in the huntingtin protein (HTT). The length of this CAG repeat correlates strongly with the age of onset as well as the rate of disease progression. The ability to identify at-risk individuals by genetic testing enabled researchers to conduct clinical studies and learn about early events in the development of HD. One of the earliest pathological changes observed in the CNS of HD patients is the appearance of neuroinflammation, preceding overt neurodegeneration or protein aggregation. Here we will review the data implicating neuroinflammation in all stages of HD, from initiation to progression. We will also explore the most recent advances in our understanding of neuroinflammation in HD including a potential role for the peripheral immune system. We will also discuss how these various biologies may lead the way to discovery of novel, innovative, and urgently needed therapies.

Keywords Huntington's disease • Huntingtin • CAG repeat • PolyQ • Neurodegeneration • Neuroinflammation • PK-11195 • Microglia • Astrocytes • Monocytes • T cells

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List of Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ApoE	Apolipoprotein epsilon
ApoJ	Apolipoprotein J
ATP	Adenosine triphosphate
B cell	Bone marrow-derived lymphocyte
BBB	Blood–brain barrier
BDNF	Brain-derived neurotrophic factor
CB2R	Cannabinoid receptor 2
CCL5	Chemokine (C–C motif) ligand 5 (also CCL5)
CNS	Central nervous system
CXCL1	Chemokine (C–X–C motif) ligand 1 (GROα, KC)
EAAT2	Excitatory amino acid transporter 2
GABA	Gama-aminobutyric acid (inhibitory neurotransmitter)
GFP	Green fluorescent protein
HD	Huntington's disease
HTT	Huntingtin's protein
HTT	Human huntingtin's gene
Htt	Mouse huntingtin's gene
Iba1	Ionized Ca^{2+}-binding adapter molecule 1 [Allograft inflammatory factor 1 (AIF1)]
IKK	IκB kinase
IL-10	Interleukin 10
IL-4	Interleukin 4
IL-6	Interleukin 6
KCNN4	K^+ intermediate/small conductance Ca^{2+}-activated channel, subfamily N, member 4
KMO	Kynurenine 3-monooxygenase
LRP	Lipoprotein receptor-related proteins
MCP-1	Monocyte chemotactic protein-1 [Chemokine (C-C motif) ligand 2 (CCL2)]
MCP-4	Monocyte chemotactic protein-4 [Chemokine (C–C motif) ligand 13 (CCL13)]
MIP-1β	Macrophage inflammatory protein-1β, CCL4 [Chemokine (C-C motif) ligand 4]
MS	Multiple sclerosis
NAD^+	Nicotine adenine dinucleotide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NMDA	*N*-methyl-d-aspartate
NO	Nitric oxide
P2X4	P2X purinoceptor 4
P2X7	P2X purinoceptor 7
PD	Parkinson's disease

PolyQ	Poly-glutamine stretch
PrP	Prion protein
Q	Glutamine
RANTES	Regulated on activation, normal T cell expressed and secreted
ROS	Reactive oxygen species (superoxide, hydrogen peroxide, etc.)
SSRI	Selective serotonin reuptake inhibitor
T cell	Thymus-derived lymphocyte
TGF-β	Transforming growth factor β
TLR4	Toll-like receptor 4
TRPM2	Transient receptor potential cation channel, subfamily M, member 2
VGCC	Voltage-gated Ca^{2+} channels

1 Huntington's Disease

Huntington's disease (HD) is a progressive, terminal, neurodegenerative disease of monogenetic origin. The first signs of disease include subtle changes in mood and cognition as well as poor coordination and unsteady gate. Cognitive, behavioral, and psychological problems as well as the uncoordinated, jerking movements (chorea) continue to deteriorate and become more pronounced, eventually leading the patient to seek medical advice. Collectively, abnormal motor movements (dystonia and chorea) accompanied by cognitive decline and personality changes suggest the possibility of Huntington's disease, a diagnosis which can be confirmed by genetic testing. The disease and symptoms continue to progress until coordinated movement becomes extremely difficult and mental abilities decline to a state of dementia. Patients eventually succumb to bulbar dysfunction and accompanying complications such as pneumonia or heart disease within 20 years of disease onset [1, 2]. There are currently no disease-modifying treatments, only medications to manage the motor symptoms (e.g., Xenazine® (tetrabenazine), neuroleptics, benzodiazepines) and psychiatric symptoms (medications used to treat similar psychiatric symptoms in the general population, i.e., SSRIs, atypical antipsychotics).

One of the earliest neuropathological changes observed in HD patients is proteinacious inclusions rich in huntingtin protein (HTT) that are found in both the nucleus and cytoplasm of striatal GABAergic neurons. Whether or not these inclusions are themselves toxic or represent a protective sequestration mechanism remains unresolved [3, 4]. The CNS pathology of HD is characterized by severe atrophy of the caudate nucleus and putamen due to extensive loss of GABAergic medium spiny neurons that project to the globus pallidus. As the striatopallidal projections are lost, secondary degeneration of the globus pallidus sets in. The basal ganglia are key regulators of motor control, mood, and higher cognitive function which accounts for the clinical manifestations. As with most neurodegenerative diseases, neuronal death and atrophy of anatomical structures are not restricted to just one area, especially as the disease reaches later stages. Cerebral cortical atrophy is also commonly observed later in disease while the cerebellar Purkinje cells are generally spared [2].

The recognition that HD was an autosomal-dominant disorder enabled scientists to discover the underlying mutation in the huntingtin gene (*HTT*). Researchers identified the disease-causing mutation as an expansion of a CAG trinucleotide repeat stretch. As CAG encodes for glutamine (Q), the expansion in the protein is also known as polyQ region [2]. They determined that the length of the expansion predicts the age of onset, which while typically around 35–45 years of age can manifest itself as early as infancy and as late as 85 years. Individuals not affected by HD have fewer than 36 CAG repeats; however, people with 27–35 repeats have a slightly increased risk that their children could pass the 36 CAG repeat threshold due to de novo repeat expansion. Carriers of 36–39 repeats have a significant risk of manifesting HD, and carriers with more than 40 repeats will get the disease with certainty. As an autosomal-dominant disease, children of HD patients have a 50 % risk of receiving the gene with the CAG expansion.

Identification of the disease-associated gene was an important step in understanding HD, but determining the molecular function(s) of the huntingtin (HTT) protein and the biological processes it initiates, coordinates, or regulates remains largely unknown. The autosomal-dominant inheritance of mutated HTT suggests a toxic gain of function since HD patients usually still have one wild-type allele and normal protein is still produced. For example, mutant HTT can recruit normal HTT into insoluble aggregates in vitro and in vivo. In contrast, ablation of *Htt* leads to embryonic lethality, suggesting a critical role of normal HTT function in development. Other in vitro and in vivo studies suggest that loss of HTT function may contribute to disease as shown by decreased cell survival and dysfunction of neurons. Because HTT is expressed throughout the body, its expression pattern fails to provide any insight into its function or the increased susceptibility of the medium spiny neurons in the striatum. The ubiquitous expression, however, implies that the polyQ repeat expansion may affect any cell type in which it is expressed (including astrocytes, microglia, and oligodendrocytes in the CNS). The impact of the mutation upon a given cell type may also depend on the specific function of HTT within that particular cell. The effects of the mutation may also manifest itself at varying stages of the disease depending on the levels of mutant HTT expression. Thus, any cell in the CNS has the potential to contribute to the etiology of HD, suggesting that the underlying disease mechanism may be non-cell autonomous as has been suggested for other neurodegenerative diseases such as Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) [5–7].

2 Neuroinflammation in Huntington's Disease

One type of nonneuronal cells that garnered interest in early neuropathological studies of brains from patients with neurological disease is microglia, the resident immune cells of the CNS. These cells were observed to have profoundly altered morphology (less ramified and more amoeboid) and increased expression of immune cell markers (e.g., HLA-DR, CD68) in neurological diseases including HD [8, 9].

Numerous studies have since been published that firmly establish altered microglial morphology and phenotype (commonly called "activated microglia") as a pathological hallmark of HD. In addition, mediators of inflammation, such as cytokines and increased oxidation products, were found to be increased in HD [10, 11]. Hence, the term "neuroinflammation" was coined to describe the *inflammation* observed in the brains of patients with *neuro*logical disease.

As the CNS resident immune cell, microglia surveil the environment for danger signals (foreign or endogenous) and continuously communicate and interact with the other cells in the brain (astrocytes, oligodendrocytes, and neurons). One mechanism is the release of molecules that are traditionally considered to be immunological signaling molecules such as cytokines and chemokines [12]. Another modality is via release of growth factors, neuropeptides, and transmitters (neuro- and glio-transmitters, such as norepinephrine, glutamate, ATP). The cells of the CNS work in concert to initiate and then modulate neuroinflammation whose goal is to remove the initial cause (infection, disease, and trauma) and ultimately restore homeostasis. The term "neuroinflammation" has therefore been extended beyond the CNS resident immune cells and come to include all cells in the CNS that contribute to the inflammatory response to neurological disease or infection.

2.1 Microglia

The primary function of microglia is to survey the CNS for any signs of danger (either endogenous or foreign) [13]. In the surveilling state (formerly referred to as the "resting" state), microglia have small cell bodies with long, thin ramified processes that constantly extend and retract, making contacts with neurons and other cells. Upon detection of danger signals, microglia can rapidly migrate to the site of injury. They become less ramified and increase the expression of cell surface antigens, resulting in the "activated" morphology observed in virtually all neurological diseases and injuries. The specific phenotype of "activated microglia," however, can be very different from one another even though they morphologically "appear" similar. Microglia have a wide-ranging arsenal of executory functions, including phagocytosis of pathogens or cellular debris, secretion of enzymes that break down the extracellular matrix, secretion of proteins that opsonize dying or damaged cells and mark them for phagocytosis, release of pro- and/or anti-inflammatory cytokines and chemokines, release of growth factors (or downregulation of their release), generation of reactive oxygen species (ROS) to destroy phagocytized pathogens, as well as release of glutamate, toxic kynurenine metabolites, ATP, and nitric oxide [13]. The nature of the insult, as well as the immediate milieu of the microglia, will all impinge on the microglia and shape their response. Hence, even if "activated" microglia are observed, one can only conclude that their phenotype is no longer surveying and that they are now "activated." One needs to be cautious in making predictions about what these microglia are actually producing or doing (or have stopped producing/doing).

Microglia can respond quickly to danger, setting in motion fast, potent mechanisms such as phagocytosis, and production of ROS to kill or neutralize phagocytized pathogens, dangerous substances, or dying cells [13, 14]. Over time, other mechanisms are engaged, such as changes in gene expression, transcription, and release of pro-inflammatory and later anti-inflammatory substances including cytokines and growth factors [13, 15]. Especially pro-inflammatory mediators (as well as withdrawal of trophic support) can easily damage or kill nearby cells (bystander damage), so that tight control and effective resolution of neuroinflammation is critical. This resolution of neuroinflammation is accomplished not only by ceasing to produce the pro-inflammatory mediators but by producing anti-inflammatory mediators (IL-4, IL-10, TGF-β) [13]. Thus, microglia are key cellular participants in all stages of neuroinflammation, initiation, resolution, and the following tissue repair. One needs to keep in mind, however, that either this can be a temporal sequence of phenotypes through which microglia pass or it could be a sequential response mediated by distinct subsets of microglia. If the inflammatory response is overshooting in amplitude or length, cells can be damaged or killed, resulting in the spillage of intracellular molecules (chemokines, ATP, heat shock proteins, mitochondrial proteins some of which are formylated like their bacterial forefathers). Since these intracellular molecules are not normally "seen" by microglia, they act as additional danger signals that activate microglia or continue to maintain the microglia in an activated state. This cascade can propagate the neuroinflammatory response or prevent its resolution, potentially starting a vicious cycle. Unrelated inflammatory diseases or conditions (including peripheral inflammation or infection) may directly or indirectly "alert" microglia in the CNS. In this alerted state, microglia are primed such that when a subsequent danger signal comes along, the response is potentiated, leading to greater bystander damage. Thus, microglia activation can be a double-edged sword, one side slaying the microbe, the other the neuron [13, 14, 16].

2.2 Microglia in HD

The first observation of activated microglia in autopsy brains from patients with HD, reported an altered microglial morphology and increased expression of HLA-DR (a component of the antigen-presenting "machinery") [8, 9]. This observation was interpreted as evidence of inflammation in the brains of these patients since HLA-DR expression is also increased on antigen-presenting cells during inflammation. Since then, other studies have demonstrated that in addition to increased expression of HLA-DR, other markers of microglial activation are also increased in HD brain, supporting the hypothesis that there is inflammation in the brains from patients with neurologic disease. These observations are complemented by PET imaging studies demonstrating increased microglial activation in HD patients (see below) [17, 18]. The first study to suggest that microglia might

actually participate in the pathological processes already in motion was the finding that there was an increased number of microglia in the caudate putamen from HD patients [19]. A subsequent study then showed an increase in the number of morphologically activated microglia in the neostriatum, cortex, and globus pallidus and adjoining white matter of HD brains vs. controls [20]. The numbers and density of microglia increased with the grade of HD pathology (i.e., Vonsattel rating scale [21]) and increasing neuronal loss. The findings were confirmed in additional studies with human HD tissue and extended to the R6/2 mouse model of HD where the number of activated microglia was also found to be increased in the striatum [20]. Importantly, this study was also the first to demonstrate the expression and, in some cells, aggregation of HTT in microglia. Aggregation of HTT is considered to be a hallmark of HD, and the observation that microglial cells contain HTT aggregates might indicate a direct cell-autonomous effect of HTT on microglial cells.

More recently, it was demonstrated that wild-type microglia localize to neurons expressing mutant HTT fragments, specifically, along dystrophic neurites but not to somata with mutant huntingtin inclusions [22]. Concurrent with neurodegeneration, microglia increased their expression of Iba1, increased in number, underwent morphological alterations (more amoeboid, less ramified), and increased the expression of the proliferation marker Ki67. Surprisingly, the inflammatory mediators IL-1β, TNFα, and IFNγ were unchanged. In contrast to other studies, however, IL-6 and complement 1q were increased once overt neurodegeneration set in, suggesting that neuroinflammation was still occurring even though different effector molecules were released [22]. Together, the data suggest that microglia recognize and respond to neurons expressing mutant HTT, perhaps to remove dysfunctional synapses or neurites.

A study by Singhrao et al. showed that in addition to an increased number of microglia, there was increased complement biosynthesis by microglia and an increase in complement activation on neurons [19]. For a long time, the complement system was ignored by neuroscientists who considered it a part of the peripheral innate immune system that provides powerful cytotoxic and cytolytic activities against a large variety of pathogens [23]. Over the years, it has become increasingly clear that complement is not only synthesized in the CNS but also participates in most CNS pathologies from acute stroke to chronic neurodegenerative diseases such as HD [24–26]. In HD postmortem samples, neurons, astrocytes, and myelin show increased deposition of C1q, C4, and C3, iC3b-neoepitope and C9-neoepitope compared with non-HD controls [19]. The authors hypothesized that increased levels of complement in HD brains contributes to disease progression, by either contributing to increased inflammatory signaling or the elimination of synapses or neurons by phagocytosis by microglia. Furthermore, the complement receptors, C3aR and C5aR, are also strongly expressed in HD caudate. Interestingly, activation of these receptors in microglia resulted in a reorganization of the actin cytoskeleton and subsequent increase in motility [27]. The increase in expression of complement

proteins in HD brains could not be recapitulated in the R6/2 mouse model. Furthermore, crossing the R6/2 mice with C3 KO mice did not change their phenotype [28]. Intriguingly, the complement system has been shown to play an important role in synaptic pruning in CNS development, homeostasis and disease (reviewed in [29]), suggesting that perhaps the complement system has a function (such as synaptic pruning or phagocytosis) not revealed in the R6/2 mouse model of HD. Thus, the role of increased complement proteins and receptors in brains from HD patients remains to be determined.

Our understanding of the role of neuroinflammation in HD was advanced in 2006 by studies using the radioligand PK-11195 that labels microglia [30, 31]. PK-11195 binds to the peripheral benzodiazepine receptor whose expression is significantly increased in activated microglia and, some studies suggest, reactive astrocytes [31]. Pavese and colleagues demonstrate increased binding of PK-11195 and presumably increased peripheral benzodiazepine receptor in the striatum of HD patients [17, 30]. The increase in PK-11195 binding was correlated with disease progression as assessed by the loss of dopamine D2 receptor binding sites in the striatum. Since most HD is inherited, genetic testing can be used to identify at-risk individuals years before disease onset. In a follow-up study, the same investigators were able to demonstrate that microglial activation is already evident as much as 15 years before onset of overt symptoms which was predicted based on CAG repeat length. The appearance of activated microglia over a decade before disease onset suggests that neuroinflammation is an early event in the disease. Furthermore, higher levels of microglial activation and decreased levels of D2 receptors were associated with a higher probability of developing clinical HD (as determined by onset of symptoms). The findings that neuroinflammation preceded neurodegeneration put the spotlight on microglia and neuroinflammation as a disease biology that had to be more than just a coincidental consequence of disease and the degenerative processes in play.

2.3 Astrocytes in HD

As with microglia, the idea that astrocytes might play a prominent role in the etiology or progression of HD took several years to gain traction even though the expression of HTT in astrocytes was demonstrated soon after the discovery of the gene mutation that caused HD [32, 33]. It was not until much later that genetic studies in mice implicated astrocytes in the pathogenesis of mutant HTT [34–38]. Restriction of mutant HTT expression to select neuronal populations, as opposed to pan-expression, actually resulted in a marked reduction of motor deficits and neuropathology in the striatum [39]. When the mutant HTT transgene was expressed in neurons and astrocytes, progression of disease like symptoms and neuropathology was exacerbated vs. those mice in which expression was restricted to neurons [40, 41]. This suggested that astrocytes, like microglia, are important contributors to neuroinflammation and pathology in HD animal models and likely in patients as well.

Like microglia, astrocytes can respond to danger signals, endogenous and foreign, in a process termed reactive astrogliosis. Their responses are also a continuum of changes that depend on the nature of the insult and signals from other cells in the CNS. These changes can result in changes to tissue structure, scar formation, and altered blood flow. Upon activation, the gene expression profile of astrocytes changes dramatically as does their phenotype and their portfolio of signaling molecules [42]. In animal models of Huntington's disease, mutant HTT expressed in astrocytes can accumulate in their nuclei and decrease the expression of glutamate transporters [35–37]. In these models, researchers demonstrated that the uptake of glutamate as well as the release of CCL5/RANTES and brain-derived neurotrophic factor (BDNF) was diminished [43]. While mRNA for the astrocyte-expressed glutamate transporter (EAAT2) was reported to be altered in HD brains in one report [44], another study failed to find any alteration in synaptosomal glutamate transport [45]. While decreased BDNF levels in HD have been largely ascribed to neuronal loss [46], BDNF is also expressed in astrocytes (and microglia) where expression of HTT decreases its release via transcriptional regulation [47]. In support of a role for astrocytic BDNF in HD, targeting BDNF overexpression to astrocytes delayed disease in animal models of HD, suggesting that this may be a therapeutic strategy for disease intervention [48, 49]. BDNF is one among dozens of genes whose transcription is regulated by NF-κB, a transcription factor found throughout the body. NF-κB plays a critical role in both microglia and astrocytes, by positively and negatively regulating transcription of various signaling molecules. For example, NF-κB can increase the transcription of pro-neuroinflammatory genes, such as cytokines, while leading to decreased transcription of neurotrophic factors. One mechanism of activating the NF-κB pathway is via stimulation of the toll-like receptor 4 (TLR4) with lipopolysaccharide (LPS). A recent study demonstrated that two HD mouse models (Hdh(150Q) and R6/2) both responded more robustly to systemic LPS with greater systemic inflammation and by producing more pro-inflammatory cytokines in the brain [50]. The hypothesis that the increased response to LPS was due to enhanced NF-κB activation was supported by observations that activated NF-κB levels were elevated in HD patients and that astrocytes from R6/2 mutant mice express higher IκB kinase (IKK) activity, which prolongs NFκB activation [50]. Similar increases in astrocytic NF-κB levels in mouse models of HD as well as HD patients suggest that enhancement of the NF-κB signaling pathway in astrocytes could contribute to neuroinflammation and HD.

Astrocytes also play a vital role in cholesterol synthesis, transport, and metabolism. Cholesterol is a vital molecule for the CNS, yet it does not cross the BBB and thus has to be synthesized locally. Almost 25 % of a person's cholesterol is within the CNS, and 70 % of that is incorporated in oligodenroglial myelin sheaths enwrapping axons [51]. Lipid imbalance as a potential cause of HD was first proposed in the 1970s but was highly controversial [52–54]. Much later it was demonstrated that expression of mutant HTT reduced the expression of genes involved in cholesterol biosynthesis in vitro and in vivo (R6/2 model) [55, 56]. However, over time dysfunction in the cholesterol synthesis pathway was replicated across 4 different HD rodent models (R6/2, YAC, Hdh^{Q111}, transgenic HD rats) [57–59].

Neurons make cholesterol much less efficiently than glia, but it is an absolute necessity for their survival and ability to function. All glial cells contribute to the overall cholesterol pool in the CNS, but the majority of neuronal cholesterol originates from astrocytes. The ABCA1 transporter on astrocytes loads cholesterol onto ApoE which carries it to neurons (and oligodendrocytes). Neurons have an array of receptors to take up the cholesterol-rich ApoE molecules. It is interesting to note that ApoE4/4 genotype is a significant risk factor for Alzheimer's and Parkinson's disease, with deficits in cholesterol transport (potentially due to reduced ability to bind to LRP class of receptors) as one proposed mechanism [60]. Oligodendrocytes (discussed below) also produce cholesterol. If the production of cholesterol is inhibited, myelination cannot occur, causing a profound phenotype, including ataxia and tremor [61]. It is interesting to note that a close relative of ApoE is ApoJ, also known as clusterin. Clusterin plays an important role in complement activation and innate immune responses, raising the question whether cholesterol metabolism and these pathways may have additional roles in neuroinflammation. Thus, cholesterol metabolism and transport is another vital metabolic process connecting the main propagators of neuroinflammation; however, little is known about neuroinflammation-dependent changes in cholesterol in HD.

2.4 Central and Peripheral Cytokines

As mentioned earlier, peripheral inflammation, infection, or disease states can alter the phenotype of microglia in the CNS and, more generally, affect the neuroinflammatory status of the CNS [34]. It is also becoming increasingly clear that the peripheral immune system significantly impacts neurological disease [62, 63]. The first evidence that the immune system may be dysregulated in HD was presented by Lebhuber et al. in 1998 [64]. In their study of 12 patients and 10 controls, they reported increased serum levels of IgA, soluble TNF receptor, soluble IL-2 receptor, neopterin, and complement C3 [64]. Another group showed that chemokines were elevated in the plasma of HD patients [65]. Eotaxin, eotaxin-3, MIP-1β, MCP-1, and MCP-4 were significantly elevated in HD patients. Of these, three (eotaxin-3, macrophage inflammatory protein (MIP)-1β, and eotaxin) correlated with advancing disease stages [65]. Björkqvist and colleagues demonstrated that HD gene carriers had elevated IL-6 levels, on average, 16 years before the predicted onset of clinical symptoms [66]. Interestingly, a more global increase in cytokine transcripts was also detected in the striatum of HD patients [11]. The Björkqvist study also examined the response of monocytes from HD subjects, in order to determine if they might be the source of the elevated cytokines observed in the plasma of patients. The monocytes not only expressed mutant HTT but also release significantly more IL-6 in response to stimulation by lipopolysaccharide. Similar patterns of cytokine release were observed in macrophages and microglia from HD mouse models [66]. Finally, IL-6, IL-10, CXCL1, and interferon-γ were significantly elevated in the serum

of HD vs. wild-type mice but were normal in HD mice receiving a bone marrow transplant from WT mice [67]. Together, these data suggest that there is dysregulation of the peripheral immune system that might parallel the neuroinflammation in the CNS and that, perhaps, this could be a contributing factor to HD pathology.

2.5 Peripheral Immune Cells

The observation that peripheral cytokine release is dysregulated in HD led people to investigate further the role of HTT in immune cells as well as HD. Studies that quantified mutant as well as total HTT protein levels in leukocytes from patients with HD demonstrated robust changes in mutant HTT expression between carriers and noncarriers and also between asymptomatic and symptomatic carriers of the *HTT* mutation in monocytes as well as T and B lymphocytes [68]. The investigators also demonstrated a significant correlation between mutant HTT levels and disease burden scores and caudate atrophy rates in monocytes and T cells in patients with HD. However, total HTT levels in leukocytes were not different between HD patients and controls or between different disease stages within the same patient. In contrast to monocytes and T cells, mutant HTT was not altered in buccal cells between any group, suggesting that the increased expression and dysfunction are specific to at least some cells of the immune system [68]. Chemotaxis was another immune cell function that was determined to be dysregulated by mutant HTT in leukocytes (white blood cells including monocytes, T and B cells, basophils, neutrophils, eosinophils, and dendritic cells) from the HD mice. Leukocytes from mutant HTT mice as well as carriers of the *HTT* mutation had a blunted chemotactic response [69]. Evidence that the peripheral cells could impact the CNS pathology in HD mouse models came from a study in which bone marrow from wild-type mice was transplanted into lethally irradiated transgenic mice (YAC128 and BACHD mice) [67]. While the bone marrow transplant only partially attenuated the hypokinetic and motor deficits in HD mice, the investigators observed increased levels of synapses in the cortex of these mice. This suggests that transplantation of peripheral immune cells could influence some of the pathophysiology in HD models. Interestingly, the group observed that in the brain of irradiated HD mice, many more microglia were positive for Iba1 as well as green fluorescent protein (GFP) than in normal irradiated mice. Since only the transplanted bone marrow cells were GFP positive, the Iba1/GFP double positive cells in the brains of these mice must have come from the periphery. The implication is that more bone marrow-derived cells could migrate into the CNS of irradiated HD mice than in irradiated wild-type mice. Once in the CNS, the bone marrow-derived cells can directly influence neuroinflammation and the pathological processes in mouse models of HD, and perhaps in patients as well [67].

Together, the findings suggest that multiple functions of immune cells are dysregulated by mutant HTT. Furthermore, the dysregulation of immune cell function is

not restricted to a single immune cell lineage, but manifest in the myeloid, lymphoid lineages, as well as yolk sac-derived microglia of the CNS. While the studies do not identify the sequelae of increased mutant HTT in T or B cells, they do raise the question whether or not the increased mutant HTT levels cause T or B cell dysfunction, much like they do in monocytes. Could T and B lymphocytes also contribute to the initiation or progression of HD? Critics of the hypothesis point out that there is no large-scale infiltration of the CNS by T cells as in multiple sclerosis (MS). In MS, it has been demonstrated in animal models as well as the clinic that T cell infiltration into the CNS plays an integral role in the pathophysiology of the disease as illustrated by Tysabri® [70]. In other neurodegenerative diseases such as PD and ALS, evidence supporting a role for T cells in the pathological process is accumulating, even though large T cell infiltrates are generally not observed [71, 72]. Recent studies, however, have demonstrated that T cells patrol the CSF and subarachnoid spaces (and perhaps even the CNS parenchyma), supporting the hypothesis that T cells are able to get into the CNS and potentially respond to antigen presentation and participate or modulate neuroinflammation [73]. The ability of T cells to control parasitic (*Toxoplasma gondii*) infections of the CNS demonstrates not only that T cells patrol the brain parenchyma but that they are able to execute their immunological functions as well. It will be interesting to see if future studies will demonstrate that mutant HTT expression in T cells results in their dysfunction and if this dysfunction contributes to the pathophysiology of HD.

3 Targeting Neuroinflammation in Huntington's Disease

An area of active investigation, not just for HD but all major neurological diseases, is the therapeutic targeting of neuroinflammation. By expanding drug discovery, efforts from the traditional neuron-focused strategies (neuroprotection, neuroregeneration, neurotransmission) to include neuroinflammation greatly increase the diversity and number of targets amenable for therapeutic intervention using small molecules and biologic. Targets, such as toll-like receptors, cytokine and chemokine receptors, purinoceptors, neuro- and glio-transmitter receptors, kinases, glutamate transporters, and catalytic enzymes (proteolytic, reactive oxygen species generators, ATP hydrolyzing), may offer tractable novel approaches to treat HD. Microglia have many of the same receptors or signaling pathways as peripheral monocytes/macrophages. This, unfortunately, is a double-edged sword, in that it is target rich and may present repurposing opportunities but may also potentially result in unwanted side effects mechanistically coupled to the target's role in the peripheral immune system (e.g., increased risk of infection). Astrocytes and oligodendrocytes have the potential advantage that they have less overlap with cells of the immune system, decreasing the risk of unwanted immune-related side effects. Other glial targets, such as neurotransmitter receptors and amino acid transporters, may also be expressed on neurons, raising the possibility of significant adverse neuronal side effects.

Currently, small molecules are the best strategy for treatment of CNS diseases as CNS-penetrant molecules can be designed or selected. While many targets would likely require a biologic as a therapeutic, delivery technologies need to be developed to increase the brain penetration, or the biologics themselves need to be optimized to achieve significant brain penetration. Until these challenges are effectively solved, many promising targets that regulate neuroinflammation will remain intractable (including adhesion molecules, immunoglobulin signaling molecules, and other targets not currently amenable to modulation by small molecules [74]). Finally, as we learn more about the role of peripheral immune cells in HD perhaps, one could target them in the periphery, without the need for a CNS-penetrant agent. Such a therapeutic could alter the phenotype of peripheral cells before they migrate into the CNS. It could also block/augment the ability of peripheral immune cells to migrate into the CNS (depending on if they are desirable or pathological). Precedent for this idea comes through modulation of peripheral immune mechanisms in the treatment of MS.

One area of intensive exploration has focused on the mechanism of action of the antibiotic minocycline, which has been reported to ameliorate neuroinflammation and subsequent pathology in many animal models of neurological disease, including HD models [75]. Experiments using minocycline were believed to target neuroinflammation presumably via inhibition of NF-κB [75, 76]. In contrast, other studies have suggested that minocycline targets caspases and neuronal apoptosis [77, 78]. The actual mechanism of action or the target of minocycline is a matter of ongoing debate and is reviewed elsewhere [79–82]. While minocycline has not been used in a clinical trial for HD, it was tested in numerous other neurological diseases and disorders exhibiting neuroinflammation. Unfortunately, the promising findings failed to translate into the clinic, as the clinical trials to date have largely failed [75].

Another potentially promising therapeutic strategy for HD is to regulate or normalize kynurenine metabolism which can produce both neuroprotective and neurotoxic metabolites. The kynurenine pathway is the primary route of l-tryptophan metabolism and the primary metabolic pathway for the formation of nicotinamide adenine dinucleotide (NAD^+) [83]. Several of the metabolites in this pathway have neuroactive properties. (For review, see [84, 85].) Schwarcz and colleagues were the first to suggest that the kynurenine pathway may play a role in the pathogenesis of HD by showing that an intra-striatal injection of quinolinic acid replicates many features of human HD in rodents [86]. Quinolinic acid is an N-methyl-d-aspartate (NMDA) receptor agonist and induces excitotoxicity [87]. Follow-up studies demonstrated that the levels of neurotoxic kynurenine metabolites were elevated in HD patients and mouse models, whereas the levels of neuroprotective metabolites were decreased [85, 88]. A subsequent report demonstrated that cultured microglial cells from the R6/2 HD mouse model synthesized increased levels of neurotoxic kynurenine metabolites [89]. The discovery that genetic ablation of kynurenine 3-monooxygenase (KMO) suppresses HTT-mediated toxicity and the fact that KMO is predominantly expressed in microglia, not neurons, made for a strong argument that microglial kynurenine metabolism might play a significant role in HD [89–91]. Microglial dysregulation of the kynurenine pathway was also the first

example of a potentially non-cell-autonomous mechanism in HD (from a neuronal point of view). KMO thus presents an attractive target for the treatment of HD as evidenced by the number of academic and nonprofit organizations working to develop KMO inhibitors [92].

Another strategy for targeting neuroinflammation is based on activation of the cannabinoid receptor 2 (CB2R) which in the CNS is only expressed on activated microglia [93]. The authors demonstrated the role of CB2Rs in regulating neuroinflammation by knocking out the CB2R in the R6/2 mouse model of HD which resulted in enhanced microglial activation, worsened disease symptoms, and shortened life span. Following injection of the neurotoxin quinolinic acid, edema and loss of medium spiny neurons were also exacerbated in the R6/2 mice lacking CB2Rs as compared to R6/2. Pharmacological activation of CB2R in R6/2 mice with intact CB2R attenuated the microglial activation and loss of medium spiny neurons following quinolinic acid lesioning [94]. The study also demonstrated that a CNS-penetrant CB2R agonist can extend the life span while suppressing motor deficits, synapse loss, and CNS inflammation in a mouse model of HD. Unexpectedly, a non-CNS-penetrant CB2R antagonist had similar effects. Since this compound does not reach the CB2R-expressing microglia in the brain, it suggested that peripheral cells were driving the neuroinflammation and pathology [95]. CB2 agonists were also protective against striatal malonate toxicity, another toxin model of Huntington's disease [96, 97]. While there is some debate about the expression of CB2 in microglial cells in vivo [93], these studies suggest that neuroinflammation in HD may be reduced by pharmacological activation of CB2 receptors.

Currently, there are no disease-modifying treatments for HD, only symptomatic medications. As discussed previously, there are many potentially promising therapeutic targets that could treat the neuroinflammation associated with HD. Even if blocking neuroinflammation in HD only would be able to slow disease progression, but not provide a cure, it would be considerable progress for a disease with currently very limited therapeutic options. While the target space for neuroinflammation in HD is rich, it still needs validation in the clinic. Since neuroinflammation is present in all neurological disease, there is the potential that discovery of a therapeutic that blocks or reduces neuroinflammation in HD may also have efficacy in other devastating neurological diseases.

4 Summary and Conclusion

Neuroinflammation is increasingly being recognized as a biological process that is intimately linked to the pathological cascades underlying HD. While symptoms are a manifestation of neuronal dysfunction or loss, neurons do not live and die in isolation within the CNS. They are in constant contact, not only with each other, but also with astrocytes, oligodendrocytes, and microglia. The polyQ expansions have been well documented to cause neurotoxicity and render neurons more vulnerable to toxic insults, but they have also been shown to alter microglial and astrocytic functions. The finding that microglial activation is increased years before overt

neurodegeneration suggests that neuroinflammation is involved early HD and, at minimum, an important contributor. This is supported by numerous studies showing that immune-related markers are similarly dysregulated in HD as well as the animal models. Furthermore, if these mechanisms are modulated, they result in robust changes in "disease" outcome in the models, suggesting that they also play a significant role in disease. Peripheral immune cells may also affect neuroinflammation upon migration into the CNS or by release of inflammatory mediators that indirectly alter neuroinflammation. While the interplay of these CNS resident and peripheral players presents a daunting complexity, it also provides a wealth of new targets for desperately needed therapeutics.

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Neuroinflammation and Immune Regulation in Ischemic Stroke: Identification of New Pharmacological Targets

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Abstract Stroke is among the most common diseases of advanced age and is becoming a steadily increasing financial healthcare problem in the industrialized world with the increasing longevity and aging of the population. The incidence of ischemic stroke is highest in the elderly population, representing one of the most common causes of disability and mortality worldwide. Over the past decades, a tremendous amount of research has been undertaken into developing effective therapeutic strategies for the treatment of acute stroke. Unfortunately, many neuroprotective agents

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that have shown successful results in treating animal models of acute stroke have failed to translate into clinical treatments. Only tissue plasminogen activator is currently licensed for use in the treatment of acute ischemic stroke. Increasing evidence shows that the central nervous system and the immune system interact in complex ways, and better insight into these interactions may be relevant to the treatment of patients with stroke and other forms of central nervous system injury. However, during recent years, promising findings suggest that systemic inflammation and neuroinflammation are central features in cerebrovascular disease. Atherosclerosis, autoimmune disease, and physiological stressors, such as infection or surgery, may be a risk factor for the initial development of cerebral ischemia. In addition, the immune system actively participates in the pathophysiological processes occurring during an ischemic stroke. Thrombosis and hypoxia trigger an intravascular inflammatory cascade which elicits an inflammatory response in the injured brain that is accompanied by a marked local inflammatory reaction that is initiated by ischemia- or hematoma-induced expression of cytokines, adhesion molecules, and other inflammatory mediators, including prostanoids, extracellular proteases, reactive oxygen species, and nitric oxide, leading to the accumulation of inflammatory cells, such as leukocytes and microglia, which is further augmented by the innate immune response to cellular damage occurring in the parenchyma. Many of these compounds are known to promote and sustain inflammatory responses at local and systemic level, producing a neuroinflammatory response and a systemic acute-phase response. The acute-phase inflammatory response after stroke is a reflection of an unspecific systemic inflammatory response syndrome. Classic acute-phase reactants and body temperature are also modified in stroke and may be useful in the prediction of events and outcome and as therapeutic targets. The activation of innate immunity after stroke sets the stage for an adaptive immune response directed against brain antigens. The pathogenic significance of adaptive immunity and its long-term effects on the postischemic brain remains unclear, but it cannot be ruled out that a persistent autoimmune response to brain antigens has deleterious and long-lasting consequences, such as the development of poststroke dementia. This immune activation causes secondary tissue injury, but it is unclear whether modulating the acute immune response to stroke can produce clinical benefits. Better understanding of the role of the postischemic-induced inflammatory response and its potential for modulation might have profound implications for patient treatment. Preclinical studies suggest that interventions that are aimed at attenuating such inflammation reduce the progression of brain damage that occurs during the late stages of cerebral ischemia. In particular, strategies that block the activity of inflammation-related enzymes reduce ischemic damage with an extended therapeutic window. Although, clinical trials using anti-inflammatory strategies did not show benefit in patients with ischemic stroke, there is a strong rationale for continuing to explore the efficacy of anti-inflammatory therapies in the treatment of the late stages of cerebral ischemia acting more on the modulation of these later events than targeting of specific steps in the ischemic cascade.

Keywords Cerebral ischemia • Neuroinflammation • Adaptive immunity • Innate immunity • Danger-associated molecular pattern molecules (DAMPs) • C-Reactive protein • Hypothermia

Abbreviations

AD	Alzheimer's disease
ANXA1	Annexin A1
APC	Antigen-presenting cells
ATP	Adenosine triphosphate
BBB	Blood–brain barrier
CBF	Cerebral blood flow
CIDS	CNS injury-induced immunodepression
CMV	Cytomegalovirus
CNS	Central nervous system
CRP	C-reactive protein
DAMPs	Danger-associated molecular pattern molecules
DC	Dendritic cells
EC	Endothelial cells
Glu	Glutamate
HMGB1	High-mobility group box 1
HPA	Hypothalamic–pituitary–adrenal
HSV	Herpes simplex virus
HIV	Human immunodeficiency virus
IGF-1	Insulin-like growth factor 1
IFN	Interferon
IL-1β	Interleukin-1β
IL-1Ra	Interleukin-1 receptor antagonist
I/R	Ischemia/reperfusion
LPS	Bacterial lipopolysaccharide
MBP	Myelin basic protein
MCs	Mast cells
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
MTDL	Multi-target-directed ligands
NK	Natural killer cells
NKT	Natural killer T cells
NO	Nitric oxide
NPCs	Neural precursor cells
PNS	Parasympathetic nervous system
ROS	Reactive oxygen species
RTL	Recombinant T-cell receptor ligand
SNS	Sympathetic nervous system

TBI	Traumatic brain injury
Th1	T helper type 1
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
Treg	T regulatory
UTIs	Urinary tract infections
UTP	Uridine triphosphate
VEGF	Vascular endothelial growth factor

1 Introduction

Stroke is the second leading cause of mortality and leading cause of adult disability worldwide [1]. As 80 % of survivors remain disabled and our society ages, the socioeconomic burden of stroke will increase further. Although progress has been made in prevention with a reduction in stroke incidence and mortality, and the introduction of specialized stroke units has improved the functional outcome of stroke victims [2], no new treatment for acute ischemic stroke has made it from bench to bedside since tissue plasminogen activator (tPA) was introduced in 1996 [3]. Intravenous tPA can only be administered within the first 4.5 h following onset of ischemic stroke, and only 5–20 % are eligible, despite admission to hospital in a timely manner [4]. Moreover, circumstantial evidence suggests potentially neurotoxic effects of tPA, by activating matrix metalloproteinases (MMP) [5] or excitotoxicity in experimental models [6], and intracerebral hemorrhage is a significant complication [7]. Most experimental therapeutic approaches have focused on specific pathogenic mechanisms causing ischemic injury, such as excitotoxicity, oxidative stress, inflammation, or apoptosis [8]. These experimental treatments have failed in large clinical trials, generating debate about the feasibility of neuroprotection in stroke therapy [8]. There are several possible reasons why the previous approaches were not successful, at the level of both preclinical development and clinical trials (Table 1) [9, 10]. Therefore, translational stroke research is at a crossroads requiring a reevaluation of traditional approaches and the development of a new conceptual framework to guide therapy [11].

In this context, there is much to learn about inflammation as a key factor in injury and repair processes following cerebral ischemia, which remains a target for developing new therapies. Emerging evidence also suggests that inflammation preceding stroke contributes to stroke risk, stroke onset, and extent of injury. The elements of the immune system are involved in all stages of the ischemic cascade, from the acute intravascular events triggered by the interruption of the blood supply to the parenchymal processes leading to brain damage and the ensuing tissue repair. In response to cerebral ischemia, the brain, through the autonomic nervous and neuroendocrine systems, exerts a potent suppressive effect on immune activation, which may increase susceptibility to intercurrent infections, which are major determinants of stroke morbidity and mortality. Therefore, the immune system is closely related

Table 1 Limitations of clinical trials in stroke [10]

Limitations
Failure to apply basic principles of immunology
Inadequate understanding of brain injury mechanisms
Exacerbation of systemic effect
Exacerbation of preexisting infection or inflammation
Failure to aquire an adequate preclinical dataset to translate experiment to clinical trial design
Failure to translate observations from phase II studies to phase III studies
Use of novel trial design elements not previously or adequately tested
Failure to adequately validate outcome measures to be used
Failure to appreciate alternative hypotheses
Conflict of interest
Failure of the fundamental hypothesis

to critical events determining the fate of the ischemic brain and the survival of stroke patients. This chapter reviews the central and peripheral inflammatory responses to cerebral ischemia, considering the balance between pro- and anti-inflammatory pathways and the prospects for the development of therapies.

2 Is Inflammation a Stroke Risk Factor or a Trigger?

A stroke risk is a personal characteristic that increases the propensity to have a stroke in someone with that characteristic when compared to someone without [12]. Some risk factors cannot be modified, other risk factors are modifiable, and their correction reduces the chance of having a stroke. The activation of the immune system may increase the risk of stroke. Numerous prospective population-based investigations demonstrated a correlation between levels of inflammatory biomarkers [such as white blood cell count, fibrinogen, D-dimer, and C-reactive protein (CRP)] and the risk of incident and recurrent stroke [13]. These observations suggest that inflammation may have a causal role in vascular injury and subsequent stroke, which would open the door for immunomodulatory agents as new tools to prevent stroke in these patients. However, observational data are notoriously prone to confounding, and animal models often do not apply well to humans. Clearly, a more detailed understanding of the complex relationship between inflammation and stroke is required to better assess the feasibility of immunomodulation as a potential tool for stroke prevention.

2.1 How Does Inflammation Increase the Propensity to Stroke?

Inflammation is a stepwise process that is fundamentally designed to enhance the resistance of the organism to infections and to lead to tissue repair after various forms of injury. Atherosclerosis, the pathological process underlying the majority of

ischemic stroke in humans, is a peculiar form of inflammation triggered by cholesterol-rich lipoproteins and other noxious factors such as cigarette smoking, diabetes mellitus, and hypertension [14]. Inflammation is the dominant process in atherosclerotic lesion formation, characterized by the accumulation of foam cells, macrophages loaded with phagocytozed lipids, and the formation of a cellular lipid core (high cholesterol ester content) that weaken the arterial wall and make atheroma prone to rupture [15]. The proliferation and accumulation of smooth muscle cells is also typical and probably secondary to the destabilization and destruction of the media layer of arterial wall. It alters vascular structure by promoting stiffening of arteries and by inducing narrowing, thickening, and tortuosity of arterioles and capillaries. It contributes to arterial remodeling with smooth muscle cell migration and proliferation with abnormal angiogenesis and destabilization of the arterial wall [16]. In the brain, these morphological changes are often associated with reductions in resting cerebral blood flow (CBF) and marked alterations in CBF regulation [17]. Vascular risk factors that impair the structure and function of cerebral blood vessels and associated cells (neurovascular unit) [18] act by vascular oxidative stress and inflammation, impairing vital adaptive mechanisms and reducing brain perfusion. Endothelial dysfunction and inflammation, microvascular disease, and macrovascular disease affect cerebral hemodynamics. Injury to the neurovascular unit alters CBF regulation, depletes vascular reserves, disrupts the blood–brain barrier (BBB), and reduces the brain's repair potential, effects that amplify the brain dysfunction and damage exerted by incident ischemia and coexisting neurodegeneration [18, 19]. The ability of the endothelium to regulate microvascular flow is compromised, while the increase in blood flow evoked by neural activity is suppressed, resulting in a mismatch between the brain's energy supply and demand [20].

These vascular alterations increase the brain's vulnerability to ischemia after arterial occlusion because they compromise the development of collateral flow which is vital to the survival of the ischemic perinfarct zone. In addition to their vascular effects, both chronic and acute systemic inflammation may enhance the intrinsic susceptibility of brain cells to injury. In mice, acute [21, 22] and chronic peripheral infections [23, 24] exacerbate ischemic brain damage, which is associated with delayed resolution of brain inflammation and augmented microvascular dysfunction after stroke, amplifying the tissue damage produced by ischemia, although the biological bases of this effect are not well understood [25, 26].

2.2 How Does Inflammation Alter Cerebral Blood Vessels?

Among potential proinflammatory mediators of atherosclerosis and stroke, infection remains one of the most plausible, although controversial, causes [27–29]. Infections may contribute to vascular risk in at least two ways [30]. First, infections could serve as risk factors through long-term effects on the vascular wall much like conventional risk factors such as diabetes or hypertension. Acute infections, alternatively, could contribute to short-term stroke risk (i.e., as a stroke trigger). Many organisms, including bacteria and viruses, have been associated with

atherosclerosis and stroke risk with regard to infection serving as a chronic risk factor [31], particularly respiratory tract infections. Case–control and prospective studies indicate that chronic infections, such as periodontitis, chronic bronchitis, and infection with *Helicobacter pylori*, *Chlamydia pneumoniae*, or cytomegalovirus (CMV), herpes simplex virus (HSV), and human immunodeficiency virus (HIV), might increase stroke risk, although considerable variation exists in the results of these studies, and methodological issues regarding serological results remain unresolved [31, 32]. However, the inconsistent results from these studies are probably a good indication that if infection plays a role, it is more likely to be in a cumulative fashion.

The aggregate burden of chronic and/or past infections rather than any one single infectious disease is associated with the risk of stroke [33, 34]. According to this hypothesis, infections contribute to the overall inflammatory milieu of the vessel lining, together with other risk factors, and individuals with the greatest exposure to different infections throughout life are most likely to develop atherosclerosis and stroke. It is also likely that individuals with a more robust inflammatory response due to polymorphisms in infection-response genes are also more likely to show vascular changes related to infection. Finally, it is plausible that more sophisticated measures of infectious burden may have a role in assessing risk of vascular disease associated with infections. Past exposure to common infections may contribute to atherosclerosis by exacerbating innate immune responses and inflammation. Future studies are needed to validate these and other approaches to measuring infectious burden, define optimal measures of infectious burden, and elucidate host and other environmental factors that modify the risk of vascular disease associated with common infections.

2.3 Inflammation as Stroke Trigger

Although the factors precipitating the ischemic event cannot be established in most instances, stroke has been associated with infectious episodes occurring within the previous 2 weeks in case–control studies [33, 35, 36]. Precisely how systemic infections exert their effect remains unclear [37]. Exacerbation of vascular inflammation and activation of the coagulation cascade are likely to play a role [38]. The added vascular dysfunction and blood clotting abnormalities, superimposed on those induced by stroke risk factors, could precipitate vascular occlusion. This view is supported by the fact that acute stroke often occurs in the setting of increased circulating leukocytes, and elevated plasma markers of systemic inflammation and vascular activation, which also predict a poor outcome [37, 38]. The identification of a short-term state of elevated stroke risk after acute infection could have direct therapeutic implications. For example, increased doses of antiplatelet agents or statins may be warranted during times of fever or infection when benefits may outweigh risks of dose-related side effects. In addition, the period during and soon after hospitalization for infection could constitute a “treatable moment” during which patients can be evaluated for cardiovascular risk and standard preventive strategies instituted.

3 Phases and Players of Ischemic Neuroinflammation

Ischemic strokes are characterized by the occlusion of a blood vessel due to a thrombus or embolus. The location and the size of the eventual infarct vary, depending on the arterial territory, whether reperfusion is established, and extent of collaterals. After the onset of cerebral ischemia, two main regions of damage can be defined according to metabolic parameters and CBF thresholds [39]. The ischemic core, characterized by profound hypoperfusion (CBF reduced to less than 12 ml/100 g/min), is defined by almost complete energy failure, resulting in necrosis. The ischemic penumbra is less critically hypoperfused (CBF maintained approximately 30 ml/100 g/min), and metabolic activity persists in this region, particularly if reperfusion can be restored quickly. The penumbra is therefore the principal target for reperfusion/neuroprotective therapy and can remain potentially viable for up to 48 h in patients.

3.1 Temporal Kinetics of the Different Mechanisms of Ischemic Neurodegeneration

In simple terms, three phases of infarct progression can be characterized (Fig. 1) [39, 40]. The acute phase starts within minutes of stroke onset. Interruption of blood flow reduces the supply of oxygen and glucose that enable the brain to generate the adenosine triphosphate (ATP) needed to support its energy demands [41].

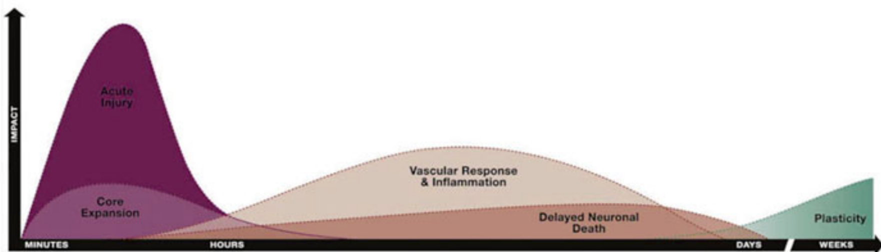

Fig. 1 The sequence of mechanisms involved in neuronal cell death following cerebral ischemia. The occlusion of a blood vessel due to a thrombus or embolus in the affected brain region induces an acute injury phase, characterized by excitotoxicity and increased free radical production. Hours to days after ischemia, an inflammatory response occurs, characterized by an increase in cytokine release and infiltration of neutrophils and leukocytes, with damage to the extracellular matrix, the blood–brain barrier, and a later phase of cell toxicity. At later stages, activated signaling cascades and transcription events by these initial mechanisms cause further tissue damage progression and induce cell death via apoptosis. A plasticity response, permitting spontaneous recovery following stroke, is also mounted following injury and involves the birth of new neurons, migration of stem cells, and the sprouting of existing fibers to form new connections

After focal ischemia, this energy deficit is most severe in areas with the lowest residual flow (ischemic core), wherein cell death (necrosis) occurs rapidly [41]. In areas of less severe ischemia (ischemic penumbra), the decrease in CBF perturbs ionic homeostasis, with an increase in intracellular calcium concentrations, and stimulates glutamate release, causing excitotoxicity and spreading depression throughout. Water shifts to the intracellular space due to osmotic gradients causing cytotoxic edema. The resulting cytotoxic edema can influence reperfusion negatively, by increasing intracranial pressure and vascular compression, potentially resulting in herniation. Generation of reactive oxygen species (ROS), which may be increased following rapid reperfusion, can damage membranes, mitochondria, and DNA, leading to misfolding of proteins and enzyme damage [41]. Reperfusion, while considered a key process to limit the evolution of infarction, may exacerbate neuronal injury through increased production of ROS and by delivering proinflammatory cells and mediators to the site of evolving injury [42]. In the subacute phase (hours to a few days after ischemia), an apoptotic and neuroinflammatory response develops, accompanied by BBB damage with interstitial tissue breakdown as a result of increased proteolytic enzyme release and activation and evolution of vasogenic edema [43]. Finally, in the chronic phase, which can last up to some months after stroke, repair and regeneration determine the extent to which neuronal function can be restored [39, 40].

3.2 Inflammation and Microvasculature: Cross Talk During Brain Ischemia

The inflammatory process begins in the intravascular compartment, immediately after arterial occlusion. The cerebral microvasculature rapidly displays multiple dynamic responses to focal ischemia including the breakdown of the permeability barrier of primary endothelial cells (EC), transudation of plasma, loss of EC and astrocyte integrin receptors, loss of their matrix ligands, expression of members of several MMP families, and the appearance of receptors associated with angiogenesis and neovascularization [44]. The ensuing hypoxia, changes in shear stress, and production of ROS trigger the coagulation cascade and lead to the activation of complement, platelets, and EC [42, 45, 46]. Ischemia triggers inflammatory signaling with intravascular and parenchymal accumulation of leukocytes [42]. Oxidative stress in EC reduces the bioavailability of nitric oxide (NO). Intravascular formation of fibrin traps platelets and leukocytes leading to microvascular occlusions [47–49]. While leukocyte infiltration into the ischemic brain is detrimental, leukocyte accumulation in the microvasculature was shown to be one of the many factors implicated in reduced reperfusion [50]. Platelet–leukocyte aggregates also contribute to microvessel occlusion and no-reflow phenomenon after reperfusion [50].

Table 2 Mediators of postischemic inflammation and their producing cells [42]

Initiation non-transcriptional (*cell type*)	Amplification transcriptional (*cell type*)	Resolution transcriptional (*cell type*)
Adhesion molecules	Adhesion molecules	Growth factors
P-selectin (*EC, PLT*)	ICAM1, VCAM1, P-selectin, E-selectin, Mac-1, VLA-1 (EC, Leuk, PVM, MG, AG)	BDNF, EPO, FGF, G-CSF, GDNF, HB-EGF, IGF-1, NGF, VEGF (*MG, AG, PVM, Macr, EC, Neu*)
Cytokines	Cytokines	Cytokines
IL-1β (*MG, PVM, MC*) IL-1α (*PLT*) TNF (*MC*)	IL-1, IL-6, IL-10, IL-17, IL-20, TNF (*EC, PVM, MG, AG, Neu*)	TGFβ, IL-10, IL-17, IL-23 (*T cells, MG, Macr, AG*)
Chemokines	Chemokines	
CCL5 (RANTES), CXCL4, CXCL7 (*PLT*) CX3CL1 (fractalkine) (*Neu*)	CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CXCL2/3 (MIP2), CXCL8 (IL-8) (*EC, PVM, MG, AG, Neu*)	
Proteases	Proteases	Proteases
Elastase, MMP8, MMP9, MT6-MMP (*Leuk*) Clotting factors (*Circ*) Complement (*Circ, EC, AG, Neu*)	MMP2, MMP9 (*EC, Leuk*) Complement (*Circ, EC, AG, Neu*)	MMP9 (*AG, Neu*) Complement (*Circ, EC, AG, Neu*)
Small molecules	Others	Small molecules
Prostanoids, leukotrienes (*EC,PLT,MG, Neu*) ATP (*Circ, Neu*) Radicals (*EC, PLT, Leuk, PVM, MG, Neu*)	iNOS (*MG, Leuk, EC*) COX-2 (*Neu, MG, Leuk, EC*) LOX (*Neu, Leuk*) PTGES (*Neu, MG, Leuk, EC*) NADPH oxidase (*MG, Leuk*)	Cyclopentenones prostaglandins Lipoxins Docosanoids (resolvins, protectins)

AG astroglia, *Circ* plasma, *EC* endothelial cells, *Leuk* leukocytes, *Macr* macrophages, *MC* mast cells, *MG* microglia, *Neu* neurons, *PLT* platelets, *PVM* perivascular macrophages, *ATP* adenosine triphosphate, *BDNF* brain-derived growth factor, *COX-2* cyclooxygenase-2, *EPO* erythropoietin, *FGF* fibroblast growth factor, *G-CSF* granulocyte colony-stimulating factor, *GDNF* glial cell-derived neurotrophic factor, *HB-EGF* heparin-binding epidermal growth factor-like growth factor, *ICAM1* intercellular adhesion molecule 1, *IGF-1* insulin-like growth factor 1, *IL* interleukin, *iNOS* inducible nitric oxide synthase, *LOX* lipoxygenase, *Mac-1* macrophage-1 antigen, *MIP* macrophage inflammatory protein, *MMP* matrix metalloproteinase, *NGF* nerve growth factor, *PTGES* prostaglandin E2 synthase-1, *RANTES* regulated upon activation, normally T expressed, and presumably secreted, *TGFβ* transforming growth factor-β, *TNF* tumor necrosis factor, *VCAM1* vascular adhesion molecule 1, *VEGF* vascular endothelial growth factor, *VLA-1* very late activation antigen-1

3.3 Early Phase: Vascular Events Triggered by Stroke

Within minutes after ischemia, the adhesion molecule P-selectin is translocated to the surface membrane of platelets and EC, and proinflammatory signals are rapidly generated (Table 2) [42]. Complement is activated and arachidonic acid metabolites are released. In the vascular wall, upregulation of E- and P-selectin on EC provides a platform for low-affinity leukocyte binding through interaction with sialyl Le^x

moieties of glycoproteins expressed on leukocytes (PSGL-1), while stronger adhesion is obtained after endothelial expression of ICAM-1 interacting with leukocyte β2 integrins (LFA-1 and Mac-1) [51]. Loss of NO promotes vasoconstriction and enhances leukocyte and platelet aggregation [52]. Upregulation of additional adhesion molecules promotes the binding of circulating leukocytes. The trafficking of inflammatory cells into the perivascular space is facilitated by the downregulation of junctional proteins that maintain the integrity of the endothelial lining and the BBB and by proteases, including MMP, that are also expressed in vascular cells and released by perivascular macrophages [53]. MMP activation further contributes to BBB breakdown and matrix proteolysis facilitating leukocyte extravasation [54], while chemotactic complement subunits (C5a), acting on mast cell complement receptors (CD88), leads to degranulation and release of histamine and proteases in the perivascular space, contributing to BBB leakiness [55–58]. Oxidative stress and induction of inflammatory mediators also contribute to permeability of the BBB. Further signals to guide leukocyte migration across the vessel wall are produced by mast cells and perivascular macrophages, secreting cytokines, such as tumor necrosis factor (TNF) and interleukin-1β (IL-1β), which in turn recruit and promote the infiltration of more leukocytes, via induction of chemokines and adhesion molecules [59, 60].

ATP is also released by vascular cells and blood cells and may promote intravascular coagulation and platelet aggregation [61]. In the brain parenchyma, within minutes after ischemia, injured cells release purines [ATP, uridine triphosphate (UTP)], as a result of neuronal and glial depolarization or escape through damaged plasma membranes [62–64]. High parenchymal ATP levels activate P_{2X7} receptors in microglia and act as early proinflammatory signals leading to the production of proinflammatory mediators (Table 2) activating microglia that develop many characteristics of macrophages (i.e., amoeboid morphology, migratory capacity, phagocytosis, and major histocompatibility complex (MHC) class II-restricted antigen presentation) [61]. Furthermore, microglia express a wide variety of neurotransmitter receptors (i.e., AMPA, kainate, adrenergic, $GABA_B$ opioid, and cannabinoid receptors) [65]. Some of these neurotransmitters, with several exceptions [65], released after ischemia/reperfusion (I/R) may counteract the emerging inflammatory response, downregulating microglial cytokine, ROS, and NO production [65], and suppress the secretory response in mast cells with the activation of their receptors [66, 67]. Finally, the disruption of neuronal–microglial interaction (CX3CL1, CD200) [68] and increases in extracellular glutamate (Glu) acting on microglial GluR1 metabotropic receptor also contribute to the proinflammatory milieu [65].

3.4 *Middle Phase: Ischemic Cell Death-Dependent Inflammation*

With the ischemic cascade progression, cell death leads to a new phase of the inflammatory response. Neurons are more vulnerable than glia and vascular cells and, when exposed to ischemia, quickly become dysfunctional and die (ischemic necrosis).

The ensuing neuronal damage is most rapid and severe in the ischemic core where cellular energy failure, attributable to lack of oxygen and glucose, occurs very rapidly. This leads to extracellular accumulation of glutamate, which in turn leads to cytoplasmic accumulation of Ca^{2+} and activation of Ca^{2+}-dependent enzymes, including calpains and caspases. These events lead to necrosis or programmed cell death, depending on the intensity of the insult and the metabolic state of the neurons.

Injured and dying cells play a key role in postischemic inflammation because they release *danger signals* which activate the immune system [69]. These *danger signals* are usually called danger-associated molecular pattern molecules (DAMPs) and represent a wide variety of molecular signals released from the intracellular compartment or generated by the action of lytic enzymes escaped from dead cells on matrix proteins [69]. Some of these signals, like the nucleotides ATP and UTP, are released by cells under stress when the cell membrane is still intact and set the stage for the subsequent immune response [70]. DAMPs and purines induce the expression of proinflammatory molecules in infiltrating leukocytes (Table 2) and prime dendritic cells (DC) for antigen presentation. Furthermore, inflammatory mediators released from parenchymal cells are likely to feed back on the vascular and perivascular compartments to reinforce and amplify the expression of cytokines, chemokines, and adhesion molecules that drive the infiltration of blood-borne cells into the ischemic tissue due to the high vascular density of the brain.

3.5 DAMPS and Ischemic Brain Injury: High-Mobility Group Protein Box 1 (HMGB1) (a DAMP with a Key Pathophysiological Role in Stroke)

DAMPs activate toll-like receptors (TLRs) and scavenger receptors on microglia, perivascular macrophages, DCs and ECs, and infiltrating leukocytes [71]. This activation induces the expression of proinflammatory molecules, such as IL-1, and primes DCs for antigen presentation. Such proinflammatory changes are initially counterbalanced by the release of neurotransmitters (Glu and purines) [71], which activate anti-inflammatory receptors on microglia, and by the presence of cell–cell interactions between microglia and adjacent neurons, which usually keep microglia quiescent [72]. Increasing concentrations of extracellular Glu activate metabotropic Glu receptors on microglia leading to a proinflammatory phenotype [73]. From one side, ATP as an early neuronal danger signal promotes the inflammatory response of resident immune cells, while from the other side, as a neurotransmitter, it may oppose these changes and counteract inflammation [74, 75]. However, as ischemic neuronal cell death progresses in the ischemic core and spreads to the penumbra, neurotransmitters are depleted, releasing this brake on proinflammatory signaling. High-mobility group box 1 (HMGB1) is a well-elucidated DAMP and is also implicated in ischemic brain injury [76–78]. HMGB1 is a nuclear protein that binds to nucleosomes and promotes DNA binding. When cells die in a nonprogrammed way,

HMGB1 is released in the extracellular medium; in contrast, apoptotic cells modify their chromatin so that HMGB1 binds irreversibly and thus is not released [79]. HMGB1, which is localized in cell nuclei in the normal brain, translocates into the cytosolic compartment and is released into the extracellular compartment in the ischemic condition. Extracellular release of HMGB1 is observed within 6 h after stroke onset, but is diminished by 12 h after the onset [76]. HMGB1 increases vascular permeability and promotes BBB breakdown [76]. The administration of anti-HMGB1-neutralizing antibody protects the BBB and reduces infarct volume [76]. Thus, HMGB1 is an essential DAMP in ischemic brain injury.

Furthermore, the loss of cell–cell interaction between neurons and microglia promotes inflammatory signaling [72]. In the normal state, microglia are kept quiescent by contact with neurons for the presence of a surface protein expressed in neurons (CD200) which interacts with its receptor CD200R on microglia enforcing a resting phenotype [80]. The disruption of this interaction due to postischemic loss of CD200 may promote microglial activation [80, 81]. Furthermore, a neuronally expressed chemokine, fractalkine (CX3CL1), may be important in maintaining microglia in preventing excessive microglial activation during cerebral ischemia [82]. Fractalkine is the only member of the CXC subfamily of chemokines and exists as both a membrane-anchored ligand and a secreted glycoprotein suggesting that it can work locally, by direct contact, as well as through distant soluble effects. Fractalkine is one of a few chemokines that is constitutively expressed at high levels in the brain and is unique in that it binds only one receptor, CX3CR1. Evidence from both in vitro and in vivo experiments demonstrates that the interaction of fractalkine and its receptor contributes to the attenuation of microglial activation and neurotoxicity under cerebral ischemia [82, 83]. Using mice lacking the microglial fractalkine receptor, it has been shown that the absence of CX3CL1-CX3CR1 signaling dysregulates microglial responses. CX3CR1-deficient mice show intense and widespread microglial activation as well as a protective inflammatory milieu, characterized by the promotion of M2 polarization markers [84]. In summary, these observations suggest that the inflammatory response after I/R starts at the vascular level, driven by non-transcriptional events triggered by hypoxia, shear stress, and ROS production. The ischemic brain tissue damage releases danger signals from cells under stress and then from necrotic cells. These signals activate purinergic receptors and pattern recognition receptors, which induce an inflammatory response in resident brain cells and infiltrating leukocytes together with the loss of immunosuppressive mechanisms.

3.6 The Innate and Adaptive Immunity Engagement

Both innate and adaptive immunity are engaged by cerebral ischemia. The inflammatory processes occurring immediately following ischemia rely on the innate immune system, because they involve the rapid activation of low-affinity receptors recognizing a wide range of targets, including DAMPs. The innate system is

germline encoded and is rapidly activated and relies on low-affinity receptors to gain wide-ranging target recognition [85]. Injury-induced inflammation is mostly dependent on TLRs, whose primary role is the initial activation of immune cell responses, mainly TLR2 and TLR4 [43]. Some endogenous TLR ligands, HMGB1, and peroxiredoxin family proteins, in particular, are implicated in the activation and inflammatory cytokine expression in infiltrating macrophages [86]. Experimental models of ischemia suggest that TLRs are involved in the enhancement of cell damage following ischemia and their absence is associated with lower infarct volumes [87].

Several cell types are predominantly associated with innate immunity, although there is considerable overlap between the roles of these cells in innate and adaptive immunity. Microglia derive from the hematopoietic system and constitute the resident immune cells of the central nervous system (CNS). They serve as sensitive sensors, through continuous extension and retraction of their processes [88], of events occurring within their environment [89, 90]. Microglia respond to cerebral ischemia with increased proliferation, motility, phagocytic activity, and the release of cytokines and ROS. However, they also contribute to the resolution of inflammation and tissue repair by producing IL-10 and transforming growth factor (TGF)-β, as well as several growth factors, including IGF-1. Perivascular macrophages, confined to the space between the vascular basement membrane and the brain surface (glia limitans) [89, 91], drive the infiltration of inflammatory cells [57]. They produce proinflammatory cytokines (IL-1β, IL-12, IL-23, and TNF), chemokines, ROS, and NO, thus promoting a T helper type 1 (Th1) immune response (M1 macrophages), or produce anti-inflammatory cytokines (IL-10 and TGF-β), IL-1ra, and arginase (M2 macrophages) [92].

Mast cells (MCs), localized in meninges and cerebral blood vessels, release vasoactive substances (histamine), cytokines (TNF), anticoagulants (heparin), and proteases (tryptase, chymase, MMP2, MMP9) from their MC granule store [55, 56] and are capable of phagocytosis, antigen presentation, and modulating the adaptive immune response [93]. Proinflammatory monocytes (producing TNF) are rapidly recruited to the site of injury where they give rise to macrophages and DC [94, 95]. DCs appear in the brain parenchyma after focal ischemia and originate from resident as well as blood-borne cells [95, 96]. They are the main interface between innate and adaptive immunity and specialized antigen-presenting cells (APC). Neutrophils adhere to the cerebral endothelium and transmigrate into the tissue [52]. They are secretory and phagocytic cells of the innate immune system with different types of cytoplasmic granules and secretory vesicles. NADPH oxidase, iNOS, MMP8, MMP9, elastase, myeloperoxidase, and cathepsins are major proinflammatory molecules stored in granules and vesicles. Receptor engagement, e.g., binding to E-selectin on EC, and IL-8 stimulation induce vesicle and granule exocytosis [97].

The immediate onset of this inflammatory cascade together with the available experimental data on patterns of signaling during early immune activation does not support a substantial role for the adaptive immune system in the early phases of cerebral ischemia, which relies on the clonal expansion of specific lymphocytes

with high-affinity receptors to specific antigens. However, the general immune activation caused by cerebral ischemia raises the questions of whether the adaptive immune system is subsequently activated and how it may contribute to the propagation and repair of brain injury. Antibodies against CNS antigens, such as myelin basic protein (MBP) and related peptides, develop after ischemic stroke, suggesting a humoral immune response to the injury, and circulating T cells become sensitized against CNS antigens, while APC are reduced in the periphery and increased in the ischemic brain both in rodent and human stroke [95, 98, 99].

After stroke, there is an increase in the number of cells in the brain that are capable of presenting antigen to lymphocytes. The accumulation of APC coincides with the peak of lymphocytic infiltration and is associated with the expression of MHC class II molecules and the co-stimulatory molecule CD80 [95, 98, 99]. More importantly, since antigens are primarily presented to lymphocytes in the secondary lymphoid organs, increased amounts of brain antigens, released from damaged cells, are able to drain to lymphoid tissues for potential presentation to lymphocytes [100]. This antigen presentation results in the production of antibodies against brain antigens and T cells sensitized to brain antigens. Oral administration of myelin antigens in experimental models results in the development of immune tolerance and reduced infarct size, though not reduced mortality [101]. Although tolerization is antigen specific, its beneficial effects are not restricted to immune responses directed at the inducing antigen, but are more widespread, a phenomenon termed bystander suppression [101]. A similar effect is also obtained with an adoptive transfer of splenocytes or CD4+ T cells from tolerized animals that can induce protection in naïve mice involving cellular immune mechanisms [101–103]. Furthermore, the Th2 cytokine response [102–104], due to IL-4 and IL-10 production by the activation of tolerized T cells in response to the antigen unveiled by the stroke, favors the formation of TGFβ-secreting Treg cells [102–104], while the administration of recombinant T-cell receptor ligand (RTL) reduces stroke volume in focal ischemia [105].

Although the available data do not provide a clear picture of how lymphocytes participate in acute infarction, mice engineered to lack selected T-cell subgroups are protected from ischemic damage to the penumbral zone around areas of infarction [106, 107]. Because B-cell-deficient mice or lymphocyte-deficient mice reconstituted with B cells are still protected from injury, the protection has been attributed to T cells. γδT cells also have been shown to contribute to the injury by releasing the proinflammatory cytokine IL-17 [108]. On the contrary, Treg are protective in the late stage of cerebral ischemia, but their effect is evident only if the injury is small [109, 110]. In conclusion, these data suggest that following macrophage activation, T lymphocytes infiltrate the ischemic brain and regulate the delayed phase of inflammation. IL-17-producing γδT lymphocytes induced by IL-23 from macrophages promote ischemic brain injury, whereas T_{reg} lymphocytes suppress the function of inflammatory mediators. The protection does not stem from an inability to propagate thrombus, and no significant differences in CBF exist between healthy and lymphocyte-deficient mice [111].

The lack of lymphocytes does not improve postischemic CBF at least in the acute phase, nor does it suppress thrombus formation [107]. Therefore, effects of lymphocytes altering microvascular perfusion or patency seem unlikely. It is possible that lymphocytes instead produce cell damage directly or through proinflammatory signaling and activation of downstream microglia and macrophages. Alternatively, early damage associated with lymphocyte infiltration of the ischemic brain may be due to natural killer T cells (NKT), which lack a TCR and, as such, do not require antigen presentation for their activation and cytotoxicity. Also, NKT or γδT cells, T cells that have a simplified TCR, do not require antigen processing, recognize aberrant cells, and particularly lack important self-recognition molecules. Because CD1-deficient mice that lack NKT cells are not protected from ischemic injury at 24 h [107], NKT cells may not be involved in the early phase of the injury. γδT cells have been implicated in ischemic brain injury, but their involvement seems restricted to the late phase of cerebral ischemia (4 days) [107, 108]. Considering the limited number of studies available, the involvement of NK, NKT, and γδT cells, lymphocyte subtypes that act in a fashion akin to innate immunity, needs further exploration. Despite the reported evidence supporting an autoimmune response against the postischemic brain, there are inconsistencies with the hypothesis that classical adaptive immunity contributes to ischemic brain injury. First, the temporal profile of the involvement of T cells in brain damage is not consistent with established concepts of adaptive immunity. The adaptive responses require an interval of 7–10 days from antigen presentation to the clonal expansion of autoreactive T cells and immune attack on the target organ, while the protective effect observed in lymphocyte-deficient mice or afforded by blocking postischemic trafficking of T cells into the ischemic brain occurs 24–48 h after ischemia [107]. Second, mice lacking co-stimulatory molecules essential for antigen-specific T-cell response appear unprotected from ischemia, and the reconstitution of lymphocyte-deficient mice with T cells targeting non-CNS antigens worsens ischemic damage [107, 111]. Finally, both CD4+ and CD8+ T cells are equally involved in ischemic injury [111], different from other autoimmune responses where prevalence for either T-helper or T-effector cell participation is present.

Collectively, these data suggest that, although an antigen-specific immune response may develop following stroke, evidence that autoreactive T cells attack brain antigens exposed by ischemic damage against which they were sensitized is lacking. Similarly, considering the evidence for humoral immune responses in stroke, the contribution of B cells to the damage needs a more in-depth assessment. Lymphocytes do play a role in the development and progression of the injury, but the mechanism of their powerful effect does not conform to the tenets of classical autoimmunity. It is unclear whether the release and presentation of CNS antigens during and after stroke results in an adaptive immune response directed against the CNS. If such an autoimmune response was directed against the brain after stroke, its long-term implications would potentially be significant. Such immune activity would be expected to impair neuronal plasticity and functional recovery and contribute to other CNS pathology, such as dementia.

4 The Inflammatory Response Is Age Dependent

The aging brain is characterized by a shift from the homeostatic balance of inflammatory mediators to a proinflammatory state. Basal mRNA expression of CD11b and Iba1, markers of activated microglia, as well as the basal mRNA expression of GFAP and S100B, markers of activated astrocytes, is higher in aged brain than in the young adult brain [112–114]. An elevated neuroinflammatory response sets the stage for an exaggerated inflammatory cytokine response in the brain after injuries such as traumatic brain injury (TBI) and stroke and may lead to more severe and persistent behavioral and cognitive deficits [68, 115, 116].

Microglial cells also exhibit an age-associated augmentation of reactivity in a variety of mammalian species [72]. In young rats, after an episode of mild cerebral ischemia, the process of microglial activation is rather slow, with microglia being fully activated at day 14. The situation is quite different in aged animals. A great number of microglia-like cells are fully activated at day 3 postsurgery, and the process reaches a maximum at day 7. Thereafter the intensity of the microglial reaction diminishes progressively with time, but is still evident even at day 28 [115]. These results have been largely confirmed in a model of intracerebral hemorrhage-induced brain injury in senescence-accelerated prone mice [117] and in a senescence-accelerated mouse prone 10 (SAMP10) [118]. Aging also exacerbates intracerebral hemorrhage-induced brain injury, with the activation kinetics of microglia/macrophages very similar to those assessed in focal cerebral ischemia and TBI. Three days after stroke, activated microglia/macrophages with OX42-positive processes and swollen cytoplasm were more abundantly distributed around and inside the hemorrhagic lesions of aged rats [119].

In most cases of acute CNS injury, the deposition of tissue debris is due to cell death, and the debris is removed by macrophages. In the CNS, the debris consists mostly of myelin, which contains several growth inhibitory molecules (such as Nogo A) that inhibit axonal regrowth [120, 121]. Therefore, delaying myelin clearance from the brain or spinal cord after acute injury may contribute to the failure of axonal regeneration. Microglia in aged rodent and human brains are subject to replicative senescence [122–124]. Importantly, in young animals myelin debris is removed more effectively than in older animals [125–128]. Furthermore, older rats show a delayed recruitment of phagocytic cells and diminished clearance of myelin after a toxin-induced demyelination lesion [129], and these deficits correlate with the slower remyelination in older animals [130]. Previous studies have shown that microglia-derived TNFα can adversely affect the survival of neural precursor cells (NPCs) and thereby limit the capacity for regeneration and repair in vitro and in vivo [131]. In light of these findings, it is conceivable that the accelerated inflammatory reaction in aged rats in response to stroke impedes the removal of cellular debris, thereby hindering poststroke tissue restoration.

5 The Systemic Acute-Phase Response

The acute-phase response comprises a variety of systemic changes in response to tissue injury, infection, and inflammation and is mediated mainly by IL-6 and IL-1. Classic *positive* acute-phase reactants that are elevated in patients with acute cerebral infarction include the plasma proteins CRP, serum amyloid A protein, and fibrinogen, neutrophil leukocytosis, and activation of the hypothalamic–pituitary–adrenal (HPA) axis. Numerous studies have investigated the time course and prognostic value of the acute-phase response in ischemic stroke. In general, acute-phase parameters correlate with stroke severity and infarct volume and predict worse outcome. CRP is the most studied acute-phase protein in stroke and will be discussed here in detail (for detailed review see also Di Napoli et al. [132]).

5.1 C-Reactive Protein

CRP is the major acute-phase protein in humans, and its concentration can rise 100-fold or more in response to injury, inflammation, or infection. Plasma CRP concentration is elevated in patients with acute stroke with or without infection [133], correlates with severity of stroke or infarct volume [134], and is an independent predictor of survival or nonfatal vascular events after ischemic stroke [13, 135]. There are limited data available to address the value of CRP measurement as a short-term prognostic marker. In one study of patients with confirmed middle cerebral artery occlusion receiving tPA within 3 h of symptom onset, patients with pretreatment levels of CRP of >7.7 mg/l were over eight times more likely to die after thrombolysis (OR: 8.51; 95 % CI: 2.16–33.5; $p = 0.002$) [136]. The utility of CRP measured after stroke as a predictor of future risk of recurrence or survival is also unsettled. In a secondary analysis of the PROGRESS trial, a multicenter secondary stroke prevention trial where patients were recruited several months after stroke or transient ischemic attack (TIA), those in the highest tertile of CRP had a modestly increased risk of recurrent ischemic stroke (OR: 1.39; 95 % CI: 1.05–1.85) [137]. In a different study, CRP levels measured at least 3 months after a first ischemic stroke or TIA were strongly predictive of increased risk of subsequent stroke or myocardial infarction (OR: 8.7) [138]. A CRP level >10 mg/l at time of discharge was significantly associated with the occurrence of a new vascular event or death at 1 year (HR: 7.4) [139]. Conversely, a CRP level ≥10.1 mg/dl when measured within 72 h of stroke predicted increased mortality over a follow-up period of up to 4 years in another study [140]. Winbeck et al. found that the measurement of CRP at 24 or 48 h, but not at admission, also predicted outcome [141]. The timing of measurement of CRP and the patient group studied are important considerations. Plasma CRP may remain elevated for up to 3 months after stroke [133, 142] implying that measurements made soon after stroke are not reflective of prestroke levels and may be less reliable for longer-term risk stratification. Based on the available data a few

years ago, the members of the CRP Pooling Project concluded that there were insufficient data to routinely recommend CRP testing for determining prognosis in ischemic stroke patients [13].

The role of CRP in the pathophysiology and outcome of ischemic stroke remains unclear. CRP in the acute phase of stroke may simply reflect the severity of tissue injury, while in the subacute/chronic phase, it may be a marker of underlying persisting inflammatory vascular risk. However, given the known proinflammatory and prothrombotic effects of CRP, it is increasingly recognized that CRP may directly mediate ischemic injury and vascular inflammation. For example, one likely mechanism of CRP-mediated injury is binding of abundant CRP to ligands exposed in dead and damaged cells, triggering substantial complement activation with the release of chemotactic factors and opsonization of cells in and around the ischemic lesion, leading to enhanced infiltration by inflammatory cells [132]. This model is supported by data indicating that CRP activates macrophages and attracts leukocytes to the damaged tissue by binding to FcgR present on phagocytic cells and leukocytes [132].

Human CRP administered intravenously in a rat model of cerebral ischemia enters the brain via a damaged BBB and binds to ligands exposed in damaged tissue and then activates complement [143]. Codeposition of human CRP and rat complement in the infarct area may lead to complement-mediated exacerbation of tissue injury. Incubation of cultured cerebral microvascular endothelial cells with CRP for 24 h significantly increased lactate dehydrogenase leakage from endothelial cells and induced significant upregulation of ICAM-1 and VCAM-1 expression. Several drugs, including statins, have been shown to be effective in reducing CRP plasma levels and in lowering future vascular events in humans. In animal models, the inhibition of CRP ligand-binding capability by 1,6-bis(phosphocholine)-hexane abrogated the increase in infarct size and cardiac dysfunction in poststroke animals [144]. Taken together, these studies suggest that CRP may be involved directly in the development of inflammation in response to cerebral ischemia.

6 Inflammation and Poststroke Infection: The Role of Immune Suppression

Infections acquired after stroke are an important contributor to morbidity and mortality [145]. Infection may worsen stroke outcome not only via its systemic effects (i.e., sepsis) but also via exacerbating brain tissue damage directly. Eighty-five percent of all stroke patients have complications, of which infection is the most frequent (23–65 %) [145]. Infection is also the most relevant complication during rehabilitation and the leading cause of death in stroke after day 1. Patients with stroke are especially at risk of pneumonia and urinary tract infections (UTIs), and such infections may independently worsen neurological outcomes and increase mortality.

6.1 Immunosuppression

Clinical data are currently emerging to support the involvement of so-called brain-induced immunodepression after acute ischemic stroke [53, 145–147]. While severity and age are strongly associated with infection [148–150], immune suppression may explain the inability of other factors (such as dysphagia or bladder dysfunction) to fully account for the high rates of pneumonia or UTIs, respectively, which are seen in survivors of stroke. Chamorro and colleagues reported a rapid increase in the plasma concentrations of circulating cytokines, with a low ratio of TNF-α to anti-inflammatory IL-10 preceding the symptoms of infection [151]. Increases in another anti-inflammatory cytokine interleukin-receptor antagonist (IL-1Ra) have also been linked to infection [152]. In terms of cellular immune activity, in a recent study of patients with acute ischemic stroke, marked signs of suppression of cell-mediated immunity were noted; these included functional deactivation of monocytes and Th1 cells, and there was some evidence that these changes occurred before the clinical manifestation of infection [22, 153]. In other studies, increases in activated circulating lymphoid cells, including those with characteristics of T-regulatory (Treg) cells, have been observed [154, 155]. Functional activity of innate immune cells from stroke patients has been monitored in terms of capacity to release cytokines following stimulation with bacterial endotoxin. The release was significantly reduced relative to age, sex, and atherosclerosis-matched controls [156]. Similar results have been noted by Haeusler et al. [148] and in subarachnoid hemorrhage [149].

These clinical observations are supported by experimental models, where a CNS injury-induced immunodepression syndrome has been described in experimental stroke models leading to spontaneous septicemia and pneumonia within three days after focal cerebral ischemia [157]. This was accompanied by extensive apoptotic loss of lymphocytes and atrophy of primary and secondary lymphatic organs. In parallel with the changes in the adaptive immune system, monocyte counts and function are compromised as well. Adoptive transfer of T and NK cells from wild-type mice, but not from interferon (IFN)-deficient mice, or administration of IFN at day 1 after focal cerebral ischemia greatly decreases the bacterial burden. A murine model of aspiration pneumonia was used to evaluate whether stroke-induced immunodepression contributed to the development of pneumonia after stroke [157]. Intranasal aspiration of only 200 colony-forming units of *Streptococcus pneumoniae* caused severe pneumonia and bacteremia in mice after transient middle cerebral artery occlusion. In contrast, 200,000 colony-forming units are needed to induce pneumonia of similar severity in sham animals. Such observations have led to the concept of CNS injury-induced immunodepression (CIDS) as an important contributor to infection [158–160].

6.2 Counter-Regulatory Response

It is interesting to speculate why poststroke immunodepression occurs, when it would seem to harm patients by increasing their risk of infectious complications. Although it may simply be a maladaptive response that stems from inherent aspects

of the design of the CNS and immune system, it may also be an attempt to control the extent of the inflammation, by mounting a homeostatic, counter-inflammatory response. In addition, it may serve to protect the CNS from the development of adaptive immune responses directed against self-antigens. Recent data indicate that the CNS undergoes regular immune surveillance by circulating lymphocytes. CNS components are not routinely presented to these lymphocytes in such a way as to sensitize them and launch an immune response against the CNS. However, increased levels of proinflammatory cytokines, such as IL-1 and IL-6, are known to be able to break tolerance [161, 162], and in the absence of countervailing factors, such antigen presentation would be expected to occur after CNS injury and compromise of the BBB. Therefore, the immunodepression seen after stroke may serve a beneficial purpose in limiting the development of such autoimmunity.

6.3 Neural and Endocrine Control

Although there is clearly potential for counter-regulatory cytokines, such as IL-4, IL-10, and TGF-β, to modulate cell responses locally, immune suppression appears to be systemic and this implies a controlling role for the autonomic nervous, or endocrine, systems, regulated by the CNS. The interaction between the CNS and immune system is bidirectional, and CNS injury has profound effects on immune function [158]. Sensors within the central and peripheral autonomic nervous systems relay information about the status of the immune system. This input is processed by the CNS, in particular the frontal premotor cortex, the hypothalamus, the pituitary, and the brain stem. This processing results in homeostatic signals being sent to various sites in the body via three major pathways of neuroimmunomodulation: the HPA axis, the sympathetic nervous system (SNS) axis, and the parasympathetic nervous system (PNS), and there is good evidence for immune control by each of these [158, 163, 164].

Neural stress seems likely to account for the earliest afferent and efferent phases of the response, but several lines of clinical and experimental evidence indicate that proinflammatory cytokines produced by inflamed tissue can directly lead to HPA axis and CNS activation. IL-6 is increased systemically after stroke [133], probably induced by proinflammatory cytokines such as IL-1, and is able to sustain the drive on the HPA axis [165]. Support for this is suggested by the observation that stroke patients treated with IL-1Ra have a normalized cortisol response as well as reversal of immune suppression [164]. Evidence for a role of the SNS in stroke patients comes from decreased baroreflex sensitivity, which is linked to increased adrenergic activity and was found to be associated with infection in intracerebral hemorrhage [150]. In a similar vein, heart rate variability is used as an index of PNS vagal tone and has been shown to be associated with reduced cytokine secretion in patients with head injury, and particularly intracerebral hemorrhage, possibly triggered by increased intracranial pressure [166].

Because the autonomic system of the CNS is *hardwired* with secondary lymphoid organs, the interruption of these circuits can result in immune dysfunction. Stroke can lead to direct damage of CNS structures involved in vegetative neuroimmunomodulation, and support for the concept of the neurogenic nature of CIDS comes from studies indicating that damage to specific areas of the brain is related to stroke-related infection [167]. In models of spontaneous or aspiration-induced infection after experimental stroke, the IFN deficiency and the development of bacterial infections were prevented by blocking β-adrenergic but not glucocorticoid activity [146, 158]. Administration of the beta-adrenoreceptor blocker propranolol also drastically reduced mortality after middle cerebral artery occlusion. These studies indicate that a catecholamine-mediated defect in early lymphocyte activation is a key factor in the impaired antibacterial immune response after experimental stroke.

6.4 Prevention of Infection and Immunosuppression

To prevent infection, preventive antibiotic strategies have been proposed. Prophylactic antibiotic treatment dramatically reduces mortality and improves outcome in mouse experiments [168], but the results of phase II clinical studies conducted in this field are contradictory at present [169]. Larger trials are needed to answer the question whether (and which) stroke patients should be treated prophylactically with antibiotics. Interestingly, several classes of antibiotics have been demonstrated to be neuroprotective. Minocycline protects brain tissue against ischemia [170, 171]. β-Lactam antibiotics also offer neuroprotection by increasing glutamate transporter expression [172]. Since the antibiotics for which neuroprotection has already been demonstrated are not suited for the treatment of infections commonly acquired by patients in stroke units, further research is needed to characterize the immunomodulatory and neuroprotective properties of suitable antibiotics. If antibiotic use is eventually shown to improve outcomes after stroke, questions will remain about the effects of such a strategy on microbial resistance patterns and hospital-acquired infections (e.g., *Clostridium difficile*).

In accord with the possible role of the SNS, intriguing clinical observations associate beta-blocker use with lower rates of pneumonia and mortality after stroke, but given the sparse nature of these data and the pleiotropic effects of beta-blockers, including effects on blood pressure, further research will be required to determine the usefulness of such widely available drugs to modulate the immune response after stroke.

Inhibition of glucocorticoids improved immune cellular parameters but failed to prevent infection in a mouse model of stroke [157]. However, the evidence for SNS and PNS immune suppression is weaker in patients, and it is difficult to see how the nervous system induces rapid suppression of circulating blood cells. It is also clear that cortisol is extremely potent in reducing immune activation and the possibility that this axis may be of some value as a therapeutic target for preventing or

reversing immune suppression has been identified by at least one drug company in respect of a patent covering cortisol antagonists for this purpose [173].

A better understanding of the interactions between the immune system and the brain will potentially result in more effective treatments of the injury and identify targets for reducing susceptibility to infection. In the meantime, physicians should be cognizant of the immunosuppressed state of their patients with stroke and should remain vigilant to expeditiously identify and appropriately treat infections in these patients.

7 Late Phase: Resolution and Repair

Inflammation is a basic mechanism by which organisms and tissues respond to injury. The inflammatory response eliminates pathogens or noxious agents and also clears debris, prior to the restoration of tissue integrity and function. The potential benefits of inflammation after stroke have received relatively little attention so far, but indirect evidence suggests that specific inflammatory reactions are neuroprotective and neuroregenerative. However, these benefits may be offset, at least partially, by the *bystander toxicity* of inflammation and by scar formation. A connective tissue response is crucial to wound closure in peripheral tissues, and fibrosis may be an important and relatively benign consequence in peripheral tissue, but is a major impediment to regeneration and plasticity in the brain. A recent example of the dual nature of ischemia-induced inflammation is the discovery of the regenerative role of MMP activation. Previously, these enzymes were almost exclusively linked to BBB disruption and lesion growth, but there is also evidence that MMPs have an important role to play in neurovascular remodeling [174].

7.1 The Role of Inflammation in Repair

Increasing evidence suggests that the resolution of inflammation is not a passive process, but is orchestrated by the interplay of a large number of mediators which actively suppress the inflammatory response. Major steps in the repair process include the removal of dead cells, development of an anti-inflammatory milieu, and generation of pro-survival factors fostering tissue reconstruction and repair [175, 176]. Microglia and infiltrating macrophages constitute the predominant phagocytes removing dead cells and tissue debris after stroke, a process orchestrated by *find-me* and *eat-me* signals [177]. *Find-me signals*, including purines released from injured cells and chemokines, attract microglia and macrophages to the site of injury [178, 179]. These phagocytic cells are then presented with *eat-me signals* associated with dying or dead cells. TGFβ and IL-10 are pleiotropic immunoregulatory cytokines that play a crucial role in the development of the anti-inflammatory milieu associated with tissue repair [176]. The production of these

cytokines is promoted by phagocytosis and occurs in concert with the removal of dead cells. TGFβ is upregulated after ischemia primarily in microglia and macrophages and, in addition to its neuroprotective properties, also has profound effects on immune cells. Although well known for its proinflammatory effects, TGFβ can suppress inflammation by inhibiting Th1 and Th2 responses and promoting Treg cell development [180]. Similarly, the immunoregulatory cytokine IL-10, produced by different cells, including Treg cells, has both neuroprotective and anti-inflammatory activities [110]. Therefore, postischemic production of TGFβ and IL-10 can facilitate tissue repair by promoting the resolution of inflammation and exerting direct cytoprotective effects on surviving cells in the ischemic territory.

Immune cells and inflammation play an important role in tissue repair and reorganization. These beneficial effects have to be considered in developing therapeutic approaches based on restraining postischemic inflammation. The concern is that counteracting the inflammatory response to ischemic injury may ameliorate the tissue damage in the acute phase, but it may compromise repair mechanisms and worsen the long-term outcome of the injury. Due to the paucity of experimental studies in the recovery phase, there is no definitive experimental evidence that anti-inflammatory treatments interfere with repair processes in the postischemic brain. The essential role of inflammation in tissue repair highlights the difficulties with approaches based on full-blown suppression of inflammation. A clear example of the duality of inflammation is characterized by IL-6, which is a major cytokine induced during the acute phase of stroke but which is a member of the neuropoietic family of cytokines, which have demonstrated the ability to promote neuronal growth and development [181]. Furthermore, in light of stroke-induced immunosuppression, the infectious complications of therapies suppressing inflammation also need to be taken into account.

Therapies based on immunomodulation, in which the overall immune response is deviated from a Th1- to a Th2-type response, also have a dark side. A similar worsening in the chronic phase has also been reported in tolerization applied to models of cerebral ischemia. Therefore, the delayed effects of humoral immunity could counteract the short-term benefit of suppression of cellular immunity. A more complete understanding of the immunology of stroke would enable the development of targeted approaches to selectively suppress the deleterious effects of inflammation. Postischemic production of growth factors helps to establish an environment that is favorable to neuronal sprouting, neurogenesis, angiogenesis, gliogenesis, and matrix reorganization [182–184]. Inflammatory cells, as well as neurons and astrocytes, are capable of producing a vast array of growth factors. For example, microglia are required for the full expression of insulin-like growth factor 1 (IGF-1) [185], a critical factor in postischemic neuronal sprouting, whereas reactive astrocytes are required for functional recovery after stroke. Vascular endothelial growth factor (VEGF), a key growth factor in postischemic angiogenesis, is produced by reactive astrocytes [186], and its action may require neutrophil MMPs, suggesting a link between inflammatory cells and angiogenesis [187]. However, VEGF administration early after ischemia, or in excessive doses, may enhance the damage [188, 189].

The role of inflammatory signaling in brain recovery has also been highlighted by studies in which the transcriptome of sprouting neurons was defined indicating involvement of MHC class I molecules and complement subunits [190]. Generally there is an increased astrocytic reaction following injury to the aging CNS [191]. Tissue morphology at 1–2 weeks after stroke in aged rats is similar to that at 4 weeks in young rats; i.e., in both instances a scar-like formation is noted, fully penetrated by reactive astrocytes. Also noted is a strong activation of oligodendrocytes, seen at early stages of infarct development in both young and aged rats that persisted in older rats, suggesting the formation of an even stronger barrier to regeneration-like phenomena. The early formation of the glial scar may impede functional recovery in aged rats. The temporal correlation between an increase in glial activity after stroke in aged rats and the decline in function lends support to this hypothesis [191].

The evidence presented above indicates that cells of the immune system serve a fundamental role in all the phases of postischemic brain recovery. But, the limited data available provide only a glimpse into the complex sequence of events that reestablish the structural and functional homeostasis of the brain after cerebral ischemia. Additional investigations of recently identified mediators instrumental to inflammation resolution and tissue repair, such as lipoxins, resolvins, protectins, progranulins, and cyclopentenone prostaglandins, are needed to fully elucidate the role of the immune system in brain repair after stroke [42].

7.2 The Role of Inflammation in Perpetuating Damage: Early Onset of Dementia

It is conceivable that sensitization to CNS antigens plays a role in the long-term outcome of stroke. Statistical analyses reveal that more than 60 % of people with stroke have some degree of cognitive impairment with a significant number having clinical dementia. Similarly, over 30 % of postmortem brains of patients with dementia also show cerebrovascular pathology resulting in a chronic mild cerebrovascular dysfunction, which may have originated as a pathological consequence of ischemic stroke or cerebral small vessel disease/silent strokes as seen in lacunar stroke [192]. Inflammation is a major debilitating feature of stroke, accounting for the majority of infarct volume, facilitated by increased vascular permeability, activation, and damage, and importantly, this inflammation, particularly the activation of cell death-mediating cytokine-induced signaling through NFκB, is known to be strongly linked to the onset of cognitive impairment. The common mediator between neurodegeneration and stroke toxicity is now thought to be neuroinflammation.

Evidence of reduced BBB integrity preceding other neurodegenerative disorders, such as Alzheimer's disease (AD), provides a strong link between cerebrovascular pathology and AD. In animal models, amyloid-β peptide-injected animals exhibited a commonality in perturbations of microvessels compared with those evident in AD brain [193]. It was suggested that amyloidogenesis promotes extensive

neo-angiogenesis leading to increased vascular permeability and subsequent hypervascularization in AD. In patients hypervascularity was corroborated in a comparison of postmortem brain tissues from AD. Brain microvessels derived from patients with AD expressed numerous factors implicated in vascular activation and angiogenesis. Signaling cascades associated with vascular activation and angiogenesis are also upregulated in AD-derived brain microvessels [194]. Original small vessels develop arteriosclerotic characteristics, weak and permeable vessel walls, and are associated with increased inflammation, while newly formed blood vessels are often immature, abnormally shaped, and nonfunctional or with blind endings creating an environment of hypoxia and continued proliferation of proinflammatory cytokines.

Current data therefore suggests a new paradigm for integrating vascular remodeling with the pathophysiology observed in AD and indeed vascular dementia [195, 196]. These vessels are demonstrated histopathologically to persist in the active state, showing expression of adhesion molecules and interleukins, making them ideal disseminators of inflammatory reaction [197]. Demonstrating the possible therapeutic benefit of amelioration of endothelial dysfunction and inflammation, Whitehead et al. [198] showed a significant protection against AD markers of pathology (e.g., APP and Tau and NFκB near to Aβ plaque deposition) following reduction of inflammation using triflusal in a combined mouse model of AD and stroke. Furthermore, although poststroke immunosuppression is deleterious in that it increases the incidence of infections [153, 158], acute infection could also negatively affect stroke outcome by upregulating co-stimulatory molecules and promoting antigen presentation [69].

Bacterial lipopolysaccharide (LPS) administered at the time of reperfusion to simulate poststroke infection worsens the outcome of experimental stroke [199] and increases postischemic brain atrophy assessed 1 month after stroke [200, 201]. This effect is associated with T-cell sensitization against CNS antigens and a Th1 cytokine response due to an increased expression of B7.1, a co-stimulatory molecule needed for efficient antigen presentation [200, 201]. According to these experimental data, poststroke immunosuppression not only increases the incidence of systemic infections, but at the same time, it could be beneficial by attenuating such delayed autoimmune response. Systemic infections can promote antigen presentation and autoimmunity against the brain, which may play a role in the long-term sequelae of the stroke.

8 Therapeutic Strategies

Recent studies have reported that targeting the inflammatory response to ischemic injury limits the expansion of the lesion and increases the survivability of neurons after stroke [43, 108, 202]. Molecular cues generated by cerebral ischemia activate components of innate and adaptive immunity that regulate both inflammatory signaling and protective immunity. Among the potential therapeutic approaches

targeting the ischemic cascade, preclinical studies in rodent models suggest that the suppression of inflammation offers unique advantages. First, these treatments could have an extended therapeutic window and appear effective in experimental stroke models. Therefore, they could be used in patients presenting too late for thrombolysis. Second, because suppression of inflammation is also beneficial in models of cerebral hemorrhage, immune-modulating agents offer therapeutic opportunities in hemorrhagic stroke and might therefore be a viable option in prehospital stroke prior to brain imaging. Thirdly, considering that inflammation may be particularly deleterious in ischemia associated with reperfusion, suppression of inflammation may be a fitting complement to reperfusion therapy using thrombolysis or intravascular thrombectomy.

Although these considerations are primarily based on animal models, which may not model in full the human disease, as mechanisms of inflammation may differ [203], inflammation is a critical pathogenic component of human stroke and remains an attractive target for therapeutic intervention. However, many drugs with anti-inflammatory properties that have proven effective in experimental models have failed to improve outcome after ischemic stroke in patients. Several reasons may explain this discrepancy [204], including use of many different stroke models (different species, permanent or transient occlusion, differing representation of gray vs. white matter). Until recently, animal studies have generally been performed in healthy young male rodents, lacking comorbidities relevant to the clinical setting (e.g., diabetes, hypertension, and infection), which impact on the ischemic cascade and inflammatory responses [205]. However, in terms of therapeutic strategies, modulation of later events like inflammation might be considered to have greater chance of success than neuroprotective agents that target much earlier steps in the ischemic cascade, such as excitotoxicity. Given the strong neuroinflammatory reaction in the brain after stroke, several studies have focused on interventional therapies that could potentially diminish inflammation. For example, intravenous immunoglobulin (IVIG) protects the brain against experimental stroke by preventing complement-mediated neuronal cell death [206].

Despite extensive programs of research to date, no therapies modulating immune/inflammatory mechanisms have translated successfully from experimental research to clinical practice (Table 3) [8]. For example, blocking antibodies directed against adhesion molecules (ICAM-1, MAC-1), or recombinant neutrophil inhibitory factor, have not been effective in clinical trials. The case of the Enlimomab Acute Stroke Trial, a phase III clinical trial of a murine monoclonal antibody to ICAM-1, led to important lessons in translational research [207]. The negative outcome of this trial has been attributed to deleterious immunoactivation resulting from the administration of a mouse antibody to humans, as reproduced in an experimental study in which murine antibodies to rat ICAM-1 were administered to rats [208]. Although there also might be other reasons for these failures, a likely contributing factor is that postischemic inflammation acts through multiple redundant pathways that cannot be effectively suppressed by blocking a single cytokine or adhesion molecule, as attempted in these clinical trials. Thus, neutralizing upstream mediators of the signaling cascade or blocking multiple inflammatory pathways might be

Table 3 Neuroprotective agents targeting neuroinflammation in acute stroke [8]

Neuroprotective agent	Mode of action	Summary of clinical trials
Recombinant human IL-1 ra (rhIL-1ra)	Interleukin-1 receptor antagonist	In the phase II clinical trial of rhIL-1ra, patients within 6 h of stroke symptom onset were randomized to either intravenous rhIL-1ra or placebo. In the rhIL-1ra-treated group, patients with cortical infarcts had a better clinical outcome. Further phase II trials of subcutaneous rhIL-1ra in ischemic stroke and subarachnoid hemorrhage are ongoing
Enlimomab	Anti-ICAM-1 monoclonal antibody	In the phase III clinical trial of enlimomab, patients were randomized to receive either the monoclonal antibody or placebo within 6 h of acute stroke onset. The modified Rankin scale was worse in patients treated with enlimomab ($p = 0.004$) and treatment was associated with higher mortality. Further development of this drug has been abandoned
UK-279, 276	Neutrophil inhibitory factor	In the Acute Stroke Therapy by Inhibition of Neutrophils (ASTIN) phase II clinical trial, patients were randomized to receive an infusion of either UK-279, 276, or placebo within 6 h of acute stroke symptom onset. No efficacy was reported on the administration of study medication and the clinical trial was terminated for futility
Cerovive (NXY-059)	Nitrone-based free radical trapping agent	The phase III clinical trial, Stroke–Acute Ischemic NXY-059 Treatment II (SAINT II) randomized patients within 6 h of acute stroke onset to an infusion of either NXY-059 or placebo. There was no significant reduction in stroke-related disability, as assessed by the modified Rankin scale ($p = 0.33$). The cerebral hemorrhage and NXY-059 Treatment (CHANT) trial also showed no treatment effect on functional outcome. Further drug development has been abandoned
Edaravone (Radicut)	Free radical scavenger	Lacunar stroke patients treated with edaravone showed significant reduction in infarct size at 1-year follow-up and early improved neurological outcomes. There was no difference in overall clinical outcomes after 1 year
Uric acid	Antioxidant	The phase II double-blinded study investigated safety and pharmacokinetics of uric acid in acute stroke patients treated with rt-PA. Levels of uric acid increased in the treatment group, with reduction in lipid peroxidation. No safety concerns were reported with uric acid treatment. Uric acid level showed a positive correlation with clinical improvement and was an independent predictor for favorable stroke outcome

(continued)

Table 3 (continued)

Neuroprotective agent	Mode of action	Summary of clinical trials
Acetaminophen (paracetamol)	Antipyretic effect	In the Paracetamol (Acetaminophen) in Stroke (PAIS) clinical trial, patients presenting within 12 h of acute stroke onset were randomized to either acetaminophen (6 g daily) or placebo for 3 days. There was no benefit seen for routine use of acetaminophen in acute stroke but post hoc analysis showed beneficial effects in patients with body temperature between 37 and 39 °C
Minocycline	Bacteriostatic antibiotic anti-inflammatory effects	Stroke patients with NIHSS >5 and symptom onset between 6 and 24 h were randomized to either once daily minocycline 200 mg or placebo for 5 days. The NIHSS and modified Rankin Scale were significantly lower in the treatment group at 90 days. The minocycline to improve neurologic outcome in stroke (MINOS) study was a dose-escalation trial, administering intravenous minocycline within 6 h of symptom onset. This was shown to be safe and well tolerated up to 10 mg/kg intravenous dosing

more effective. For example, blocking upstream components of inflammatory signaling, such as IL-1, complement, TLR, or scavenger receptors, is highly protective in experimental models. Blockade of IL-1, using treatment with its IL-1Ra, reduces infarct volume and parameters of inflammation in a range of experimental models of cerebral ischemia, including comorbid animals [209]. Treatment with IL-1Ra in patients with ischemic stroke [210] or subarachnoid hemorrhage [211] appears safe and is undergoing further evaluation in phase II trials in these patient groups. Furthermore, minocycline, an agent with multiple neuroprotective actions including broad anti-inflammatory properties, has shown promise in clinical trials [170, 212]. Another strategy is to develop approaches in which the immune system is directed to suppress the deleterious effects of inflammation while enhancing its protective potential.

Ischemic tolerance or preconditioning is a phenomenon in which a sublethal injurious stimulus protects an organ against a subsequent lethal stimulus. Ischemic preconditioning is a procedure whereby brief episodes of ischemia to the brain (local preconditioning) or other organs (remote preconditioning) are protective against a subsequent, more severe ischemic insult [41, 213, 214]. One factor that may mediate the neuroprotective effect of ischemic preconditioning is inflammation [215, 216]. For example, the administration of low doses of LPS protects the brain from ischemic damage [217]. Likewise, a mild systemic inflammation elicited prior to stroke in a rat model for periodontitis has a neuroprotective effect by reducing the infarct volume in a rat model for cerebral ischemia [218].

Although ischemic tolerance is well known to protect the brain by simultaneously suppressing multiple pathways in the ischemic cascade, modulation of the postischemic immune response has emerged as one of its key effector mechanisms [71, 219]. It was hypothesized that the reduction in the infarct volume was due to a reduction in the number of macrophage-like cells that when present cause an enlargement of the infarcted area [218]. Postischemic TLR4 signaling, redirecting toward production of IFNβ, reprograms the immune system to suppress the production of proinflammatory cytokines and the infiltration of inflammatory cells [220]. However, the full expression of the tolerance depends on several factors based on the conditions that stimulate NF-κB-dependent inflammatory mediators. The timing and threshold of IL-1, TNF, and their receptors, iNOS-derived NO and ROS, and the extent of microglial activation in specific brain regions are also required, indicating that the protection does not rely just on the suppression of deleterious inflammatory mediators, but on a fine balance between pro- and anti-inflammatory signaling [221–224].

One of the challenges, therefore, is to learn how to modulate the immune system to replicate the beneficial inflammatory milieu induced by preconditioning. Tolerization may provide the opportunity to achieve this goal. However, due to the need to establish tolerization prior to injury, this approach, like preconditioning, would be more appropriate for stroke prevention in high-risk patients than acute stroke treatment. One strategy could be the induction of immune tolerance through mucosal exposure to myelin antigens or E-selectin promoting a protective Th2 response through multiple pathways that suppress the deleterious effects of inflammation [103]. Another strategy is based on the administration of RTL. RTL suppresses the infiltration of inflammatory cells and provides neuroprotection even if administered after the onset of cerebral ischemia [104]. Similarly, the administration of the immunomodulatory copolymer poly-YE ameliorates neurological dysfunction without reducing injury volume, an effect attributed to increased production of growth factors and hippocampal neurogenesis [225]. However, the full translational potential of treatments based on immunomodulation has not been established.

Therapeutic window, efficacy in both sexes and higher-order species, aging, and the presence of cardiovascular risk factors need further exploration. The critical effector of the tissue damage in autoimmune diseases by IL-17-secreting T cells (Th17 cells) [226] and in cerebral ischemia (γδT cells) [108] raises the possibility that counteracting IL-17 could be beneficial in cerebral ischemia [226]. Similar strategies could also boost the protective roles of Treg, although a negative role of these cells has also been proposed [227]. Although, at the moment, the role of lymphocytes in ischemic injury is poorly understood and the full implications of suppressing the action of specific T- and B-cell populations remain to be defined, these approaches may be desirable because they target the delayed phase of the injury and have a particularly wide therapeutic window.

Hypothermia is another potent neuroprotective strategy that engages central and peripheral mechanisms leading to a marked reduction in ischemic brain injury in experimental stroke. Brain cooling has been reported to improve recovery from

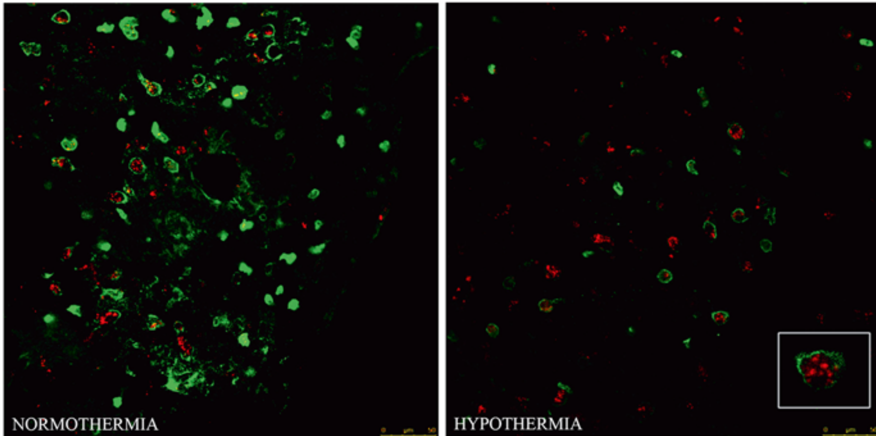

Fig. 2 Long-term hypothermia causes a reduction of inflammatory reaction. Exposure to hypothermia led to a large reduction in the number of phagocytic cells in the penumbra. Two-day exposure to hypothermia led to a large reduction in the number of phagocytic cells (ANX1-positive, green) in the penumbra. Nuclei are shown in *red*

traumatic brain injury (TBI) in rats [228] and from focal cerebral ischemia in baboons [229]. In animal studies of focal ischemia, short-term hypothermia consistently reduces infarct size [230–233]. However, efficient neuroprotection requires long-term, regulated lowering of whole-body temperature and detailed mechanistic analysis. The exposure of aged rats after stroke to H_2S-induced hypothermia for 48 h causes a 50 % reduction in infarct size without obvious neurological deficits or physiological side effects [234, 235]. In fact, animals recover within minutes after 48-h exposure, and behaviorally, rats kept under hypothermic conditions scored significantly better in tests that require complex sensorimotor skills, such as the rotarod and inclined plane tasks, suggesting a cytoprotective effect of prolonged hypothermia with subsequent beneficial effects on behavioral recuperation. Additionally, after the first week poststroke, rats kept under hypothermia began gaining weight at a higher pace than did control rats [234].

Transcriptionally, hypothermia was associated with decreased levels of inflammation-related mRNAs coding for caspase 12, NF-κB, and grp78. Unlike other caspases that have functions related to apoptosis, caspase 12 may be proinflammatory and is implicated in cytokine processing and the regulation of inflammation [236]. In addition to the role of caspases in the processing of cytokine precursors into mature, proinflammatory cytokines, new evidence supports a role for caspase 12 as a dominant negative regulator of inflammation [237]. We hypothesized that a reduction in the levels of caspase 12 leads to decreased levels of other inflammatory mediators such as NF-κB and grp78 mRNAs, as underscored by our finding that the number of phagocytic cells is reduced in the penumbra of rats subjected to prolonged hypothermia (Fig. 2) [234]. It should be noted that NF-κB is normally bound by the inhibitory protein, I-κBα, and sequestered in the cytoplasm.

Upon degradation of I-κBα, NF-κB translocates to the nucleus and initiates the activation of NF-κB signaling cascade. More recently, it has been shown that the loss of I-κBα in the brain worsened tissue damage following a TBI. Moreover, astrocytes, but not neurons, exhibit prominent NF-κB activity [238].

A recent comprehensive physiological, biochemical, immunohistochemical, and gene expression characterization of the brain response to long-term gaseous hypothermia identified annexin A1 (ANXA1) as a prominent target of the anti-inflammatory actions of H_2S-induced hypothermia in the peri-infarcted area of aged rat brains. Two days of hypothermia greatly diminished the number of co-localized PMN-ANXA1 cells [235]. These data identify ANXA1 as an important component of phagocytosis that appears to link actin accumulation to different stages of phagosome formation [239]. The anti-inflammatory role of ANXA1 has been explained by its ability to prevent the infiltration of neutrophils when they adhere to inflamed endothelium. In stroke, the leakage of neutrophils into the infarct site could follow two pathways: (1) directly from the bloodstream via the disrupted BBB or (2) infiltration of neutrophils that bind to the inflamed endothelium. Both mechanisms may be operating [240, 241]. Yet a third mechanism could be that hypothermia itself reduces the number of circulating leukocytes and may diminish their phagocytic capacity [242]. Similarly, mild hypothermia has been found to delay the migration of phagocytic cells toward the lesion in a rat model of stab injury [243]. Gaseous hypothermia utilizing H_2S enables precise temperature control that is achieved by the simple inspiration of a mixture of air and H_2S.

The disagreeable odor of H_2S can be circumvented by using injectable formulations that yield therapeutic doses of the gas, a number of which are already in clinical trials. The organism adapts to prolonged exposure to H_2S and increasingly counteracts the pharmacological effects of the gas by gradually raising both temperature and blood pressure. It thus appears that H_2S-induced hypothermia has a pleiotropic effect by (1) reducing the metabolic rate, (2) inducing a hibernation-like state, (3) reducing the epileptic forms of EEG activity, and (4) inducing a sleep deprivation state. Therefore, the ability of ANXA1 to control and contain inflammation may play a pivotal role in postischemic recovery. H_2S-induced hypothermia, by simultaneously targeting multiple points of intervention, could have a higher probability of success in treating stroke.

However, many questions still must be answered regarding the use of therapeutic hypothermia for ischemia in clinical practice, such as the H_2S concentration, optimal target temperature and duration, the therapeutic window in humans, and cost-effectiveness [244]. The concentration of H_2S used is well below the toxicity limit for humans (80 ppm). A more serious problem is the translation to animals with larger body size. Recently it has been reported that inhaled H_2S did not induce hypothermia in sheep or pigs [245]. However, the beneficial effects of H_2S may extend beyond the cooling effect. Therefore, a better understanding of the pathophysiology of the ischemic injury processes on which hypothermia acts will serve to further promote the use of this promising method to reduce the mortality and morbidity caused by stroke.

Although the relative importance of the many physiological effects of hypothermia in neuroprotection remains unclear, hypothermia improves neurological outcome in patients with cardiac arrest and in children with hypoxic–ischemic brain injury. Hypothermia counteracts excitotoxicity, inflammation, and apoptosis and promotes tissue homeostasis by producing growth factors and *cold shock proteins* endowed with cytoprotective and repair-promoting properties. Mild hypothermia (33 °C) in stroke patients receiving tPA is feasible, but did not improve outcome in one trial, possibly because too few patients were studied and cooling was started relatively late after stroke [246]. Moreover, hypothermia has also potentially detrimental complications, causing, for example, pneumonia and malignant cerebral edema during rewarming, which need to be controlled. Therefore, more studies and new strategies to minimize the complications of cooling and rewarming are needed.

The brain is endowed with a rich complement of central and peripheral defense mechanisms that are unveiled by acute injury, preconditioning stimuli, or exercise. Pharmacological interventions or other therapeutic approaches that reproduce or mobilize these coordinated neuroprotective programs could have a transformative effect on the treatment of ischemic stroke, which has remained stagnant for almost two decades. This would represent a paradigm shift in stroke therapy: from interventions targeting individual pathogenic mechanisms to protect neurons to interventions that engage multifunctional genetic and epigenetic programs directed at maintaining the homeostasis of the brain tissue as a whole. Therapies with cytokine antagonists, hematopoietic growth factors, minocycline, hypothermia, or remote post-conditioning are steps in this direction, but their efficacy in human stroke remains to be proven. Recently identified factors that contribute to the genomic response that confers ischemic tolerance, such as Polycomb proteins and sirtuins, raise the possibility of using epigenetic approaches to induce tolerance to cerebral ischemia. Although these treatments are still in the early preclinical stage, they offer the opportunity to enrich our armamentarium for the fight against stroke.

Future pharmacotherapy should focus on amelioration of vascular dysfunction in order to control both acute and chronic CNS/neuroinflammation. Current drugs under consideration include NSAIDs and multi-target-directed ligands (MTDL) [247]; however, a recent study investigated over 600 relevant trials demonstrating that interventions with aspirin, traditional nonsteroidal anti-inflammatory drugs, COX-2 inhibitors, or steroids had more side effects and a trend for higher death rates with a final recommendation against use in therapy for AD [248]. Hence, alternatives need to be considered and one of these may be calpain inhibitors.

Calpains are cysteine proteases produced in large quantity after brain injury or stroke. Inhibition of calpains is known to significantly reduce inflammation through blocking of NFκB [249] while protecting directly against beta-amyloid production and tau hyperphosphorylation as demonstrated in a mouse transgenic model of AD [250]. This dual mode of action could make them of particular interest in the treatment of vascular-based brain pathologies. Activation of cell signaling via microglia and astrocytes also plays a major role in stimulation of inflammation, involving intermediates such as TNF-α, MCP-1, adhesion molecules, and interleukins. Molecules with the ability to inhibit cytokine production from these cells could

also be therapeutically useful. For example, epigallocatechin-3 [251], arctigenin [252], and telmisartan [253] all effectively inhibited neuroinflammation and concomitantly improved memory deficiency, cognitive impairment, and/or neurological outcome in various in vivo models of AD and cerebrovascular disease. Furthermore, antibodies against beta-secretase cleavage site of amyloid precursor protein significantly improved cognitive abilities in conjunction with reduced neuroinflammation in a mouse triple transgenic model of AD [254]. Clinical trials involving specific neuroinflammatory modulators as protectors against development of AD are ongoing.

9 Conclusion and Overview

The relationship between the CNS and the immune system is complex and remains incompletely understood. It has particular salience after stroke and other forms of CNS injury, which trigger immune processes that seem to be both beneficial and harmful. A major frontier in stroke research involves efforts to better understand these interactions to develop new strategies and drugs that will prevent and reduce the burden of stroke. The realization that the immune system and inflammation are central to the pathophysiology of stroke has raised the prospect of new therapeutic approaches to counteract ischemic injury. However, our understanding of the cross talk between the immune system and the ischemic brain is still rudimentary and, as suggested by failed clinical trials, not adequate to guide therapeutic interventions. Modulation of adaptive immunity may afford the opportunity to deviate the post-ischemic immune response away from tissue damage and toward protection, an approach very effective in stroke models. However, immunomodulation can also have deleterious effects that need to be considered. Nevertheless, the remarkable impact that modulation of the immune system has on stroke damage and repair justifies the aggressive pursuit of basic and clinical investigations seeking to unravel the fundamental processes governing the interaction of the ischemic brain with the immune system. Learning how to mimic or engage endogenous neuroprotective mechanisms may provide new directions in stroke research and open new avenues in the treatment of this devastating disease.

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Part III
Infectious Diseases

HIV/NeuroAIDS

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Abstract Early after primary infection of human immunodeficiency virus (HIV), the HIV virus invades the central nervous system resulting in motor and cognitive dysfunction ranging from mild impairment to frank dementia. Chronic HIV infection can result in neurodegenerative disease, overall termed neuroAIDS. HIV infection leads to neuronal dysfunction and neurodegeneration via the activation of brain macrophages/microglia, the presence of viral proteins, and/or inflammatory factors generated in response to viral infection as well as alterations in glia and the blood–brain barrier. Although HIV does not productively infect neurons, neuronal injury and loss play a central role in the neurological decline and HIV-associated neuropathology. Early in the AIDS epidemic, patients exhibited high viral loads and elevated markers of immune activation as well as neurological dysfunction in cognition, motor performance, and behavior. Despite successful reduction of viral burden using antiretroviral therapies, the persistence of a milder less severe form of HIV-associated neurocognitive disorder (HAND) remains. The presence of several risk factors has been associated with the development of HAND, which include host factors, HIV disease and viral factors, comorbidity factors, as well as CNS penetrance and toxicity of cART. As HAND has been associated with increased mortality, decreased quality of life, and poor adherence to treatment, even this milder form of HIV-associated neurocognitive impairment is clinically relevant. This chapter on NeuroAIDS highlights the progression in the field and our current understanding of HIV infection of the CNS, HIV-induced neurodegeneration, and HAND pathogenesis.

Keywords Macrophage/microglia • Inflammation • Neurodegeneration • HIV-associated neurocognitive disorders • cART • CNS • Blood–brain barrier

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1 Introduction

Worldwide the human immunodeficiency virus (HIV) infects an estimated 35 million people [1], and in the United States over 1.1 million individuals are infected with HIV [2]. HIV invades the central nervous system (CNS) early after initial retrovirus infection and can result in neurodegenerative disease, overall termed neuroAIDS. The incidence of HIV-associated dementia (HAD) has been reduced via effective use of combination antiretroviral therapies (cART). However, despite the successful control of viral replication in the blood, a milder less severe form of HIV-associated neurocognitive disorder (HAND) persists, and poor prognoses continue to correlate with HIV infection of the nervous system. In fact, neurological complications are observed in approximately 30 % of persons with asymptomatic HIV infection and in more than 50 % of adults with acquired immunodeficiency syndrome (AIDS) according to the National Institute of Neurological Disorders and Stroke [3]. This chapter reviews the progression to our current understanding of the clinical features, neuropathology, pathogenesis, and treatments of neuroAIDS.

2 HIV Invasion of the CNS

In the mid-1980s, the “Trojan horse” hypothesis was adapted from studies on other lentiviruses and proposed to explain HIV entry into the CNS as a passenger in cells that are trafficking to the brain [4–7]. Evidence supports this model since HIV enters the CNS early in the course of infection soon after peripheral infection of circulating cells including T cells and monocytes, which express the major HIV receptors CD4 that aid attachment and entry of the virus into the cell [8]. In addition to CD4 receptors that are necessary for cellular infection, coreceptors are required, and such coreceptors have been identified as chemokine receptors. The coreceptor CXC-chemokine receptor 4 (CXCR4) appears to be the most important for HIV entry into lymphocytes, while CC-chemokine receptor 5 (CCR5) is important for monocytes, macrophages, and microglia [9]. HIV strains have been characterized for their varying phenotypes based on their usage of the CCR5 or CXCR4 coreceptors and are designated as R5- and X4-viruses, respectively [9]. Such coreceptor use can change in vivo, with initial infection occurring with R5 viruses but with the development of dual-tropic viruses that use both CCR5 and CXCR4 as well as viruses that utilize CXCR4 exclusively.

Brain infection, as it occurs in myeloid cells, is largely with R5 viruses. Infected $CD4^{+}$ T cells and monocytes can transmigrate the blood–brain barrier (BBB) from the peripheral circulation and propagate infection within the CNS [5, 10, 11]. Cellular migration into the brain through the BBB during HIV infection seems to be regulated by several factors including monocyte chemoattractant protein-1 (MCP-1), a microglial and astrocytic chemokine, and vascular cell-adhesion

molecule-1 (VCAM-1), an adhesion molecule [12, 13]. Additionally, the inflammatory cytokine tumor necrosis factor-α (TNF-α) has been suggested to play a role in HIV entry across the BBB into the brain [14]. Another possible mechanism of HIV neuroinvasion is the entry of cell-free HIV particles from the blood into the brain via migration between the brain microvascular endothelial cells [15, 16]. Upon entry into the CNS, infected cells come into direct contact with perivascular macrophages, microglia, and astrocytes. Monocytes differentiate into macrophages after establishing residence in the CNS, and this process allows for brain infiltration by intracellular HIV. In fact, the main cell populations responsible for productive HIV infection in the brain are monocyte-derived macrophages and microglia [17, 18]. The presence of multinucleated giant cells (MGNCs) in brain tissue is one of the characteristics of HIV encephalitis (HIVE) and, while often associated with HAD patients, is not itself pathognomonic. MGNCs are formed by the fusion of infected cells that express surface viral envelope glycoproteins with uninfected cells (likely a microglial and macrophage cell) that express CD4 and an HIV coreceptor [19]. Perivascular macrophages and microglia both express CD4 and the major HIV coreceptors CXCR4 and CCR5 supporting their role in MGNC formation [20].

3 Macrophages and HIV Infection

Macrophages are terminally differentiated, nondividing phagocytes, derived from circulating monocytes that play an important role in the innate and adaptive immune response [21]. Macrophages promote HIV infection through several distinct routes including (1) active production of HIV, (2) transmission of HIV to T cells, (3) recruitment of T lymphocytes to sites of infection via secretion of cytokines, and (4) facilitation of HIV entry into the brain [22–24]. Additionally, macrophages may act as HIV reservoirs, storing HIV particles in internal compartments [25]. The CNS provides an optimal HIV sanctuary (infection site which is difficult to reach by antiretroviral drugs) as it contains infected macrophages, which mediate long-term virus persistence [26].

As previously mentioned, HIV neuroinvasion is mediated by infected monocytes and macrophages crossing the BBB; these cells can then transmit the virus to brain macrophages, microglia, and possibly astrocytes [27]. Neurons are not infected. The presence of activated macrophages/microglia correlates strongly with the severity of neurocognitive impairment, suggesting that brain inflammation is a key driving force behind dementia. Activated macrophages/microglia secrete cytokines amplifying the population of activated cells and increasing neuroinflammation creating a vicious cycle [28, 29]. Additionally, several HIV proteins themselves activate macrophages/microglia and are neurotoxic in both cell-associated and soluble forms [30]. Although macrophages are suspected to play a pivotal role in the development of HAND, the precise viral and cellular factors responsible for HIV-induced neuronal damage remain obscure.

4 HIV-Induced Neurodegeneration

As discussed above, the primary cell types of the CNS that are productively infected by HIV are macrophages and microglia. Studies have found that HIV can infect astrocytes although these cells rarely produce virus [31, 32]. Astrocytes may function as HIV reservoirs, similar to macrophages, and transmit HIV to other cells in the CNS [33, 34]. Evidence for HIV infection of oligodendrocytes and neurons in vivo is lacking. However, significant neuronal injury and apoptosis can be observed in brains from HIV patients [35].

Two major hypotheses exist to explain how HIV-infected macrophages/microglia and perhaps astrocytes disrupt normal neuron function including (1) the direct injury hypothesis involving the production of neurotoxic viral proteins and (2) the indirect “bystander” injury hypothesis where neurons are damaged as a consequence of inflammation, through production of neurotoxic molecules by infected and/or activated nonneuronal cells [18]. The functional properties of viral proteins such as Tat, Vpr, Nef, and the envelope proteins gp120 and gp41, all of which can be secreted, have been shown to have neurotoxic properties [36]. Neurotoxic viral proteins have been shown to excessively stimulate neurons resulting in excitotoxicity, to disrupt neuronal membranes, or to induce apoptosis.

Neurons are not only damaged by exposure to HIV proteins, but indirect effects on neurons involve neurotoxic cytokines that are produced and released by activated glial cells (both infected and uninfected) [37]. Activated macrophages, microglia, and astrocytes produce proinflammatory cytokines including TNF-α, interleukin-1β (IL-1β), and interferon-γ, which promote inflammatory signaling cascades resulting in neuronal toxicity [27]. In the brain and/or cerebrospinal fluid (CSF) of HIV patients with HAD, increased expression of cytokines including transforming growth factor (TGF)-β, IL-1α, IL-1β, IL-6, and TNF-α has been reported [38, 39].

Chemokines and their receptors have also been implicated to mediate the pathogenesis of HAD due to their role in HIV entry, inflammatory processes, and normal neuronal physiology. The expression of chemokines and chemokine receptors has been observed to be altered in HIV-infected brains. Increased expression of β-chemokines and several receptors, such as CCR3 and CCR5, has been reported in the brains of HIV patients [40, 41]. The α-chemokines CXCL10 and CXCL12 are also found at increased levels in HIV-positive brains [42]. The neurodegeneration resulting from HIV infection is caused at least in part by dysregulation of inflammatory cytokines and chemokines and recruitment of immune cells (monocyte-macrophages) to the CNS. The activation of macrophages/microglia in the brain due to HIV infection, viral proteins, and/or inflammatory factors generated in response to viral infection leads to dysfunction of neurons as well as alterations in glia and the BBB, thus driving neuropathogenesis and the establishment of HAD.

5 From AIDS Dementia Complex (ADC) to HAND

In the 1980s early in the AIDS epidemic, neurological symptoms were identified in patients with advanced AIDS, who exhibited high viral loads and elevated markers of immune activation [43, 44]. The first systematic description of the CNS complications of AIDS occurred in 1983 in a case series of 50 patients, and the observation of possible AIDS-related dementia was noted [43]. Clinically, the AIDS dementia complex (ADC) was recognized as a novel pathology, and diagnosis was based on the pattern of neurological dysfunction in cognition, motor performance, and behavior [45]. The main characteristic is cognitive impairment, which results in mental slowness and poor memory and concentration. Motor dysfunction and behavioral changes can also be present.

While the progression of dysfunction is variable, if left untreated, patients become nearly vegetative, and the disease can be fatal. Pathological changes were most prominent in subcortical structures and included diffuse white matter pallor and astrocytosis, multinucleated cell encephalitis, and vacuolar myelopathy [46–49]. The first comprehensive study of HAND deficits in 1987 provided strong evidence of neurocognitive impairment across all stages of HIV disease from medically asymptomatic to AIDS [50]. Early reports of improvement of neurological disease in ADC patients by zidovudine monotherapy (AZT) showed that antiretroviral therapy might be effective in treatment of AIDS neurocognitive disease, and it suggested that HIV infection was indeed the cause of ADC [51]. Diagnostic guidelines were described by the AIDS Task Force of the American Academy of Neurology (AAN) in 1991 for classifying the neurological manifestations of HIV infection associated with two levels of severity: (1) HAD with motor, behavioral/psychosocial, or combined features and (2) minor cognitive motor disorder (MCMD) described as a less severe presentation of HIV-associated neurocognitive impairment that did not meet the criteria for HAD.

The advent of combination antiretroviral therapy (cART), which has decreased viral expression and allowed HIV-infected individuals to live longer with milder symptoms, contributed to the changing epidemiology of HIV infection requiring new criteria for HAND diagnosis published in 2007 [52]. Despite the restricted entry of many drugs to the CNS where infection formerly led to HAD, standard cART regimens have greatly decreased the incidence of severe HAD forms, and a shift to a higher prevalence of milder neurocognitive impairment in treated patients has occurred [53, 54]. The neurological manifestations of HIV infection are now referred to as HIV-associated neurocognitive disorders (HAND). Advances in neuroimaging techniques including magnetic resonance spectroscopic imaging (MRSI) have allowed for the detection of changes in the brains of HIV-positive individuals with milder subcategories of HAND, which include symptomatic minor neurocognitive disorder (MND) and asymptomatic neurocognitive impairment (ANI) [52, 55]; however, these imaging techniques are not themselves diagnostic, nor are there any other biomarkers available to diagnose HAND. Therefore, neurocognitive testing is necessary. Currently, the focus of neuroAIDS research has shifted from severe ADC to the milder forms of HAND, including ANI and MND.

6 HAND in the cART Era

Before the introduction of cART (commonly referred to as highly active retroviral therapy (HAART) regimens) in 1996, the prevalence of the devastating cognitive/motor disorder known as HAD (previously known as AIDS dementia complex) was estimated in the early 1990s to be as high as 20–30 % of individuals with advanced HIV disease. Although standard cART regimens have significantly decreased the incidence of HAD, HAND in its milder forms (ANI and MND) remains common [52, 53]. In fact, the prevalence of HAND remains high in the cART era despite effective viral suppression in CSF and plasma [56]. The development of the milder forms of HAND has been explained to occur as a result of slow progressive neurodegeneration due to low-level viral replication, longer survival of patients, and insufficient CNS penetration of certain antiretroviral drugs [57, 58]. Several lines of reasoning have been proposed to explain the persistence of HAND despite successful cART including the presence of the following risk factors associated with the development of HAND: (1) host factors, (2) HIV disease and viral factors, (3) comorbidity factors, and (4) CNS penetrance and toxicity of cART, each of which will be discussed in detail, and the differences occurring following the introduction of cART are illustrated in Fig. 1.

6.1 Host Factors

In the early years before the introduction of antiretroviral therapy, HIV replication was uninhibited and progressed to AIDS with few patients surviving beyond 1–3 years. The majority of AIDS patients were young adults with profound immunodeficiency, and the cause of death was related to underlying CNS pathology in about 35 % of the autopsies, second to pulmonary failure [55]. The advent of cART resulted in improved longevity of patients and older individuals (≥50 years of age) now comprise over 25 % of HIV-positive patients in the United States. The diagnosis of AIDS is often overlooked in older HIV-infected individuals; thus, they are more likely to have higher plasma viral load at HIV diagnosis and tend to progress to advanced AIDS more rapidly [59]. Several features of HIV infection are risk factors for developing Alzheimer's disease including immune dysfunction, inflammation, and hyperlipidemia in the elderly population [60, 61]. Increased age is a strong risk factor for HAND and combined with the cognitive changes that occur in normal aging presents major difficulties in the assessment of neurocognitive impairment in HIV-positive seniors [60]. As patients live longer, neurocognitive impairment is further complicated by longer cumulative exposure to CNS HIV infection, the use of drugs of abuse, psychiatric disturbances, and normal neurocognitive decline with aging.

In addition, there is a well-documented phenomenon of immune reconstitution inflammatory syndrome (IRIS), in which the now recovering immune system in

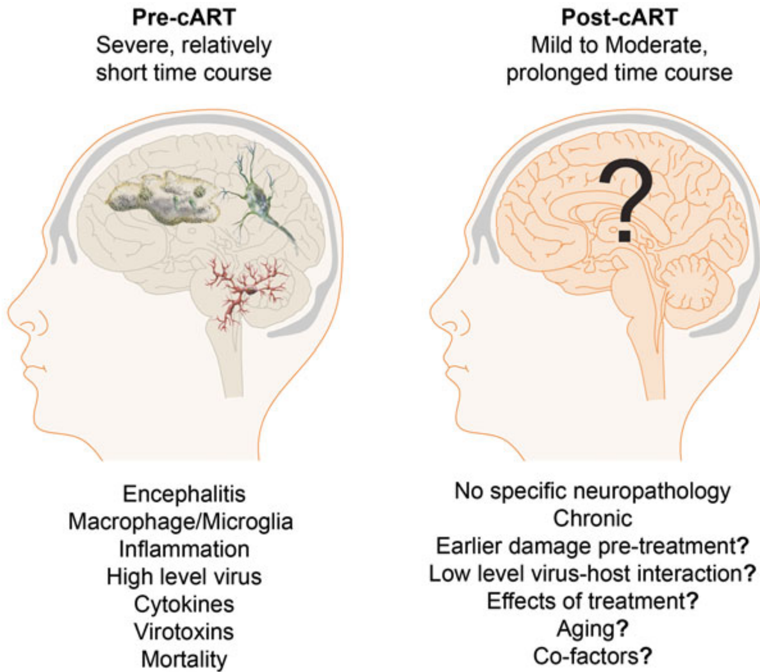

Fig. 1 Pathogenic mechanisms leading to HAND. Before the advent of antiretroviral therapy, the work of many helped unravel the processes contributing to encephalitis and the neurocognitive deficits and frank dementia that occurred in HIV-infected individuals (*left*). However, the nature of the insults that are responsible for less severe deficits now found is largely unknown (*right*)

cART-treated HIV-infected individuals shows a pronounced reactivity to an infection, and this immune reaction itself is damaging to the host. While these are often directed against opportunistic pathogens, IRIS can occur in the brain in the absence of such an opportunistic infection and may be directed against HIV itself in the CNS [62]. Both before and after the introduction of cART, neurocognitive impairment has been linked to the nadir CD4 cell count [63–65], leading to the possibility of a "legacy effect" in that current neurocognitive dysfunction is due to permanent damage linked to events that occurred during earlier immunosuppression. Still it remains unclear whether immune restoration and/or the "legacy effect" is a major factor in the persistence of HAND despite successful cART.

However, polymorphisms that modify the function or expression of immune response genes and factors as well as neurotransmitters have been identified that may affect the progression to neurocognitive impairment [66–69]. Host genetics therefore can play a role in the progression of neuroAIDS, and in the current era of cART treatment, studies are needed to assess the role of these or other genetic factors.

6.2 HIV Disease and Viral Factors

The progression of HIV disease is correlated with the development of neurocognitive disorders even in the cART era. Therefore, early initiation of cART may reduce the risk of developing HAND and prevent early CNS injury, and studies are beginning to show a protective effect of early initiation of therapy [70]. Extensive genetic variation of the HIV virus exists within individual hosts, making HIV one of the most rapid evolving viruses to date. In fact, most infected cells contain two or more different proviruses [71]. This within-host genetic viral variation poses a problem for successful clinical treatment since the HIV virus shows strong positive selection resulting in mutations to evade the host immune response [72]. The emergence of HIV strains capable of using coreceptors other than CCR5 such as the X4 strain seems to occur later in infection [73].

The evolution of HIV is also seen among different hosts. The different subtypes of HIV virus show different fitness levels and rates of neurovirulence. Some data suggest that some HIV clades might be less (clade C) or more (clade D) neuropathogenic and clade D is associated with an increased risk for dementia compared to clade A [74]. Recombination of different subtypes might accelerate disease progression and provide mechanisms to evade drug therapy, vaccine treatment, and/or immune pressure [75, 76]. However, as different clades are predominant in different geographical areas throughout the world, it can be difficult to control for the myriad other factors that can affect CNS disease other than the clade itself.

Clearly, the use of cART in reducing viral load has been the most important advance in decreasing the severity of CNS disorders. The development of drug resistance in HIV has been a setback in AIDS treatment. HIV reservoirs such as the CNS play an important role in this process by replenishing the main pool of replicating virus even long after cART initiation [77]. The rapid evolution of drug-resistant HIV mutants is facilitated by the accumulation and exchange of drug-resistant mutations between viruses from different reservoirs [78]. These factors must be considered as the field moves forward with treatments for HIV, and the CNS reservoirs remain important targets for drug design.

6.3 Comorbidities

The use of substances of abuse and/or presence of psychiatric illness, which themselves are associated with neurocognitive impairment, has been suggested to explain the high prevalence of HAND in the cART era. Drug abuse is a major cause of morbidity in young adults, and this population is highly susceptible to contracting HIV infection. HIV-infected drug abusers have higher viral loads, increased immunosuppression, and experience more severe neurological disease [79]. The degree to which drug abuse contributes to the neurocognitive and motor dysfunction that is observed in HIV patients is largely unknown since drug histories are often not reliable, and drug abusers are frequently noncompliant with medications [80]. Drugs

commonly used by HIV-infected individuals include cocaine, methamphetamine, and opioids, all of which experimentally can potentiate HIV replication and augment the neurotoxic effects of HIV viral proteins [81, 82]. For example, the development of HAND has been linked to the effects of cocaine and methamphetamine on increased dopamine, HIV replication, and macrophage infiltration [81]. Additionally, cocaine and methamphetamine disrupt endothelial cell tight junctions enhancing monocyte migration across the BBB, increasing the ability of HIV to enter the brain [83]. Coinfection with other viruses such as hepatitis C (HCV) is also a risk factor since it is associated with neurocognitive dysfunction [84].

6.4 CNS Penetrance and Toxicity of cART

One of the factors that may be resulting in the high prevalence of HAND is inadequate CNS penetrance of cART. Studies have revealed that antiretroviral drugs that have greater ability to enter the brain through the BBB can improve neurocognitive function [85]. These cART regimens with high CNS penetration effectiveness (CPE) scores correlate with lower CSF HIV RNA levels and have been termed neuro-HAART [86]. Concerns have been raised about the possibility of neuro-HAART toxicity contributing to the persistence of HAND despite the proven neuropsychological benefits. Studies have shown that nucleoside reverse-transcriptase inhibitors can disrupt mitochondrial function, and nucleoside analogs are known to cause mitochondrial toxicities [87, 88]. Despite greater benefits in neuropsychological function over other treatments, a quadruple nucleoside-analog regimen resulted in an increased level of myoinositol/creatine ratio, a metabolic marker of increased cerebral microglial activation, which is associated with HIV cognitive dysfunction [88]. Indirect toxicity of cART is also proposed to raise common HIV inflammatory markers [89]. The improvement of CNS penetrance will be important in order to deplete viral reservoirs and maintain viral latency in the CNS, but the possibility of toxicity will have to be addressed.

7 Basis for HAND in the cART Era

Before the advent of treatment, especially in the setting of HIVE, prolific work was performed on brains of patients with HAD, as well as on in vitro and animal models, demonstrating untoward effects of a number of cytokines, other molecules produced during inflammation, and viral proteins on the brain, neurons, and glia. However, in the largest study to date on neuropathological analysis of brains from neurocognitively characterized HIV-infected individuals concluded that there was "no observed relationship between parenchymal HIV brain pathology and HIV-associated neurocognitive disorder [90]." Instead, a correlation was found with Alzheimer's type II gliosis (the presence of a type of enlarged astrocyte thought to be metabolically

hyperactive) and minimal nondiagnostic abnormality, a term that certainly connotes a lack of specific mechanistic pathogenesis. While a recent study did identify a link between latent HIV infection (presence of HIV DNA and not RNA) and neurocognitive abnormalities, another study found that it was the brain HIV RNA that correlated best [91], with a lower effect found for the level of brain HIV DNA [92]. In this regard, it is of interest to point out that the viral protein Tat, highly linked to neuropathogenesis in many studies [93], can continue to be produced by HIV-infected macrophage/microglia even in the presence of antiviral therapy.

Clues from recent gene array studies of brains from HIV-infected individuals focused on those with more severe CNS abnormalities. In one, a distinct effect of cART treatment was found in those with neurocognitive impairment in reducing abnormal gene expression; however, remaining altered genes were involved in a number of pathways including immune responses [94]. In a second study, two different types of gene expression patterns were found in those with significant neurocognitive impairment, one associated with HIVE and the other in the absence of HIVE, the latter of which showed changes in transcriptions associated with endothelial cells [95]. Data from this second study was used in another analysis, which revealed similar altered pathways in HAND and Alzheimer's disease [96].

The work performed on brains with HIVE as well as its in vitro and animal models have provided a framework for the current knowledge and research on HAND in the era of cART. Lessons from the effects of the virus, inflammatory molecules, and subsequent damage to neurons could indeed still underlie the neuropathogenesis, however instead of high levels for a small number of months or years, much lower levels for decades. While speculative, alterations in the endothelial cells of the BBB, an ongoing, low-level CNS immune response to HIV (as well as effects of viral proteins such as Tat) remaining in the brain, residual continued glial activation, and/or mechanisms associated with aging-related neurodegenerative disorders may provide additional potential avenues to explore in determining the pathogenesis of HAND in the era of cART.

8 Conclusions

In this review, we have highlighted the developments in the neuroAIDS field that have led to our current understanding of HIV infection of the CNS, HIV-induced neurodegeneration, and HAND pathogenesis. However, much of our knowledge comes from studies related to HIVE and HAD, and continued research is needed which aimed to elucidate the molecular mechanisms underlying neuronal dysfunction in the current era of cART treatments. Furthermore identification of risk factors associated with the development of HAND is necessary in order to develop means to prevent and treat neuroAIDS. As the CNS provides a reservoir for HIV during the cART era and the continued presence of HIV in the brain can be linked to HAND, it is crucial to develop means of treatment to control and hopefully eliminate HIV from the brain.

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Herpes Simplex Virus Infections of the Central Nervous System

Richard J. Whitley

Abstract Herpes simplex virus infections of the central nervous system are associated with significant morbidity in spite of efficacious antiviral therapy. Herpes simplex virus, type 1 (HSV-1), causes focal neurologic findings that are characteristic of temporal lobe localization. Herpes simplex encephalitis occurs in a biphasic age distribution with one-third of the cases less than 20 and the majority of remaining cases over 50. The diagnostic test of choice is the detection of HSV DNA by PCR in the cerebrospinal fluid. Acyclovir is the treatment of choice and is administered for 14–21 days intravenously at a dose of 10 mg/kg every 8 h. Neonatal HSV infections are more frequently caused by HSV-2 than HSV-1, although the number of cases of the latter is increasing. Infection is most frequently acquired intrapartum by contact with infected maternal genital secretions. Approximately 50 % of all newborns with neonatal infection will have central nervous system involvement. Importantly, HSV-2 infections of the central nervous system in neonates have a poorer outcome than those attributable to HSV-1. Therapy of neonatal infection is achieved with high-dose acyclovir that is administered at 20 mg/kg/every 8 h for 14–21 days. Six months of oral acyclovir post-intravenous treatment has resulted in an improved neurologic outcome for children with central nervous system infection. Likely, in the future, combination antiviral approaches will be employed for both adult and pediatric disease in order to improve neurologic outcome.

Keywords Herpesvirus • Herpes simplex encephalitis • Acyclovir • Polymerase chain reaction • Latency • Neonatal herpes virus infection

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1 Introduction

Eight herpesviruses routinely cause human disease. There are three subfamilies: alpha (herpes simplex virus 1 (HSV-1), HSV-2, and varicella-zoster virus (VZV)), beta (cytomegalovirus (CMV), human herpesvirus-6 (HHV-6), and HHV-7), and gamma (Epstein–Barr virus (EBV) and Kaposi sarcoma herpesvirus (i.e., HHV-8)). Members of the alpha herpesvirus subfamily are characterized by a very short reproductive cycle, prompt destruction of the host cell, and ability to establish latency, usually in sensory ganglia. Its two leading members—HSV-1 and HSV-2 as causes of CNS disease—are the subject of this chapter.

2 History

Infections caused by HSV have been recognized since the time of ancient Greece. Greek physicians used the word *herpes* to mean "creeping" or "crawling" in reference to skin lesions. Likely, this word was used to describe various skin conditions ranging from cancer to shingles and probably even fever blisters. The Roman scholar Herodotus associated mouth ulcers and lip vesicles with fever [1]. He called this event *herpes febrilis*. Genital herpetic infections were described first by Astruc, a physician to the French royalty [2].

The transmissibility of these viruses was established unequivocally by passage of virus from human lip and genital lesions to either the cornea or the scarified skin of the rabbit [3]. Goodpasture [4] further demonstrated that material derived from the lesions of herpes labialis consistently produced encephalitis when inoculated onto the scarified cornea of rabbits.

Since the first suggestions of herpes simplex encephalitis (HSE) by the Mathewson Commission in 1926 [5] and subsequent description of the histopathologic changes [6], HSV is reported as the most common cause of sporadic fatal encephalitis in the United States [7]. Intranuclear inclusion bodies consistent with HSV infection were first demonstrated in the brain of a neonate with encephalitis [6] in 1941, as is described later in this chapter. Virus was subsequently isolated from this brain tissue [6]. The first adult case of HSE providing similar proof of viral disease (i.e., intranuclear inclusions in brain tissue and virus isolation) was described in 1944 [8]. The most striking pathologic findings in this patient's brain were apparent in the left temporal lobe, where perivascular cuffs of lymphocytes and numerous small hemorrhages were identified. This temporal lobe localization is characteristic of adult HSE, and it differs from the patchy diffuse encephalitis of neonates with HSV brain infection.

In the mid-1960s, Nahmias and Dowdle [9] demonstrated two antigenic types of HSV. Viral typing allowed the demonstration that HSV-1 was virtually uniformly responsible for herpes encephalitis in older children and adults. In contrast, infection of the newborn brain is attributable to HSV-1 or HSV-2 but more frequently the latter.

3 Pathology and Pathogenesis

Recent detailed reviews highlight the importance of these organisms as models of viral replication and as pathogens for human infection [10–13].

3.1 Pathology of CNS Disease

HSE results in acute inflammation, congestion, and/or hemorrhage, most prominently in the temporal lobes and usually asymmetrically in adults [14] and more diffusely in the newborn. Adjacent limbic areas show involvement as well. The meninges overlying the temporal lobes may appear clouded or congested. After approximately 2 weeks, these changes proceed to frank necrosis and liquefaction.

Microscopically, involvement extends beyond areas that appear grossly abnormal. At the earliest stage, the histologic changes are not dramatic and may be nonspecific. Congestion of capillaries and other small vessels in the cortex and subcortical white matter is evident; other changes include the development of petechiae. Vascular changes that have been reported in the area of infection include areas of hemorrhagic necrosis and perivascular cuffing. The perivascular cuffing becomes prominent in the second and third weeks of infection. Glial nodules are common after the second week [15, 16]. The microscopic appearance becomes dominated by evidence of necrosis and inflammation; the latter is characterized by a diffuse perivascular subarachnoid mononuclear cell infiltrate, gliosis, and satellitosis neuronophagia [14, 17]. In such cases, widespread areas of hemorrhagic necrosis, mirroring the area of infection, become most prominent. Oligodendrocytic involvement and gliosis (as well as astrocytosis) are common, but these changes develop very late in the disease. Although found in only approximately 50 % of patients, the presence of intranuclear inclusions supports the diagnosis of viral infection, and these inclusions are most often visible in the first week of infection. Intranuclear inclusions (Cowdry type A inclusions) are characterized by an eosinophilic homogeneous appearance and are often surrounded by a clear, unstained zone beyond which lies a rim of marginated chromatin.

3.2 General Observations on the Pathogenesis of Human Disease

The pathogenesis of human disease depends on intimate, personal contact of a susceptible individual (namely, one who is seronegative) with someone excreting HSV. Virus must come in contact with mucosal surfaces or abraded skin for infection to occur. With viral replication at the site of infection, the capsid is transported by neurons to the dorsal root ganglia, where after another round of viral replication, latency is established. Transport of the virion is by retrograde axonal flow [18]. In some instances, replication can lead to severe CNS infection; however, more often a host–virus interaction results in latency. After latency is established, reactivation

can occur, with virus shedding at mucocutaneous sites appearing as skin vesicles or mucosal ulcers or being completely asymptomatic. Occasionally, primary infection can become systemic, affecting other organ systems besides the CNS and the peripheral nervous system. Such circumstances include disseminated neonatal HSV infection with multiorgan involvement, multiorgan disease of pregnancy, and infrequently dissemination in patients undergoing immunosuppressive therapy. Multiorgan disease is likely the consequence of viremia in a host not capable of limiting replication to mucosal surfaces.

3.3 Pathogenesis of Latency

All of the herpesviruses have the ability to become latent, persist in an apparent inactive state for varying durations, and be reactivated by a provocative stimulus, as yet unidentified [11, 19–24]. As a biologic phenomenon, latency has been recognized since the beginning of the twentieth century [19, 21–23, 25–31]. In 1905, Cushing [32] noted that patients treated for trigeminal neuralgia (by sectioning a branch of the trigeminal nerve) developed HSV lesions along the innervated areas of the sectioned branch, as suggested previously by Goodpasture [33]. Several investigators have demonstrated that microvascular surgery of the trigeminal nerve tract for tic douloureux resulted in recurrent herpetic lesions in more than 90 % of seropositive individuals [34–37]. Axonal injury and attempts at excision of lesions have been associated with recurrences [38, 39]. Reactivation of latent virus appears to depend on an intact anterior nerve route and peripheral nerve pathways [40].

Recurrences occur despite both cell-mediated and humoral immune responses and can be either symptomatic or asymptomatic. Recurrences are spontaneous, but there have been associations with physical or emotional stress, fever, exposure to ultraviolet light, tissue damage, and immune suppression [23, 30, 41, 42]. Viral DNA can be detected in neuronal tissue in the absence of cutaneous lesions [22, 27, 43–48]. Latent virus has been retrieved from the trigeminal, sacral, and vagal ganglia of humans [19, 25, 26, 44, 48].

3.4 Pathogenesis of Encephalitis

The pathogenesis of HSE in older children (older than 3 months) and adults is only partly understood. Both primary and recurrent HSV infections can cause disease of the CNS. From studies performed by the National Institute of Allergy and Infectious Diseases (NIAID) Collaborative Antiviral Study Group (CASG), approximately one-third of the cases of HSE are the consequence of primary infection. For the most part, the patients with primary infection are younger than 18 years. The remaining two-thirds of cases occur in the presence of preexisting antibodies, but

only approximately 10 % of patients have a history of recurrent herpes labialis. Patients with preexisting antibodies are considered to have HSE as a consequence of reactivation of HSV [49]. When the DNA from the peripheral nervous system (labial) and CNS isolates are compared by restriction endonuclease analysis, the isolates are usually identical; however, this is not always the case. The virus isolated from the peripheral site can be different from that retrieved from the CNS [50]. Thus, the issues of reactivation of virus directly within the CNS, the potential for enhanced neurotropism of certain viruses, and the selective reactivation and access of one virus by the trigeminal route or other routes to the CNS remain for further elucidation.

The route of access of virus to the CNS in primary infection is a subject of debate, especially in humans. Studies performed more than five decades ago defined pathways for HSV access to the brain in animals, including both the olfactory and trigeminal nerves among others [51]. However, which of these nerve tracts uniformly leads to HSV infection in the CNS of humans is not clear. The anatomic distribution of nerves from the olfactory tract into the limbic system, along with the recovery of virus from the temporal lobe (the site of apparent onset of HSE in the human brain), suggests that viral access to the CNS via this route is a tenable hypothesis. Reports in the literature have found electron microscopic evidence that suggests this has been the case in some individuals with HSE [52–55]. Animal model data support the contention that the olfactory tract provides one neurologic avenue for viral access to the CNS and causes localization of the infection in brain regions analogous to medial temporal structures in humans [56, 57]. Definitive proof of such progression in humans is lacking.

Reactivation of HSV, leading to focal HSE, is a similarly confusing problem from the standpoint of pathogenesis. Evidence of latent virus within infected brain tissue exists [58]; however, virus reactivation at that site remains purely hypothetical. Reactivation of virus peripherally (namely, in the olfactory bulb or the trigeminal ganglion) with subsequent neuronal transmission to the CNS has been suggested [51, 57, 59, 60]. Nevertheless, a relevant observation is that with recurrent herpes labialis, whereby reactivation of virus from the trigeminal ganglia occurs, HSE is a very uncommon event. Furthermore, HSE does not occur more frequently in immunocompromised patients. In addition, individuals who are seropositive for HSV and have brain tissue examined for the detection of HSV DNA by PCR will have detectable DNA in multiple areas of the brain and not just localized to the temporal lobe [25].

Host immunity plays an important, but undefined, role in the pathogenesis of HSE. Possibly, the CNS is particularly prone to HSV infection because intraneuronal spread may shelter virus from host defense mechanisms. HSE is no more common in the immunosuppressed host than in the normal host; however, when it does occur, the presentation is atypical, with a subacute but progressively deteriorating course [61].

More recently, a host genetic deficiency has been found to play a role in recurrent HSE but certainly does not exist in all patients [62].

4 Epidemiology

4.1 Herpes Simplex Virus, Type 1

The epidemiology of HSV infections is multifaceted. Because the focus of this book is CNS inflammation and infection, only a brief review of non-CNS HSV infections follows. The reader is referred to more complete reviews [13, 63–65]. HSV infections are distributed worldwide and have been reported in both developed and developing countries, including remote Brazilian tribes [66]. Animal vectors for human HSV infections have not been described; therefore, humans remain the sole reservoir for transmission of these viruses to other humans during close personal contact. There is no seasonal variation in the incidence of infection. Because infection is rarely fatal, and because these viruses become latent, more than two-thirds of the world's population can have recurrent HSV infections and can transmit HSV during episodes of reactivation. HSV disease ranges from totally asymptomatic in most patients to sporadic, severe, and life-threatening disease in a few infants, children, and adults. With clinical illness, oropharyngeal disease, namely gingivostomatitis, usually is the manifestation. The identification of primary gingivostomatitis that was proven to be caused by HSV infection [67, 68] led to the definition of the natural history of infection, including the appearance of neutralizing antibodies [69], absence of virus shedding in children younger than 6 months [70], and a higher rate of occurrence among individuals of lower socioeconomic status. Contemporary surveys document the viral shedding data, ranging from 2 to 5 % [71–78].

Antibody surveys have helped clarify the epidemiology of HSV infection. Geographic location, socioeconomic status, and age all influence the acquisition of HSV infection [67, 79–82]. In developing countries, seroconversion occurs early in life. In Brazilian Indians, HSV antibodies are detectable in more than 95 % of children by the age of 15 years [83]. Similarly, serologic studies performed in New Orleans demonstrated acquisition of antibodies in more than 90 % of children by the age of 15 years [84]. In developing countries, such as Uruguay, or in lower socioeconomic populations in the central United States, the appearance of antibodies occurred at similar but lower frequencies [84–87]. By 5 years of age, approximately one-third of patients had seroconverted; this frequency increased to 70 to 80 % by early adolescence.

Middle-class individuals of industrialized societies acquired infection later in life. Seroconversion occurred during the first 5 years of life in 20 % of children; there was no significant increase until the second and third decades of life, at which time the prevalence of antibodies increased to 40 and 60 %, respectively [88, 89]. One study of university students demonstrated that seroconversion of susceptible individuals occurred at an annual frequency of approximately 5 to 10 % [90–92]. In summary, primary infection occurs very early in children of underdeveloped countries and in those of lower socioeconomic classes; however, in developed countries and more affluent classes, primary infection is delayed until adolescence or, perhaps, even adulthood. The frequency of direct person-to-person contact is the major mediator of acquisition of infection.

4.2 Herpes Simplex Virus, Type 2

Because HSV-2 infections are usually acquired through sexual contact, antibodies to this virus are rarely found before the age at onset of sexual activity. Although most genital HSV infections are caused by HSV-2, an ever-increasing proportion is attributable to HSV-1, now as high as 50 % of all new primary infections [93–97]. Approximately 1.5 million new cases of HSV-2 occur annually in the United States [98]. Genital HSV infections are not reportable in the United States [99]. Current estimates of infected individuals with genital herpes in the United States range from 40 to 60 million [99–101].

Women have the highest rates of infection, particularly prostitutes and others with multiple sex partners, including those with HIV infection. The incidence of genital HSV infections in both indigent women and those of middle and upper socioeconomic classes is significantly lower than the incidence found among women attending sexually transmitted disease clinics [102]. As with HSV-1 infections of the mouth, HSV-2 can be excreted in the absence of symptoms at the time of primary, initial, or recurrent infection [103, 104]. The actual frequency of asymptomatic excretion of HSV-2 in women by culture is approximately 3 to 5 % of all days, and by polymerase chain reaction (PCR) 15 to 20 %. Furthermore, some individuals can start and stop shedding multiple times during the same day [105]. Its occurrence creates a silent reservoir for transmission of infection [105, 106]. The appearance of HSV-2 antibodies reflects the time of exposure or more simply the acquisition of infection and is positively correlated with the onset of sexual activity [86, 87, 107]. However, crowded living conditions may indirectly contribute to antibody prevalence [108, 109]. If HSV-2 type-specific antibodies are sought in healthy women, there is a wide discrepancy in prevalence, ranging from averages of 10 % in England and Italy to 25 % in the United States and 77 % in Uganda [110, 111]. Up to 50 to 60 % of lower socioeconomic populations in the United States and elsewhere develop antibodies to HSV-2 by adulthood [11]. The reader is referred to a review for worldwide seroprevalence of HSV-2 [112]. Seroprevalence is a function of age, number of sexual partners, race, and marital status [113–115].

4.3 Latent Genital Herpes Simplex Virus Infections

Latent genital infection with subsequent reactivation is the largest reservoir for transmission of HSV-2. As with HSV-1 infection, recurrent HSV-2 infection can be either symptomatic or asymptomatic; however, recurrence is usually associated with a shorter duration of viral shedding and fewer lesions [93]. Several studies have implicated a frequency of recurrence as high as 60 % [107, 116]. The type of genital infection, HSV-1 versus HSV-2, is predictive of the frequency of recurrence [116–118], with HSV-1 infection recurring less frequently than HSV-2 [119, 120].

5 Herpes Simplex Encephalitis

5.1 Background

HSV infections of the CNS are among the most severe of all viral infections of the human brain. Currently, HSE is estimated to occur in approximately 1 per 250,000–500,000 individuals per year. At the University of Alabama at Birmingham, the diagnosis of HSE was proven by brain biopsy in an average of ten patients per year, for an incidence of approximately 1 in 300,000 individuals, an incidence similar to those in Sweden and England [121, 122]. With the advent of PCR for diagnostic purposes, HSE accounts for 10 to 20 % of viral infections of the CNS [123].

The economic cost of HSE is considerable, as estimated in 1983 for hospitalization alone of adults to be more than $25 million [124, 125]. The total medical cost is considerably higher because of the long-term care and support services required for many of the survivors.

HSE occurs throughout the year and in patients of all ages, with approximately one-third of cases occurring in patients younger than 20 years but older than 6 months and approximately one-half in patients older than 50 years [126]. Whites account for 95 % of patients with biopsy-proven disease. Both sexes are affected equally.

The severity of disease is best determined by the outcome of patients who have received either no therapy or an ineffective antiviral medication, such as idoxuridine or cytosine arabinoside. In such situations, mortality is in excess of 70 %; only approximately 2.5 % of all patients with confirmed disease (9.1 % of survivors) returned to normal function after recovery from their illness [127–131]. Because brain biopsy with isolation of HSV from brain tissue was the method of diagnosis in these early studies, a far broader spectrum of HSV infections of the CNS actually was thought to exist. However, with the more recent use of PCR for diagnosis of HSE, virtually all patients have a focal neurologic disease, suggesting a limited spectrum of disease [132].

5.2 Diagnosis

Several aspects relating to the diagnosis of HSE merit discussion: (a) the clinical presentation in regard to the sensitivity and specificity of various clinical characteristics, (b) the historical use of brain biopsy to establish the diagnosis, (c) conditions that mimic HSE, and (d) noninvasive means of diagnosis. Data from the NIAID CASG compare presentation and outcome for brain biopsy-positive and brain biopsy-negative patients [126].

Most patients with biopsy-proven HSE presented with a focal encephalopathic process, including (a) altered mentation and decreasing levels of consciousness with focal neurologic findings, (b) CSF pleocytosis and proteinosis, (c) the absence of bacterial and fungal pathogens in the CSF, and (d) focal electroencephalographic

(EEG), computed tomographic (CT), and/or magnetic resonance image (MRI) findings [126]. The frequency of headache and CSF pleocytosis is higher in patients with proven HSE than in patients with diseases that mimic HSE. Nearly uniformly, patients with HSE present with fever and personality change. Seizures, whether focal or generalized, occur in only approximately two-thirds of all patients with proven disease. Thus, the clinical findings of HSE are nonspecific and do not allow for empirical diagnosis of disease predicated solely on clinical presentation. Although clinical evidence of a localized temporal lobe lesion is often thought to indicate HSE, various other diseases can mimic this condition.

Examination of the CSF is indicated in patients with fever and altered mentation, provided it is not contraindicated because of increased intracranial pressure. In patients with HSE, CSF findings are nondiagnostic, being similar in patients with confirmed disease or diseases that mimic HSE. Both the CSF white blood cell (WBC) count (lymphocyte predominance) and the CSF protein level are elevated. The average CSF WBC count is 100 cells/μL; the protein averages approximately 100 mg/dL. Sequential evaluation of CSF specimens from patients with HSE indicates increasing cell counts and levels of protein. The presence of CSF red blood cells is not diagnostic for HSE and indeed is absent in 30 % of cases. Approximately 5 to 10 % of patients have a normal CSF formula on first evaluation.

Noninvasive neurodiagnostic studies support a presumptive diagnosis of HSE. These studies have included EEG, CT, and MRI. Focal changes of the EEG are characterized by spike and slow-wave activity and periodic lateralized epileptiform discharges, which arise from the temporal lobe [133–136]. Early in the disease, the abnormal electric activity usually involves one temporal lobe and then spreads to the contralateral temporal lobe as the disease evolves, usually over 7–10 days. The sensitivity of the EEG is approximately 84 %, but the specificity is only 32.5 %. CT scans initially show low-density areas with mass effect localized to the temporal lobe, which can progress to radiolucent and/or hemorrhagic lesions [137, 138]. Bitemporal disease is common in the absence of therapy, particularly late in the disease course. When these neurodiagnostic tests are used in combination, the sensitivity is enhanced; however, the specificity remains inadequate. None of these neurodiagnostic tests is uniformly satisfactory for diagnosing HSE. MRI detects evidence of HSE earlier than CT scan [139].

PCR detection of HSV DNA in the CSF has become the diagnostic procedure of choice. Brain biopsy is of value in confusing clinical presentations not been substantiated by follow-up studies of patients in the NIAID CASG.

5.3 Serologic Evaluation

Several strategies using antibody production as a means of diagnosing HSE have been utilized [71]. Because most encephalitic patients are HSV seropositive at presentation, seroconversion per se is usually not helpful because fever alone can reactivate labial herpes, resulting in antibody elevations. A fourfold rise in serum antibody was neither sensitive nor specific enough to be useful. A fourfold or greater

rise in CSF antibody occurred significantly more often within a month after onset of disease in patients with biopsy-proven HSE: 85 % versus 29 %. By 10 days after clinical presentation, however, only 50 % of brain biopsy-positive patients had a fourfold rise in CSF antibody. This test is useful only for retrospective diagnosis. The use of a ratio of serum to CSF antibody of 20 or less did not improve sensitivity during the first 10 days of disease.

5.4 PCR Detection of Viral DNA

PCR detection of HSV DNA in the CSF is the diagnostic method of choice [140–147]. Data from the NIAID CASG defined the sensitivity and specificity as 94 and 98 %, respectively. These CSF specimens were obtained from patients with biopsy-proven or biopsy-negative disease. Notably, the specificity would have been higher except that some tissue specimens were fixed in formalin, which killed infectious virus. HSV DNA persisted in 80 % of tested CSF specimens for 1 week or more.

5.5 Diseases That Mimic Herpes Simplex Encephalitis

In a compilation of the NIAID CASG data, 193 (45 %) of 432 patients undergoing brain biopsy for a focal encephalopathic process had HSE [148]. The remaining patients were evaluated for diseases that mimic HSE [148]. Thirty-eight had disease amenable to other forms of therapy, including brain abscess, tuberculosis, cryptococcal infection, and brain tumor. An additional 19 patients had diseases that were indirectly treatable, and another 38 patients had an alternative diagnosis established for which there was no current therapy, usually other viral infections. Thus, those diseases that mimic HSV infection of the CNS and that require immediate medical intervention should be considered if the PCR is negative for HSV DNA.

5.6 Therapy

The first antiviral drug reported as efficacious therapy of HSE was idoxuridine; however, it was soon proven both ineffective and toxic [127]. Subsequent therapeutic trials defined vidarabine as a useful medication for the management of biopsy-proven HSE [130, 131]; however, it has been replaced by acyclovir in the physician's armamentarium. During these studies, the variables of age, disease duration, and level of consciousness at the onset of therapy were proven major determinants of clinical outcome. Patients younger than 30 years and with a more normal level of consciousness (lethargic as opposed to comatose) were more likely to return to normal function than older patients, especially those who were semicomatose or

comatose. From these data, older patients (older than 30 years), whether comatose or semicomatose, had mortality rates that approached 70 %, a figure very similar to that encountered in the placebo recipients of the previously cited studies. If therapy is to be effective, it must be instituted before the onset of hemorrhagic necrosis of a dominant temporal lobe and significant deterioration of consciousness.

Acyclovir is superior to vidarabine for the treatment of HSE [149]. The NIAID CASG study defined a mortality of 55 % at 6 and 18 months after the initiation of treatment for vidarabine recipients versus 19 and 28 %, respectively, for the acyclovir group. Late deaths were not a consequence of either persistent or reactivated HSV infection but occurred in patients who were severely impaired as a consequence of their disease. Acyclovir decreases mortality to 19 % 6 months after therapy. Importantly, 38 % of patients, irrespective of age, return to normal function.

Previous studies indicated that age and level of consciousness influenced long-term outcome. A more objective reflection of level of consciousness is the Glasgow Coma Scale (GCS). Scores that approached normal predicted enhanced survival. When GCS score and age were assessed simultaneously, a GCS score of 6 or less predicted a poor therapeutic outcome, irrespective of the agent administered or of the age of the patient [149].

Regarding morbidity for acyclovir recipients, 38 % of patients returned to normal or with minor impairment, 9 % of patients had moderate sequelae, and 53 % of patients were left with severe impairment or died. Relapse of HSE has been reported, though not well documented, in a few patients following the administration of vidarabine [150–152] and acyclovir [152, 153]. Many patients were not afebrile at the conclusion of treatment, suggesting that a longer duration of therapy to a minimum of 14–21 days may be desirable.

Of acyclovir recipients, 10 % experienced an increased BUN level, and 6 % developed a creatinine level in excess of 2 mg/dL. No clinical evidence of toxicity was detected. The current therapy of choice for the management of HSE is acyclovir. This drug is administered at a dosage of 10 mg/kg every 8 h (30 mg/kg per day) for 14–21 days.

6 Neonatal Herpes Simplex Virus Infections

6.1 History

In 1941, Smith, Lennette, and Reames [6] reported the first case of HSV infection of the CNS, as noted earlier. This case occurred in a newborn with neonatal HSE. In 1952, Zuelzer, Wolf, and Stulbery [154] reviewed eight cases of disseminated HSV infection in neonates with involvement of most organs, including the brain in many instances. This report was followed shortly by others indicating the association between HSV infection of the newborn and necrotizing encephalitis, including the isolation of HSV in cell cultures from brain tissue.

6.2 Pathology and Pathogenesis

6.2.1 Pathology

Although the pathology of HSE is discussed earlier in this chapter, a few characteristics appear more commonly in the newborn. Gross examination of the brain often reveals encephalomalacia and hydranencephaly, which are the consequence of extensive hemorrhagic necrosis. Porencephaly, hydranencephaly, and multicystic lesions are often sequelae in neonates who survive for several weeks or months following neonatal HSV infection of the brain. The microscopic appearance of the brain is characterized by a mononuclear inflammation, necrosis, and hemorrhage.

6.2.2 Pathogenesis

In utero disease is likely a consequence of transplacental infection and usually involves skin, brain, eye, liver, and adrenals. More commonly, the fetus comes in contact with infected maternal genital secretions at the time of delivery. Viral replication in the newborn either remains limited to the portal of entry—namely, the skin, eye, or mouth—or progresses to involve various other organs, including the brain (resulting in encephalitis), causing life-threatening disease. Host mechanisms responsible for control of viral replication at the site of entry are unknown. For babies with encephalitis, intraneuronal transmission of virus provides a privileged site that may be impervious to circulating humoral and cell-mediated defense mechanisms. Thus, transplacental maternal antibodies may be of less value in the prevention of encephalitic forms of neonatal HSV infections. Disseminated infection is a consequence of viremia or secondary to extensive cell-to-cell spread, as occurs with pneumonitis after aspiration of infected secretions.

Neonatal HSE illustrates the two major pathogenic routes for virus access to the brain, namely, hematogenous and intraneuronal. For example, hematogenous spread of virus usually occurs with disseminated disease, and diffuse involvement of the brain ensues in 60 to 80 % of patients. In contrast, neuronal transmission probably results in the focal CNS disease encountered in babies with encephalitis only when no distal organ involvement is documented [155].

6.3 Times of Transmission of Infection

Neonatal HSV infection is acquired at one of three times: in utero, intrapartum, or postnatally. Regardless of the time or route of acquisition, the newborn is at risk of CNS disease. Certainly, the mother is the most common source of infection for the first two of these routes of transmission of infection to the newborn.

6.3.1 Intrauterine Infection

In utero acquisition of HSV infection is becoming increasingly documented [156–159]. Manifestations of disease acquired in utero include chorioretinitis, cutaneous aplasia, hydranencephaly, and encephalomalacia [160]. Risk factors associated with intrauterine transmission of infection are unknown; however, both primary and recurrent maternal infection can result in infection of the fetus in utero. In utero infection is the consequence of either transplacental or ascending infection.

6.3.2 Intrapartum Infection

The most common time of transmission of infection from mother to the fetus is intrapartum. Transmission occurs when the infant comes in contact with infected maternal genital secretions at delivery, accounting for 80 % of cases [161].

Prospective assessment of HSV excretion in the genital tract at delivery indicates that shedding can occur in 0.5–1.3 % of women [162]. Factors that influence intrapartum acquisition of infection by the fetus include: (a) type of maternal infection (primary vs. recurrent) [93, 163], (b) maternal antibody status [164–166], (c) duration of ruptured membranes [164], and (d) placement of a fetal scalp monitor in a woman excreting HSV [167, 168].

Primary infection is associated with (a) larger quantities of HSV replicating in the genital tract (>10^6 viral particles/0.2 mL of tissue culture inoculum) and (b) a period of viral excretion that on average persists for 3 weeks. In contrast, in women with recurrent genital infection, HSV is shed for an average of only 2–5 days and at lower concentrations (approximately 10^3/0.2 mL of tissue culture inoculum). Because of the larger quantity of virus and the longer period of viral excretion, primary maternal infection is associated with a higher rate of transmission to the fetus—estimated between 30 and 50 % [93, 163, 169]. Reflecting the type of maternal infection, the delivery of transplacental maternal antibody to the fetus influences both the severity of disease in the newborn and the likelihood of fetal infection [164–166, 170]. Lastly, placement of a fetal scalp monitor in women excreting virus has been shown to lead to fetal infection. Monitor placement should be discouraged in women with a history of genital herpes or visualized lesions.

The duration of ruptured membranes is reported to be an important indicator of risk for acquisition of neonatal infection. Recent data suggest that cesarean section decreases the incidence of infection in women with lesions present at delivery [169].

6.3.3 Postnatal Infection

The third route of transmission is postnatal acquisition [171–178]. Documented sources include the mother (including the breast as a source of virus [171–173]), the father (labial lesions) [174, 175], nosocomial transmission (nursery personnel or other babies) [176–178], and as a consequence of the Jewish tradition of circumcision, known as mitzba ba pa (sp, CDC).

6.4 Incidence and Presentation of Neonatal Infection

The incidence of neonatal HSV infection is about 1 in 3,000 (0.03 %) deliveries [155]. Overall, two-thirds of children with neonatal HSV infection develop disease of the CNS, and the disease may remain localized to the brain or become disseminated to involve various other organs. If untreated, newborns with disseminated disease have a mortality of 80 %, and newborns with disease limited to the CNS have a mortality of approximately 50 %.

Classification of newborns with HSV infection is mandatory for prognostic and therapeutic considerations [164, 179]. Babies with congenital infection, by definition, must be identified within 48 h of birth. Those babies who are infected (either during delivery or postnatally) are divided into three categories: (a) those with disease localized to the skin, eye, or mouth; (b) those having encephalitis with or without skin, eye, and/or mouth involvement; and (c) those having disseminated disease involving multiple organs, such as CNS, lung, liver, adrenals, skin, eye, and/or mouth. This chapter focuses on CNS disease and considers prospectively acquired data obtained through the NIAID CASG. All babies, irrespective of disease classification, should be considered at risk for CNS complications of infection. The presentation and outcome of infection (particularly prognosis after therapy) according to category vary significantly with regard to both mortality and morbidity.

6.5 Intrauterine Infection

Intrauterine infection is usually apparent at birth and is characterized by a triad of findings: (a) skin vesicles and/or scarring (cuteus aplasia), (b) eye disease (chorioretinitis, optic atrophy), and (c) brain disease (microcephaly, encephalomalacia, or hydranencephaly). Retinitis alone can be a presenting sign and should alert the pediatrician to the possibility of intrauterine HSV infection, although HSV infection is a less common cause of chorioretinitis relative to other congenital infections. The frequency of occurrence of intrauterine HSV infection has been estimated to range between 1 in 100,000 (0.001 %) and 1 in 200,000 (0.0005 %) deliveries [156].

6.6 Disseminated Infection

Disseminated HSV infection has the worst prognosis with regard to mortality. Children with disseminated infection usually present to tertiary medical centers for therapy between 9 and 11 days of life; however, signs of infection are, on average, usually present 4–5 days earlier.

The principal organs involved following disseminated infection are the liver, brain, and adrenals; however, infection can involve various other organs, including the larynx, trachea, lungs, esophagus, stomach, lower gastrointestinal tract, spleen, kidneys, pancreas, and heart. Constitutional signs and symptoms include irritability,

seizures, respiratory distress, jaundice, bleeding diatheses, and shock, in addition to a characteristic vesicular exanthem that is often considered pathognomonic for neonatal HSV infection.

The vesicular rash, as described later in this chapter, is particularly important in the diagnosis of HSV infection. Notably, about 20 % of children with disseminated neonatal HSV infection will not develop skin vesicles during the course of their illness [161, 164, 180]. In the absence of skin vesicles, the diagnosis becomes exceedingly difficult because the clinical signs are often vague and nonspecific, mimicking those of neonatal sepsis. Mortality in the absence of therapy exceeds 80 %; if therapy is instituted before CNS disease ensues, outcome is usually good. The most common cause of death in babies with disseminated disease is either HSV pneumonitis or disseminated intravascular coagulopathy.

Evaluation of the extent of disease is imperative, as with all cases of neonatal HSV infection. The clinical laboratory should be used to define hepatic enzyme elevation (serum alanine aminotransferase and AST), direct hyperbilirubinemia, neutropenia, thrombocytopenia, and bleeding diatheses. Unless contraindicated, examination of the CSF is imperative. In addition, chest roentgenograms, abdominal X-rays, EEG, and CT or MRI of the head can be judiciously and serially employed to determine the extent of disease. The radiographic picture of HSV lung disease is characterized by a diffuse interstitial pattern that progresses to a hemorrhagic pneumonitis. Pneumatosis intestinalis can be detected when gastrointestinal disease is present. Encephalitis is a common component of disseminated infection, occurring in about 75 % of these newborns. Serial evaluation of the CSF and noninvasive neurodiagnostic tests, as defined later in this chapter, will help assess the extent of brain disease.

6.7 Encephalitis

Infection of the CNS alone or in combination with disseminated disease presents with findings indicative of encephalitis. Overall, nearly 90 % of babies with brain infection caused by HSV have evidence of an acute neurologic syndrome. Brain infection can occur in one of two fashions: either as a component of multiorgan disseminated infection or as encephalitis only, with or without skin, eye, and mouth involvement. Nearly one-third of all babies with neonatal HSV infection have only the encephalitic component of disease.

Clinical manifestations of these two types of encephalitis include seizures (both focal and generalized), lethargy, irritability, tremors, poor feeding, temperature instability, bulging fontanel, and pyramidal tract signs. Whereas babies with disseminated infection often have skin vesicles in association with brain infection, the same is not true for babies with encephalitis alone. In this latter group, only approximately 60 % have skin vesicles at any time during the disease course [161, 164, 180–182]. Cultures of CSF yield virus in 25–40 % of all patients. Anticipated findings on CSF examination include pleocytosis and proteinosis (as high as 500–1,000 mg/dL). Although a few babies with CNS infection, demonstrated by brain biopsy, have been reported to have no abnormalities of their CSF, certainly this is very uncommon.

Serial CSF examinations provide a useful diagnostic approach because the infected newborn with brain disease demonstrates progressive increases in its protein content. The importance of CSF examinations in all infants is underscored by the finding that even subtle changes have been associated with significant developmental abnormalities [183]. An EEG, CT, or MRI can be very useful in defining the presence of CNS abnormalities. Death occurs in 50 % of babies with localized CNS disease who are not treated, and it is usually related to involvement of the brainstem. In the absence of antiviral therapy, with rare exceptions, survivors are left with neurologic impairment, and the long-term prognosis after either disseminated infection or encephalitis alone is particularly poor. Up to 50 % of surviving children have some degree of psychomotor retardation, often in association with microcephaly, hydranencephaly, porencephalic cysts, spasticity, blindness, chorioretinitis, or learning disabilities. Whether visceral or CNS damage can be progressive after initial clearance of the viral infection is unclear, but it is a possibility suggested by long-term assessment of children with skin, eye, or mouth disease [161, 164, 184] and more recently by a study of a group of babies with more severe disease [185].

Several points warrant reiteration. Clinical manifestations of disease in children with encephalitis alone are virtually identical to those findings that occur with brain infection in disseminated cases, in spite of the presumed differences in pathogenesis. For babies with encephalitis only, approximately 60 % develop evidence of a vesicular rash characteristic of HSV infection. Thus, a newborn with pleocytosis and proteinosis of the CSF but without a rash can easily be misdiagnosed as having bacterial or other viral infection unless HSV infection is carefully considered. In such circumstances, a history of genital lesions in the mother or her sexual partner may be very important in the incrimination of HSV as a cause of illness.

6.8 Skin, Eye, and/or Mouth Infection

Infection localized to the skin, eye, and/or mouth is associated with virtually no mortality. When infection is localized to the skin, the presence of discrete vesicles remains the hallmark of disease. Clusters of vesicles often appear initially upon the presenting part of the body that was in direct contact with the virus during birth. With time, the rash can progress to involve other areas of the body as well. Vesicles occur in 80 % of children with skin, eye, or mouth infection. Children with disease localized to the skin, eye, or mouth generally present at about 10 to 11 days of life. Those babies with skin lesions invariably suffer from recurrences whether therapy is administered or not. Although death is not associated with disease localized to the skin, eye, and/or mouth, approximately 30 % of these children eventually develop evidence of neurologic impairment in the absence of antiviral therapy, which can result in significant neurologic morbidity [160, 161, 184].

Infections involving the eye may manifest as keratoconjunctivitis or later chorioretinitis. The eye can be the only site of HSV involvement in the newborn [160]. Findings include keratoconjunctivitis, microphthalmia, or retinal dysplasia. In the presence of persistent disease and no therapy, chorioretinitis can result. Chorioretinitis

can be caused by either HSV-1 or HSV-2 [186–188]. Keratoconjunctivitis, even in the presence of therapy, can progress to chorioretinitis, cataracts, and retinal detachment. Cataracts have been detected on long-term follow-up of proven perinatally acquired HSV infections [189].

Long-term neurologic impairment has been encountered in children whose disease appeared localized to the skin, eye, and/or mouth. The significant findings include spastic quadriplegia, microcephaly, and blindness. Despite normal clinical and CSF examinations at the time these children completed antiviral therapy, neurologic impairment became apparent between 6 months and 1 year of life. In retrospect, when CSF from these babies was subjected to PCR analysis, evidence of HSV DNA was detected in virtually all of these children, indicating an asymptomatic infection of the CNS [190].

6.9 Diagnosis

The appropriate use of laboratory tools is essential if a diagnosis of HSV infection is to be achieved [191]. Virus isolation remains one of two definitive diagnostic methods. If skin lesions are present, a scraping of skin vesicles should be made and transferred (in appropriate virus transport media) to a diagnostic virology laboratory. Typing of an HSV isolate must be done for prognostic purposes.

Cytologic examination of cells from the maternal cervix or from the infant's skin, mouth, conjunctivae, or corneal lesions has a sensitivity of only approximately 60–70 % and, therefore, should not be the sole diagnostic determinant for infection in the newborn [11, 192]. Cellular material obtained by scraping the periphery of the base of lesions should be smeared on a glass slide and promptly fixed in cold ethanol. The slide can be stained according to the methods of Papanicolaou, Giemsa, or Wright before examination by a trained cytologist. Deployment of Giemsa or, alternatively, Tzanck smears likely will not demonstrate the presence of intranuclear inclusions. Intranuclear inclusions and multinucleated giant cells are indicative, but not diagnostic, of HSV infection. The use of HSV monoclonal antibodies for rapid diagnosis has gained widespread acceptance. These fluorescence studies should be performed by laboratories experienced in the procedure.

Serologic diagnosis of HSV infection is not of great clinical value. Therapeutic decisions cannot await the results of serologic studies. The inability to differentiate transplacentally acquired maternal immunoglobulin G from endogenously produced antibodies makes the assessment of the neonate's antibody status both difficult and of little value during acute infection. Commercially available serologic tests are now capable of distinguishing HSV-1 from HSV-2 antibodies. These assays are based on differences in glycoprotein gG1 and gG2 [193]. These are the only antibody assays that should be used. Serial antibody assessments may be useful if a mother without a history of HSV infection has a primary infection late in gestation and, therefore, transfers little or no antibody to the fetus.

The use of CT and MRI scans to define CNS disease is essential, even in the child who appears normal.

6.10 PCR Detection of Viral DNA

The other definitive diagnostic method is PCR detection of viral DNA, as discussed earlier in this chapter [140, 194, 195].

6.11 Treatment

6.11.1 Background

Of all the perinatally acquired infections, the one most likely to be amenable to successful therapy is that caused by HSV. Of children presenting with disease localized to the skin, eye, and/or mouth, approximately 70 % will progress to involve the CNS or result in disseminated infection [181]. When such events occur, the likelihood of an adequate outcome, even with efficacious drugs, is not optimal because many of these children will either die or be left with significant neurologic impairment. The following paragraphs summarize our knowledge of therapy [184, 196–199].

First, the overall mortality rate for babies with encephalitis or disseminated infection 1 year after treatment with high doses of acyclovir (20 mg/kg every 8 h for 21 days) is lower than that of prior studies of neonatal HSV infection that used lower doses [200]. There are no differences in either adverse effects or laboratory toxicity.

Second, irrespective of the therapeutic modality employed, there has been a significant increase in the number of babies who returned to normal function. This can be accounted for largely by the introduction of therapy before the development of encephalitis or disseminated disease [200]. Of the babies entered in a controlled trial comparing vidarabine with acyclovir in neonatal herpes simplex virus infection (Whitley, et al.), more than 48 % have disease localized to the skin, eye, and mouth [200]. This represents a threefold increase in the number of babies with skin, eye, and mouth involvement, when compared with that of previous studies and historic data ($p < .001$). The change in spectrum of disease presentation is most likely related to earlier diagnosis. The number of babies with encephalitis has remained fairly constant at approximately 30 %, whereas the number of babies with disseminated disease has decreased to 22 %. Nevertheless, improved morbidity by disease classification is unchanged for encephalitis.

Third, available data indicate that therapy has not been initiated any earlier in the most recent neonatal HSV studies [201] as compared to earlier studies [200]. However, the mean duration of disease for all children (irrespective of disease classification) entered into these studies was 4–5 days; therapy can, therefore, be instituted even earlier in the disease course. This "window" for earlier administration of therapy is significant if further advances in therapeutic outcome are to be achieved.

The existing database from the NIAID CASG has provided insight into those factors that influence outcome [202]. Those factors that have a major impact on outcome include disease classification, level of consciousness, time of initiation of therapy, virus type, and the virus type and frequency of skin recurrences for babies whose disease is localized to the skin, eye, and mouth. Our understanding of these

data implies that limitation of disease before there has been extensive multiorgan involvement or disease of the CNS is associated with the best prognosis. This information will be useful in developing therapeutic strategies and in counseling parents of children with neonatal HSV infection.

6.11.2 Long-Term Suppressive Therapy with Oral Acyclovir

The use of oral acyclovir therapy for prolonged periods for 6 months has recently been shown to improve neurologic outcome such that over 60 % of children with CNS disease returned to normal function. This finding implies the chronic replication of HSV in the brain [203].

6.11.3 Long-Term Follow-Up

Children with neonatal HSV infection require frequent and detailed long-term follow-up. Children with CNS or disseminated disease are at risk for neurologic impairment. Management of resultant seizure disorders is standard. Even children with skin, eye, and/or mouth disease are at risk for neurologic impairment and must be followed carefully.

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Congenital Cytomegalovirus Disease

Mark R. Schleiss

Abstract Of the myriad of congenitally and perinatally acquired infections that can impair the neurodevelopment of the infant, cytomegalovirus (CMV) is the most important. In the developed world, congenital CMV infection occurs in approximately 1 % of all pregnancies. Long-term neurodevelopmental disabilities include developmental delay, cerebral palsy, seizure disorders, and sensorineural hearing loss. This chapter summarizes the epidemiology and impact of congenital CMV on brain development. Hypotheses regarding the pathophysiology of CNS injury are reviewed. Prospects for intervention are also summarized.

Keywords Cytomegalovirus • Congenital cytomegalovirus infection • Cytomegalovirus vaccine • Cytomegalovirus neuropathogenesis • Sensorineural hearing loss • Inflammatory response • Cytomegalovirus immune evasion

1 Epidemiology of Congenital CMV Infection

Human cytomegalovirus (CMV) is a ubiquitous betaherpesvirus that replicates only in human cells. CMV infections are generally asymptomatic in immunocompetent individuals but produce a mononucleosis syndrome (heterophile negative) in approximately 10 % of primary infections in older children and adults [1–3]. Similar to other herpesviruses, CMV becomes latent after primary infection, but it may reactivate from latency, particularly in the setting of immune suppression, leading to disease in HIV-infected patients [4], or in solid organ or hematopoietic stem cell

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transplant patients [5, 6]. Acquisition of infection typically requires intimate contact with body fluids (blood, urine, saliva, breast milk). There is no seasonality to infection. Patient populations with increased rates of primary infection include breastfeeding infants, sexually active adolescents, and childcare providers in group day care [7–13]. Seroprevalence is higher among nonwhites and among individuals of lower socioeconomic status [14].

From a public health perspective, the most important medical impact of CMV is the damage caused to the developing central nervous system (CNS) of a fetus when infection occurs in utero. A recent meta-analysis of published studies concluded that the overall birth prevalence of congenital CMV infection was approximately 0.65 %, although this study also noted that congenital infection rates varied considerably among different study populations [15]. This corresponds to over 60,000 congenital infections annually in the United States and Europe. Some of the risk factors for congenital CMV infection include nonwhite race, low socioeconomic status, premature birth, and neonatal intensive care unit admittance. The risk of fetal transmission appears to increase with gestational age, but neurological outcomes are more severe when infection occurs during the first trimester [16, 17]. However, viral transmission can occur during the entire gestation period, and adverse neurological outcomes may still be observed in the setting of infections acquired in late gestation [18], although CNS injury is much less common when fetal infections are acquired during this time point in pregnancy [19].

The prevalence of congenital CMV infection within a given population correlates directly with maternal CMV seroprevalence [20]. Indeed, preconception immunity to CMV does not confer complete protection against fetal transmission in subsequent pregnancies, although the risk of congenital CMV clearly is greater in the setting of a primary infection during pregnancy. Overall, transplacental transmission of virus occurs in about one-third of mothers with primary CMV infection [21–23], and approximately one-half of these infections acquired in utero result in a symptomatic clinical syndrome [24]. Fetal infection occurs in up to ~1.5 % of pregnancies in which there is preconception immunity [15], either due to reactivation of latent infection or, probably more commonly, due to maternal reinfection with novel strain variants of CMV [21, 25, 26]. Strain variation among clinical isolates of CMV is substantial, and the presence of preexisting maternal immunity does not appear to fully protect against different strains [27–29]. Importantly, preconception maternal immunity unfortunately does not completely protect the infected fetus from neurological injury and sequelae [30]. Indeed, in a study of 300 children with confirmed congenital CMV, investigators at the University of Alabama, Birmingham, found that the incidence of progressive hearing loss was not different in children born to mothers with preexisting immunity when compared to women who gave birth to infected newborns in the setting of a primary maternal infection during pregnancy [31]. Such observations complicate the conceptualization and design of a preventative CMV vaccine (reviewed later in this chapter). These observations suggest that infection prevention strategies should not only be targeted to seronegative but also to seropositive pregnant women.

Congenital CMV infection is the major infectious cause of birth defects and childhood neurodevelopmental disorders. Among the primary clinical manifestations associated with congenital CMV infection, the most devastating are those involving the developing CNS since, in contrast to other end-organ injury, CNS injury is generally believed to be irreversible. The presence of symptoms at birth in an infant with congenital CMV is an important harbinger of brain involvement and potential neurodevelopment sequelae. CNS injury and attendant long-term neurodevelopmental deficits are substantially more common in infants with symptoms at birth. The most commonly observed symptoms of CMV infection at birth are intrauterine growth retardation (IUGR), purpura, jaundice, hepatosplenomegaly, microcephaly, hearing impairment, and thrombocytopenia [32]. While clinical signs due to abnormalities of the reticuloendothelial system (like anemia, hepatosplenomegaly, jaundice) are transient, neurological deficits either are evident at birth and typically persist for life or tend to become evident (as sensorineural hearing loss) in early childhood. Only 10–15 % of children with congenital CMV infection exhibit clinical signs at birth, although even children who appear asymptomatic at birth are at risk for neurodevelopmental sequelae [33]. Most children (60–90 %) with symptomatic infection, and 10–15 % of those with asymptomatic infection, develop one or more long-term neurological sequelae, such as mental retardation, psychomotor retardation, cerebral palsy, developmental delay, sensorineural hearing loss, and ophthalmologic abnormalities [32, 34, 35]. Current estimates indicate that approximately 8,000 children in the United States are affected each year with one or more neurological sequelae related to in utero acquisition of CMV infection. CMV exerts a far greater impact on neurodevelopmental outcomes than that of other, better-known childhood disorders, such as Down syndrome (4,000/year), fetal alcohol syndrome (5,000/year), or spina bifida (3,500/year), although public awareness of CMV remains low [36–38]. In light of the public health significance of CMV-related long-term neurological disabilities, increased attention needs to be devoted to the study of the neuropathogenesis of this infection. Accordingly, the development of effective interventions, such as vaccines, would have a substantial and major public health impact on the prevalence of childhood disabilities [39].

2 Pathogenesis of CNS Injury Induced by Congenital CMV

Given the intrinsic limitations of performing histopathological studies on brain tissue from infants with symptomatic congenital CMV infection, the pathogenesis of CNS injury must be indirectly investigated. Brain imaging studies, cell culture models of infection, and observations from the study of cytokine and host inflammatory responses in children with CMV-induced brain injury (including cerebral palsy) have provided insights into the pathogenesis of CNS injury. In addition, animal models of perinatal and congenital CMV infection have provided important additional information about mechanisms of pathogenesis. These studies are considered in this section of the review.

2.1 Imaging Studies

A number of imaging modalities have contributed to our understanding of the natural history and pathogenesis of congenital CMV. Of particular interest are the imaging studies that have been reported of the developing brain in the CMV-infected fetus. Serial ultrasonograms or cranial CT scans have proven useful in detecting the overt pathological alterations in the fetal brain of symptomatic children and can accurately predict development of cognitive and motor deficiencies [40–42]. Importantly, the absence of detectable lesions in an asymptomatic congenitally infected newborn does not provide complete reassurance against the eventual diagnosis of CNS injury, since infants with normal CNS imaging are nevertheless at risk for developing hearing loss later in life [41].

Fetal imaging studies can demonstrate structural brain abnormalities as early as 28 weeks of gestation, using either MR images or ultrasonograms. T2- and T1-weighted MRI scans of CMV-infected fetal brains have demonstrated white matter abnormalities reflective of acute responses to infection, such as the loss of intermediate zone layer, focal necrosis, and hemorrhage. Chronic lesions due to CMV infection can also be demonstrated, including ventricular dilatation, white matter gliosis, atrophy (volume loss), parenchymal cysts, ependymal cysts, calcifications, and cortical malformations, most notably polymicrogyria [43]. Fetal sonographic studies obtained between 22 and 37 weeks of gestation have also demonstrated structural brain changes attributable to CMV. Transvaginal ultrasonograms have been reported to show abnormal periventricular hyper-/hypoechogenicity, ventricular adhesions, cystic formation around the ventricles, ependymal protrusions, abnormal sulci formation, and hypoplasia of the corpus callosum [44]. Fetal imaging studies have been useful for establishing timelines for determining the embryologic sequence of CNS infection, and these findings may in turn be useful in predicting neurodevelopmental prognosis [45]. Lanari and colleagues recently compiled an elegant summary of the pattern of neurodevelopmental injury as a function of timing of acquisition of brain infection in utero. This review noted that lesions occurring prior to 18 weeks gestational age commonly include lissencephaly with thin cerebral cortex, cerebellar hypoplasia, ventriculomegaly, periventricular calcification, and delay in myelination. At 18–24 weeks, migrational abnormalities may occur, including polymicrogyria, schizencephaly, and periventricular cysts. Third trimester infections may be associated with central nervous system (CNS) lesions that may include delayed myelination, dysmyelination, calcification, and white matter disease [46].

Neonatal imaging of children with symptomatic CMV infection is typically associated with structural brain abnormalities similar to those described in the infected fetus. The most frequent of these is the presence of intracranial calcifications, present in approximately 70 % of cases [47]. Abnormal cranial ultrasonograms (demonstrating periventricular or parenchymal calcifications, or increased ventricular size) can be performed in the neonatal period in symptomatic congenitally infected infants and are able to both identify children with overt, acute CNS

injury as well as those at risk for later neurological deficits [41]. Ultrasonography, however, may miss more subtle CNS pathology in the neonate. Brain MRI of children with congenital CMV has revealed multiple intracranial pathologies, including white matter lesions, neuronal migration, and myelination abnormalities; polymicrogyria; cerebellar, cortical, and hippocampal dysplasia or hypoplasia; periventricular cysts; and ventriculomegaly [48, 49]. The finding of subtle white matter lesions with or without polymicrogyria and in combination with anterior temporal lobe cysts was described in a study of congenital CMV identified by PCR-based screening of Guthrie newborn screening cards [50]. Another recently reported study assessed the diagnostic and prognostic value of cerebral MRI in comparison to ultrasonography in predicting neurodevelopmental outcome in newborns with congenital CMV. Of note, MRI provided additional information beyond that which could be identified by ultrasound (white matter abnormalities in three cases, lissencephaly/polymicrogyria in one and a cyst of the temporal lobe in another one) in four infants who had abnormal findings in both exams. Even more significantly, three newborns had normal ultrasound exams, but had abnormal MRI exams documenting white matter abnormalities and, in one case, cerebellar hypoplasia [51]. Further studies will be required to identify the prognostic role of MRI, particularly with respect to the finding of white matter lesions currently not identifiable by ultrasonography. Figure 1 demonstrates an example of CNS pathology in an infant with symptomatic congenital CMV infection.

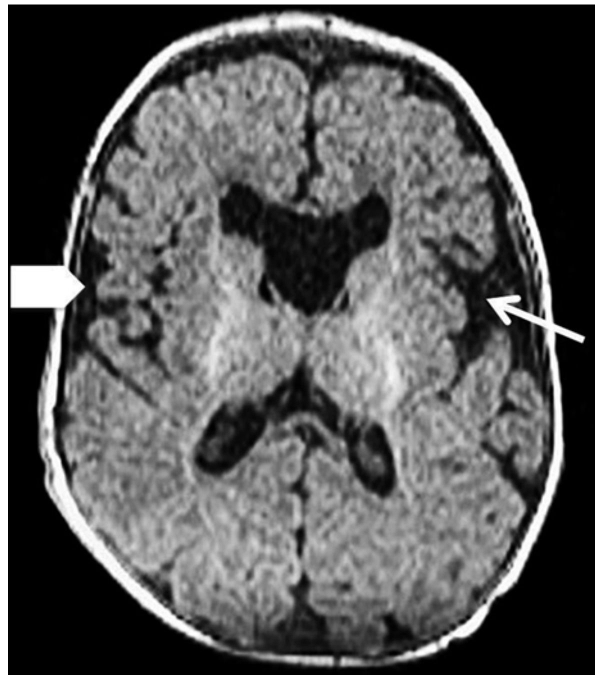

Fig. 1 T1-weighted brain MRI of infant with congenital CMV infection. Axial view is demonstrated. Findings include ventriculomegaly, loss of brain volume with prominence of sulci (*arrow*), pachygyria (*solid arrowhead*) on the surface, and very thin cortex. This infant went on to manifest a seizure disorder and profound neurodevelopmental delay

2.2 Cell Culture Models of CMV Infection

Although the developing brain is the major target for end-organ damage in the setting of congenital CMV infection, the precise cellular targets of infection remain incompletely characterized. Inclusion bodies in the brain have been detected during postmortem histological analysis of fatal cases of congenital CMV infection [52], but little or no histological data identifying the different cell types infected during congenital CMV infection has been reported. Cell culture models of human brain cells are therefore vital in attempting to elucidate the pathogenesis of fetal CNS injury. Both primary human cell culture systems and studies with brain-derived cell lines have demonstrated that practically all cell types in the brain have some degree of susceptibility to CMV infection. The current state of knowledge about CNS targets of infection, including the permissiveness of various cell types for full viral replication and the putative mediators of injury, is summarized in Table 1. Brain microvascular endothelial cells [53–55], astrocytes [56], neuronal cells [57], oligodendroglial cells [58], microglia/macrophages [59], and neural progenitor/stem cells (NPCs) [60] all have a propensity for CMV infection. However, these different cell types vary in their ability to support a complete viral replication cycle, with the permissivity of any given cell type for completion of the viral infection cycle limited by host and viral transcription factors and other elements regulating viral gene expression [47].

Of the cell types of the brain that can be infected with CMV, the astrocyte, the major cell type constituting about 70 % the brain, is the cell type most supportive of productive CMV replication. Primary human fetal astrocyte cultures support cytopathic viral replication, immediate early (IE) gene expression, and β-promoter (early gene) activity, and infectious virus is readily detectable in cell supernatants [56]. Notably, these cells, in association with brain microvasculature endothelial cells (BMVEC), form the blood–brain barrier, a structure that maintains the highly regulated solute, immunologic, and cellular microenvironment in the CNS [61]. Lytic viral replication is supported by BMVEC, which in turn promotes monocyte activation, migration, and infection in the CNS [62].

In contrast to astrocytes, primary differentiated human neurons have generally been found to be refractory to CMV replication, with some exceptions as noted below. Highly purified primary neuronal cultures (>90 % neurons) contain a small percentage of dividing astrocytes that support viral replication, but viral gene products cannot be detected in neurons [56]. The block in the viral replicative cycle appears to be at the level of the major immediate early promoter (MIEP), and not due to a defect in viral entry [60]. Experiments with undifferentiated human oligodendroglioma (HOG) cells, representative of immature oligodendrocytes, demonstrate that oligodendrocytes, like neurons, may not be fully permissive for CMV infection. However, CMV IE, US11, and glycoprotein B (gB) gene expression is induced in HOG cells upon differentiation with phorbol-myristate acetate (PMA), without production of viral progeny [58]. Some studies utilizing neural progenitor cells (NPCs) have demonstrated, following in vitro differentiation and enrichment, that neurons can support productive replication of CMV [63, 64]. Taken together, it appears that the state of cell differentiation as well as its functional status may modulate permissiveness

Table 1 CNS targets of CMV infection and pathogenic mechanisms. Impact of CMV infection on major cell types in the developing fetal CNS is summarized, and potential mechanisms mediating neuropathogenesis are noted

Cell type	Permissivity for infection	Cytokines and inflammatory mediators induced	Potential role(s) in neuropathogenesis
Astrocytes	Fully permissive for lytic replication	Predominant cytokine produced in infected astrocytes is TGF-β; CCL2, CXCL8, CCL3, and CCL5 are also produced	Plays key role in intercellular signaling and neuronal development; modulates synaptic activity within the nervous system; astrocyte signaling plays key role in microglial activation
Microvascular endothelial cells (BMVEC)	Fully permissive for lytic replication	Key cells in promoting spread; possible site of *trans*-endothelial entry of CMV into the brain	BMVEC participate in formation of blood–brain barrier (BBB); infection promotes monocyte activation, migration, infection
Pericytes	Fully permissive for lytic replication	CXCL8/IL-8, CXCL11/ITAC, CCL5/RANTES, TNF-α, IL-1β, and IL-6	Support BMVEC cells; contribute to BBB; infection contributes both to viral dissemination in CNS and neuroinflammation
Microglia	Abortive; not permissive for lytic replication	Respond to CMV by producing TNF-α, IL-6, CXCL10, CCL2, CCL3, CCL5	Reservoir for latent genome; origin from myeloid precursors; activation may play role in perturbation of neural cell development, oligodendrocyte maturation
Neural progenitor/stem cells (NPCs)	Fully permissive for lytic replication	CMV interferes with migration, proliferation, and differentiation into neurons and astrocytes; modifies cell cycle; modifies metabolism	Disruption of normal cellular processes of NPCs by CMV may be responsible for most structural and migratory abnormalities seen during congenital infection
Neurons	Uncertain; minimal evidence for permissive infection	Block in replication at level of immediate early transcriptional machinery; virus enters but does not complete life cycle	NPCs can be induced to undergo differentiation in cell culture, and under these conditions neurons may support productive viral replication

of neurons and oligodendrocytes to CMV brain infection in cell culture systems. The implications for in utero neuropathogenesis remain to be elucidated.

Microglia, the end-differentiated resident brain macrophages, also do not appear to support productive CMV infection [56]. However, CMV DNA has been demonstrated in infected microglial cells in the absence of detectable viral IE proteins [65, 66]. It has been proposed that brain microglia are replenished from bone marrow-derived

precursors that migrate into the brain [47]. It has been suggested that myeloid precursor cells may be a site for CMV latency and a vehicle of viral dissemination in the host [67–70]. Although myeloid precursors and monocytes are not typically productively infected by CMV [70], they support productive CMV infection at certain stages of differentiation [71]. In addition, endothelial cell-adapted viral strains have been shown to infect both macrophages and dendritic cells [72], a process which requires the CMV gene products essential for endothelial tropism, UL128, 130, and 131 [73]. These gene products have recently emerged as key candidates for CMV subunit vaccines [74]. It has been suggested that macrophages originating in the vascular space may be an important vehicle for trafficking of virus into the CNS in the developing fetal brain [47].

NPCs have emerged as cells of particular interest in the pathogenesis of congenital CMV-induced brain injury. These cells are predominantly located in the subventricular zone and subgranular zone of the hippocampus in the mammalian brain [47]. NPCs possess the ability to migrate, proliferate, and differentiate into neurons, astrocytes, and oligodendrocytes. Figure 2 provides a schematic model of the central role of these cells in CMV-induced neuropathogenesis. In the setting of CMV brain infection (including histopathological observations discerned from fatal congenital infections), it is well recognized that virus preferentially infects cells in the ventricular or subventricular regions [52, 75]. This anatomic distribution suggests the possibility that CMV replication may be particularly well adapted to replicate in the neural stem/precursor cells residing in this region. Several studies have demonstrated that human CMV replicates efficiently in undifferentiated human neural precursor cells in cell culture [60, 63, 64, 76, 77]. It has been proposed that the extent to which these cells are infected in utero may determine the outcome of CNS sequelae associated with congenital CMV infection [47].

CMV infection of human neural precursor cells appears to inhibit their differentiation into both neurons and astrocytes, an effect that may be mediated by virus-induced apoptosis in cells undergoing differentiation [76–78]. It has been proposed that CMV replication may inhibit neural precursor cell proliferation by altering cell cycle mechanisms [60, 79] and may perturb expression of genes related to neuronal metabolism and neuronal differentiation in NPCs [78]. Indeed, disruption of these cellular processes in neural precursor cells may account for a large portion of the structural and migratory abnormalities seen during congenital human CMV brain infection [47, 78]. A recent study in a cell culture model suggests that this susceptibility does not diminish with advancing brain development. In this study, NPC cultures derived at different gestational ages were evaluated after short (3–6) or extended (11–20) in vitro passage for viral entry efficiency, viral gene expression, virus-induced cytopathic effect, and release of progeny virus. Extended passage cultures showed evidence of increased viral entry and more efficient production of infectious progeny, suggesting that CMV infection in fetal brain may continue to result in neural cell loss even with advancing brain development [80]. These observations suggest that persistent CMV infection may continue to negatively impact brain development postnatally. Extended, long-term infect of NPCs postnatally pro-

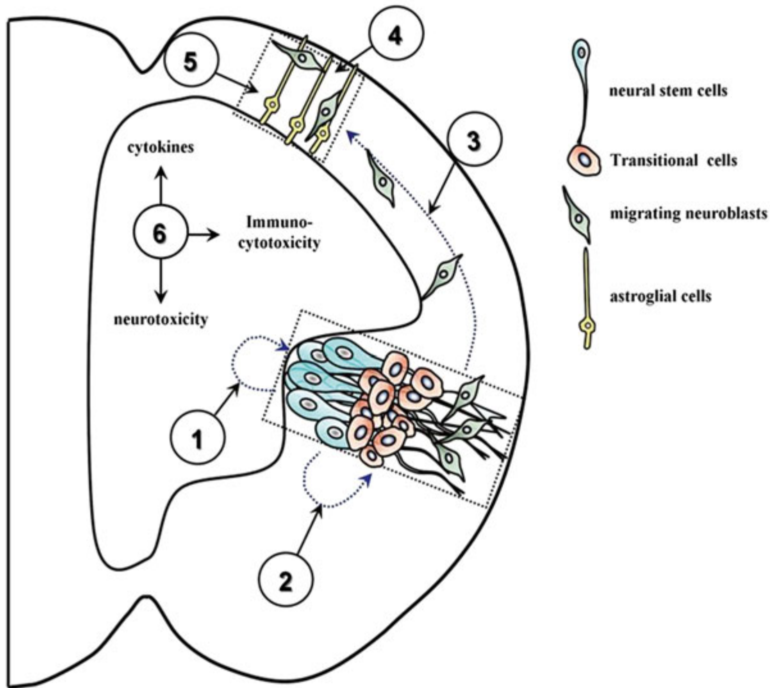

Fig. 2 Schematic representation of mechanisms of brain injury following infection with CMV. Neural stem cells (in *blue*), found along the lateral ventricular wall of the brain, are involved in the development of new neural circuits in the developing brain. These cells differentiate into new brain cells (astrocytes, oligodendrocytes, and neurons), either directly or via an intermediate transitional progenitor cell (*red cells*). Formation of new neural circuitry involves migration of neuroblasts (*green cells*), through a directed pathway that is supported in part by astroglial cells. CMV may potentially affect any or all of these stages: (*1*) Infection of neural stem cells may disrupt their ability to maintain a self-renewing cycle; (*2*) Differentiation of neural stem cells via the transitional cells and eventually neuroblasts may also be disrupted by CMV; (*3*) Brain infection affects the migratory patterns of neuroblasts, particularly during cortical and cerebellar development; (*4*) This presumably alters the migratory patterning of other specific brain structures, causing improper layering of the neocortex; (*5*) Since glial cells are also susceptible to CMV, functions of glia in directing neuronal layering patterns may be affected. (*6*) Finally, infection can induce a myriad of inflammatory mediators, including cytokines and chemokines, and elicit inflammatory cell infiltration. *Reproduced from* Cheeran et al., Neuropathogenesis of congenital CMV infection: Disease mechanisms and prospects for intervention. Clin Micro Rev 2009: 22, 99–126

vides a mechanism by which CMV could to continue to exert an impact on neurodevelopmental processes in early childhood in the context of congenital infection of the CNS. This aspect of CMV replication in NPCs provides reinforcement for clinical trials examining the impact of extended, long-term (6 months) valganciclovir therapy in infants with congenital CMV infection [81].

2.3 Role of Inflammatory Response

In addition to the direct damage to specific cell types conferred by fetal brain infection with CMV, the inflammatory response to infection also is also increasingly becoming recognized as a major contributing factor in the pathogenesis of brain injury. Autopsies of prenatally infected fetuses with CMV have confirmed the presence of a significant inflammatory response in addition to viral inclusions in the brain [82–86]. Upon autopsy, one study recently found that nearly all fetal organs infected with CMV had evidence of inflammatory infiltrate and found that the level of organ damage was associated with the level of inflammation; intriguingly, in addition to the damaging direct effects of viral brain infection and the attendant inflammatory response, hypoxic brain injury due to severe CMV placentitis was also postulated as a contributing factor in brain injury [83]. Tissue-specific viral load has been proposed to impact the magnitude of the inflammatory response. In one study, it was noted that tissue viral load was correlated to immune response; low CMV viral load elicited only a modest immune response with mild brain damage, while tissue containing high viral load had high levels of cytotoxic CD8+ T-lymphocytes, which are associated with immune-related structural damage [82]. In total, this evidence suggests that direct fetal infection with a neurotropic pathogen like CMV not only increases the risk of neurological sequelae mediated by factors such as lytic infection, disruption of neuronal migration, and increased apoptosis, but also through a cascade of events triggering a pathological fetal immune response.

A current view of mechanisms of neuroinflammation in adults revolves around the concept of "reactive microgliosis" [87]. Microglia can be activated in several different patterns, including classical activation (M1 phenotype), alternate activation (M2a phenotype), or acquired deactivation (M2b phenotype) [88, 89]. While the M1 phenotype appears to promote a deleterious, pro-inflammatory status, it has been proposed that the M2 phenotype could favor brain repair [90, 91]. In addition to promoting a pro-inflammatory milieu, developmental brain damage mechanisms can also be driven by microglial activation [92–95]. Systemic and brain inflammation driven by activated microglia in turn impacts the development of different neural cell populations, and influences oligodendrocyte maturation [96] and survival [97, 98]. CMV-driven release of cytokines, particularly IL-6, could impact proliferation and function of neural stem cells [99]. Migrating neurons can be compromised by the release of inflammatory factors released by activated microglia, potentially leading to neuronal cell death or abnormal neuronal migration, one of the hallmarks of CMV infection of the developing fetal brain [100].

A number of studies have attempted to define the precise cytokine responses associated with fetal brain infection with CMV, predominately using cell culture models. In one model of cultured human glial cells, derived from 16- to 20-week-old fetal brain tissue, response to CMV infection is heralded by expression of a number of immune mediators, including chemokines and cytokines [101]. Microglial cells respond to CMV infection by producing TNF-α and IL-6 as well as CXCL10, CCL2, CCL3, and CCL5 [102, 103]. Many of the cytokine responses of microglial cells appear to be driven by innate responses mediated by pattern

recognition receptors on microglia that recognize pathogen-associated molecular patterns [104]; for CMV, these ligands appear to be envelope glycoproteins gB and gH, which signal through Toll-like receptor (TLR) 2, and double-stranded RNA molecules generated during infection, which signal via TLR3 [105–109]. Chemokines and cytokines are also elicited by CMV infection of astrocytes. As noted, the chemokine response in astrocytes includes CCL2, CXCL8, CCL3, and CCL5 [102, 103]. In contrast, the cytokine response to CMV in astrocytes appears to be restricted to TGF-β, an anti-inflammatory cytokine [110]. Primary brain vascular pericytes have recently been shown to be a source of cytokine production in the context of CMV infection, including CXCL8/IL-8, CXCL11/ITAC, CCL5/RANTES, TNF-α, IL-1β, and IL-6 [111].

In addition to inducing the production of chemokines and cytokines following CMV infection of the fetal brain, the viral genome itself encodes homologs of several immunomodulatory proteins, including homologs of CXC (*UL146*, *147*) and CC (*UL128*) chemokines [112]. CMV also encodes functional homologs of chemokine receptor-like G-protein coupled receptors [113]. The role that such gene products play in the pathogenesis of fetal brain injury is unknown. CMV also encodes a functional homolog of the IL-10 gene. Transcription of this gene product has been shown to inhibit CXCL10 production in human microglial cells, a possible mechanism by which CMV genes contribute to evasion of host immune clearance [102].

3 CMV Fetal/Neonatal Brain Infection: Prospects for Intervention

Currently, there are limited interventions for the treatment and prevention of fetal and neonatal CMV brain infections. In women with primary CMV infections complicated by intrauterine transmission, the use of an anti-CMV high-titer immunoglobulin (HIG) has been associated with improved neurodevelopmental outcomes, including regression of fetal cerebral abnormalities for fetuses treated in utero [114, 115], although these studies are uncontrolled and proof of efficacy is still uncertain. A controlled, multicenter trial of ganciclovir in infants with congenital CMV infection and neurological findings at birth indicated that 6 weeks of intravenous therapy was associated with improved short-term and long-term audiologic outcomes [116]. In a follow-up study in which infants were administered serial neurodevelopmental screening examinations, ganciclovir therapy was associated with fewer developmental delays at 6 and 12 months, compared with untreated infants [117]. Based on these encouraging observations, a trial is currently being conducted that will compare 6 weeks to 6 months of therapy, using oral valganciclovir [81], toward the goal of ascertaining whether any additional neurodevelopmental benefit might be realized by longer treatment courses.

Ultimately, the best prospects for control of congenital CMV rest with the development of an effective preconception vaccine. Several candidate vaccines are in various stages of preclinical development, and some have advanced to clinical trials [118, 119]. Most vaccines currently being examined in human studies target envelope

glycoprotein B (gB) and various combinations of T-cell targets, including the pp65 (ppUL83) tegument protein and the major immediate early gene product-1 (IE1) [120]. The gB vaccine has demonstrated modest efficacy in a phase II study in young women of child-bearing age [121]. As previously noted, vaccine development has been complicated by the increasing recognition that women can become reinfected with new, novel strains of CMV during pregnancy and that these strains, in turn, can be transmitted to the fetus, resulting in attendant neurological injury and long-term sequelae [25, 122]. Ultimately, development of a CMV vaccine would therefore probably need to focus not only on prevention against primary infection in seronegative women, but also reinfection in seropositive women, in order to fully protect all infants against the neurodevelopmental sequelae of congenital infection.

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Japanese Encephalitis: A Tale of Inflammation and Degeneration in the Central Nervous System

Kallol Dutta and Anirban Basu

Abstract Over more than a century, many regions in Southeast Asia have been under the grip of a disease called Japanese encephalitis, which is caused by a vector-borne flavivirus. The origins of the disease can be traced to the frequent and multiple epidemics in the islands of Japan during the early twentieth century, which then rapidly spread to other countries. Currently, an estimated three billion people live in the JE endemic region, making this one of the most dreaded arboviral diseases. The JE virus is highly neuroinvasive, and once it reaches the central nervous system, it replicates rapidly in neurons and ultimately causes death. This initiates an inflammation cascade involving the glial cells which further complicates matters in the brain. The virus can specially arrest cell cycle in neuroprogenitor cells, thereby inhibiting their maturation. All these features cause debilitating symptoms and can be fatal in children. Survivors generally suffer from various neuropsychiatric sequelae and require prolonged rehabilitation measures to regain normalcy. There is no specific antiviral therapy available. To date, all therapeutic countermeasures have been supportive. Even though vaccination has led to a marked decrease in the incidence of this disease in several countries in the endemic zone, still epidemics of varied proportions are reported almost every year. In this chapter, our efforts have been focused on providing a general idea about the virus, a detailed analysis of the pathology of the disease, an insight into basic research in unraveling the molecular mechanisms of host responses, and a brief idea about the multiple efforts made over the years to find a cure for the disease.

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1 Prologue

The following paragraph is inspired by true events:

The first ray of sun kisses the dew covered grounds in a small idyllic village, somewhere in the eastern regions in India. This usually is the signal for beginning of an arduous life in the village where rice cultivation is a primary vocation for many. Here, every member of a family has their daily share of chores, but some cannot help. A man and his wife get ready to leave for work. Before leaving their hut, the man brings a "charpoy" out and lays it in the morning sun. Next, he carries out a little girl and places her on the "charpoy." After murmuring a few tender words of affection, the couple leaves for their daily toil. This girl, their daughter, was once a vivacious young child but now a mere memory of her former self. She is a telling victim of the dreaded JE virus that is commonly referred to as the "child-killer" in her part of the world. As the virus infected and multiplied in her body, it inflamed the tissues in her brain. Miraculously, she survived but the virus left her brain permanently damaged. Her ability to speak or comprehend is entirely lacking; the disease has paralyzed her limbs, thereby confining her to the cot. More importantly, the disease has crippled the family economically. In their part of the world, where the monthly family income can be less than $40, having a child with special needs leads to untold difficulties and a dark future.

2 What Is Japanese Encephalitis?

In the late nineteenth century, the islands of Japan were ravaged by a new kind of brain fever we commonly refer today as Japanese encephalitis (JE). To differentiate it from the then common von Economo disease or encephalitis lethargica or encephalitis A (an atypical form of encephalitis), this was initially termed as encephalitis B. The first outbreak suggestive of JE was recorded in 1871, and major epidemics occurred in 1924, 1935, and 1948. The largest of these outbreaks was in the year 1924 in which reportedly 6,000 people were affected with nearly 60 % case fatality ratio [1]. The virus was first isolated from human brain tissue in that year, and in 1934, the disease was transmitted experimentally to primates by intracerebral inoculation of infected human brain lysate [2]. The virus was classified as a member of the genus *Flavivirus* (family Flaviviridae) named after the prototype yellow fever virus (Latin; *flavus* = yellow). The genus consists of over 70 other closely related viral species [3].

The JE virion consists of a single strand of positive-sense RNA of about 11 kb, inside a nucleocapsid, and is surrounded by a glycoprotein-containing envelope.

The RNA comprises a short 5′ untranslated region (UTR), a longer 3′ UTR, and with a single open reading frame between them. It codes for a single polyprotein, which is translationally and posttranslationally cleaved by viral and host proteases into three structural proteins (core, C; pre-membrane, PrM; and envelope, E) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The C protein (12–14 kDa) is highly basic and combines with the RNA to form the nucleocapsid. The prM is closely associated with the E protein, forming a heterodimer, and is thought to act as a "chaperone" to it, impairing its function until after virion release. NS1 is a glycosylated protein; NS3 and NS5 are hydrophilic, whereas NS2A, NS2B, NS4A, and NS4B are hydrophobic. NS3 and NS5 are believed to be enzymatic components of the viral RNA replicase. They are localized in the cytoplasm and remain associated with intracellular membranes. NS5 possesses a methyltransferase-polymerase interface, while NS3 is actually a multifunctional viral enzyme that contains helicase and NTPase activities in its central region and a protease activity in its N terminus when associated with the viral cofactor NS2B [4–9]. The viral replication is initiated by the replication complex through a process of RNA-dependent RNA polymerization in the perinuclear endoplasmic reticulum membranes. Nonstructural proteins 3 and 5 are components of the replication complex, which associates with the 3′ noncoding region of genomic RNA to initiate viral replication. The core protein and NS5 interact with heterogeneous nuclear ribonucleoprotein A2 in the host cell to regulate replication [10]. The host cell DEAD box helicases DDX3 and DDX5 have been reported to regulate such processes by interacting with viral core NS3 and NS5 proteins [11, 12]. NS3 and NS5 interact with heat shock protein 70, eukaryotic elongation factor 1 alpha, and ras-related nuclear protein to form the replicase complex [13]. JEV is also reported to utilize the host cell ATP production machinery to drive its replication. Host cell fructose-bisphosphate aldolase A binds with JEV UTRs to drive viral replication, knocking down aldolase A reduced viral translation, genome replication, and viral production significantly [14].

The actual origin of this virus remains unclear, but comparative phylogenetic analysis with other flaviviruses points to a common ancestor of African origin [15]. However, owing to the fact that the Indonesia-Malaysia region is the only area where all the genotypes of the JE virus (JEV) are represented and also as the only region where the most divergent genotypes (IV and V) that are thought to represent the oldest lineages have so far been found, it could be assumed that the origins of the JEV lies there [16]. Among the five genotypes of the virus, IV and V are thought to be the oldest, from which the newer genotypes I, II, and III have evolved [17]. It has been observed over the ages that genotypes I and III occur mostly in epidemic regions, whereas II and IV are associated with endemic disease [18], but not always. There have been instances in the past where genotype III has been isolated from both endemic and epidemic regions [19, 20]. Also, the emergence of new genotypes in regions that are commonly associated with other genotypes are being reported frequently. For example, South Korea, Thailand, China, and India have been under the influence of genotype III virus for many decades, but later, the introduction of genotype I in their epidemic areas has been reported as well [21, 22]. It is now believed that genotype I is the dominant genotype in the entire Asian continent [23].

By 1938, it was established that the disease is spread by mosquito bites, especially those belonging to the *Culicine* species. However, it was not until the late 1950s when the entire enzootic life cycle of the virus was described [24–31]. The virus reportedly replicates within the salivary gland of the mosquito. The virions remain entrapped in intracellular vacuoles that are later released into the apical cavity of salivary gland cells through the fusion of these vacuoles with the apical plasma membrane. This process is associated with primary resynthesis of saliva in mosquitoes following their blood-feeding activity. Another type of shedding involves virus particles, either singly or in mass, being released directly through the apical plasma membrane [32]. An ideal nursery for these mosquitoes is stagnant water, such as in rice paddy fields which are found in most endemic regions. The majority of the population in rural Asia has been believed to be infected with the virus by early adulthood [33, 34]. Birds such as herons and pond egrets and bats serve as virus reservoirs or maintenance hosts, but the virus regularly spills over into pigs, members of the family Equidae (e.g., horses and donkeys), and humans. Interestingly, the Asiatic cattle egret's range dramatically expanded across Asia in the nineteenth century following changing agricultural practices [35], which coincides with the evolution and spread of the more recent JEV genotypes. Pigs are considered as the amplification hosts as viremia with resultant high titer. Due to the close proximity of pigs with human dwellings, these animals are considered a main component in the transmission cycle with respect to human infection [36]. Although the virus has occasionally been isolated from human peripheral blood [37], viremia is usually brief with low titers. As a result, humans are considered dead-end hosts from whom transmission does not normally occur.

According to reports, the current realm of JE endemic region extends from the islands of Japan in the east to Pakistan in the west [38] and parts of Russia (erstwhile USSR) in the north [39] to northern parts of Australia in the south [40, 41]. The rapid spread of the virus (it took only about a century to encompass the current endemic zone) is attributed to rapid globalization, climatic changes, and changes in agricultural practices [36, 42]. Based on the available facts, a further spread into the African continent or Mediterranean Europe cannot be effectively ruled out. Recently, studies have reported the detection of JEV RNA from mosquitoes in Italy [43] and also from birds in Italy and Spain which seem to support the hypothesis of long-distance spread of JEV from endemic countries [44, 45]. It is not uncommon for short-term travelers from non-endemic regions to be infected with JEV while visiting endemic countries [46–48]. However, since humans are considered as dead-end hosts of the virus, spread from infected humans is never a threat in virgin territories. The Americas are so far not considered to be under the threat of this disease. The only reported cases of Americans affected by JE are from visitors to endemic regions or servicemen posted in those regions [49, 50]. But if the virus ever makes it to the North American continent, it will not be lacking avian reservoirs, as some native species of birds have been shown to be capable of maintaining the virus within their bodies [51].

3 JE and Human Infection

Even after more than a century of its discovery, a lot remains unknown about the pathogenesis of the JEV. How the virus reaches the central nervous system (CNS) following peripheral inoculation or how it evades the host's immune system still remains enigmatic. Our inability to unravel its mysteries has led to vast and untold misery to masses. The prologue is but only one single story that haunts the lives of thousands of people across the entire Southeast Asia. Approximately 67,900 JE cases typically occur annually (overall incidence: 1.8 per 100,000), of which only about 10 % are reported to the World Health Organization. Approximately 33,900 (50 %) of these cases occur in China (excluding Taiwan), and approximately 51,000 (75 %) occur in children aged 0–14 years (incidence: 5.4 per 100,000). An estimated 55,000 (81 %) cases occur in areas with well-established or developing JE vaccination programs, while about 12,900 (19 %) occur in areas with minimal or no JE vaccination programs [52]. The estimated global impact from JE in 2002 was 709,000 disability-adjusted life years (DALYs), which is a measure of the disease burden expressed as the number of years lost due to ill health, disability, or early death. However, as the disease usually occurs in epidemics and there is considerable fluctuation in estimates of its global impact, the interpretation of these data needs to be carefully evaluated. In 1999, JE caused 1,046,000 DALYs; in the two subsequent years, it caused 426,000 and 767,000 DALYs, respectively. Underlying factors that might explain these fluctuations are contextual determinants (mainly environmental factors) and spillover effects into the human population, which trigger epidemics [53].

Adults living in the epidemic zones are often asymptomatic for this disease. However, it is reported that they may become symptomatic in cases of spread to virgin territories. Even though humans are incidental dead-end hosts, mother-to-child transmission of the virus has been reported [54, 55]. Of course, the clinical impact of such observations is yet to be evaluated stringently, but it is known that JEV persists in the human brain for 8–15 years after the onset of encephalitis [56]. Whether the virus reactivates during immunosuppression or has any long-term neuropathologic effects is yet unknown.

The neuropathology associated with JEV involves a combination of direct neuronal damage and indirect damage mediated by the generation of an inflammatory milieu in the CNS. In the following sections, we shall try to link available laboratory research reports along with clinical case studies so as to gain a better understanding of this disease.

3.1 The Journey from the Periphery to the CNS

Despite the enormity of the disease, not much is known about the exact mechanism of spread to the CNS [57]. However, studies regarding other flaviviral infections or in vitro studies have led us to believe that following intracutaneous inoculation via

a mosquito bite, the virus is taken up by Langerhans dendritic cells in the skin which carry the virus into the nearest draining lymph nodes [58]. From there, the virus enters the general circulation via the thoracic duct, where it probably infects cells of myeloid lineage. From the general circulation, the JEV is hypothesized to enter the CNS. There are three possible mechanisms by which the virus is thought to enter the CNS by crossing the blood–brain barrier (BBB):

(a) Passive transport across the endothelium
(b) Active replication in endothelial cells of the BBB
(c) By a "Trojan horse" mechanism in which the virus is carried into the brain by infected peripheral inflammatory cells [59]

Investigations in mouse models [60] and study of human autopsy samples [61, 62] have confirmed that the JEV infects and is able to replicate in peripheral organs such as the lymph nodes, spleen, kidney, and lungs before crossing the BBB. Hematogenous spread of the virus from the periphery to the CNS is supported by the observation that in intranasally JEV-inoculated monkeys, virus replication was widespread in the CNS, but not always identified in the olfactory bulb [63]. The hematogenous route into the CNS is also supported by observations that led to isolation of the virus from blood clots collected during the acute phase of infection [64]. As perivascular cuffing is a commonly reported observation in human infections of JEV, this could probably lead to influx of peripheral inflammatory cells into the CNS parenchyma. Inflammatory cells invading the parenchyma are shown to be predominantly macrophages with small numbers of T cells [65], though the role of these cells in transporting the virus from the periphery into the CNS remains ambiguous. Some in vitro studies have reported that JEV is capable of surviving within cells of monocyte/macrophage lineage for prolonged time periods [66, 67]. Also, it is known that JEV activates macrophages to secrete proinflammatory cytokines which a recent study proposes to be due to viral interaction with CLEC5A (a member of the myeloid C-type lectin family expressed on macrophages and neutrophils) thereby inducing DAP12 phosphorylation, a transmembrane adapter known to play a significant role in cytokine signaling [68].

Though human data are lacking, studies in a mouse model of JE have shown that there is upregulation of the cellular adhesion molecules ICAM and VCAM in the brain [69], which may be important in initiating adhesion and migration of neutrophils and macrophages [70]. This was also associated with elevated levels of MMP-9 in the brain which could also contribute to increased BBB permeability. Taken together, these findings could indicate a "Trojan horse" role of these cells at a later stage of infection. Further strengthening this hypothesis are the observations of the disruptive role of secreted cytokines on the BBB. In a mouse model, macrophage-derived neutrophil chemotactic factor was shown to alter the BBB permeability, in a dose-dependent manner [71]. Tumor necrosis factor alpha (TNF-α) and interleukin (IL)-8 which are involved in polymorphonuclear cell recruitment have also been reported to be elevated in the cerebrospinal fluid (CSF) and serum of humans with JE and are higher in fatal than nonfatal cases [72, 73].

3.2 JEV Entry into Host Cells

How JEV actually infect neurons is not well understood either. There are a number of studies arguing about the mechanism of viral entry into host cells. Generally, most viruses are known to enter host cells either by membrane fusion or via receptor-mediated endocytosis or via direct insertion of genetic material. Now, the exact mechanism that is employed by the JEV may vary depending on the type of cell that it intends to infect. The entry of the virus into neurons has been reported to be via a clathrin-independent but caveola-mediated endocytosis mechanism which requires the involvement of dynamin and plasma membrane cholesterol at low cellular pH [74]. JEV binding to neuronal cells leads to rapid actin rearrangements and an intact and dynamic actin cytoskeleton. The small GTPase RhoA has also been reported to play an important role in viral entry [75]. However, in a different non-neuronal cell type such as fibroblasts, the mechanism can be entirely different in that entry could well be a clathrin-dependent one. Even in cells of epithelial lineage, JEV has been reported to enter through receptor-mediated endocytosis involving both clathrin-dependent and caveola-dependent pathways with low pH-triggered membrane fusion followed by replication in intracellular membrane structures [76]. In another study, vimentin, a class III intermediate filament protein, has been shown to be involved in viral entry in a porcine stable cell line [77]. An interesting observation utilizing neural stem/progenitor cells is the role of lipid rafts. Lipid rafts are specialized membrane domains enriched in certain lipid cholesterol and proteins that can be utilized by the virus to gain entry into the stem cells in the neurogenic regions of the brain [78]. Lipid rafts have also been shown to be associated with Hsp70 that expressed on cell surface to facilitate JEV entry, albeit this mechanism was reported in a non-neuronal cell line [79]. Although microglial cells are believed to be sparingly infected by the virus in vivo, in vitro studies have pointed out multiple receptor proteins, especially the surface glycoprotein CD4, which may mediate the entry of JEV [80]. This observation could be of significance as the role of peripheral monocyte/macrophages in viral dissemination has been discussed in the earlier section.

3.3 Neuropathology of JE

Over the last century, multiple investigations, involving human autopsy samples or animal models or even in vitro studies with neuronal cell lines, have shown conclusively that JEV infection leads to massive neuronal death. Initial human autopsy studies had identified severe damage to nerve cells and the brain parenchyma including minute necroses, softening, and perivascular cuffing. The majority of the lesions were observed in the diencephalon and the mesencephalon [62]. These observations were confirmed from investigations of several autopsy samples in the early 1960s. It was observed that these changes were scattered widely from the cerebrum, cerebellum, and brain stem to the spinal cord and most prominently in the cerebral cortex, thalamus, and substantia nigra. Direct neuronal death due to JEV infection has been

shown to be apoptotic in nature. The tumor necrosis factor receptor (TNFR)-associated death domain (TRADD) has been suggested to be the crucial signal adaptor that mediates all intracellular responses from TNFR-1. Using an in vitro approach, it has been shown that the altered expression of TNFR-1 and TRADD following JEV infection regulates the downstream apoptotic cascades [81, 82]. Recent evidence also indicates that the NS3 helicase and protease domains of JEV may activate caspase-9-/caspase-3-dependent and caspase-9-/caspase-3-independent cascades to trigger cell death [83]. However, even though the infected neurons eventually die, evidence suggests a possible intracellular antiviral response or an innate immune response against the virus. In cultured neuroblastoma cell lines, it has been reported that the far upstream element (FUSE) binding protein 1 binds with JEV 5′ and 3′ untranslated regions and functions as a host anti-JEV defense molecule by repressing viral protein expression [84]. The innate immune reaction is mounted following viral recognition through retinoic-acid-inducible gene I (RIG-I) and its downstream adapter stimulator of interferon gene (STING; less commonly known as MPYS or ERIS or TMEM173 or MITA) [85, 86]. The role of toll-like receptors in the mounting of an innate immune defense mechanism against JEV in neurons is yet unknown but possible. Another interesting observation that points to the virus's immune evasion strategy was the activation of autophagy in neuroblastoma cell lines. It was found that JEV infection leads to the accumulation of microtubule-associated protein 1 light chain 3-II protein and GFP-LC3 puncta in vitro and an increase in autophagosomes/autolysosomes in vivo. The fusion between autophagosomes and lysosomes was shown to be essential for viral replication [87].

Even in the early days of investigation, it was known that this disease was accompanied by inflammation in the CNS. The overall inflammatory changes in the CNS involved a marked increase in the number of glial cells. This led early investigators to believe that Japanese encephalitis was a "generalized toxic inflammation." However, it should be kept in perspective that this was before the isolation of the causative agent for the disease, i.e., the JEV. The brain is a unique organ where the movement of cells or molecules is restricted by the BBB. The selectively permeable nature of the barrier makes the brain somewhat "immunocompromised," even though that concept has been critically challenged in the past decade or so. Though the fact that immune cells from the periphery do infiltrate into the CNS at different stages of the disease, the initial inflammation is now known to be due to the activity of resident immune cells, i.e., the glia. Microglia and the astrocytes have been reported to play crucial roles following JEV infection. In animal models as well as in vitro models of JE, it has been reported that there is microglial activation characterized by distinct morphological changes along with heightened release of proinflammatory cytokines and chemokines such as TNF-α, IL-6, MCP-1, IFN-γ and IL-1β, and other mediators. Region-specific analyses showed that the release of these mediators was highest in the hippocampus region [88]. This inflammatory milieu in the brain has severe detrimental effect on neurons, leading to their death. Neuronal death also acts as a stimulator for further microglial activation, thereby creating a vicious cycle. Even though it is difficult to ascertain the extent of direct viral killing or a "bystander" death, the net effect of JEV infection remains neuronal death. Astrocytes, on the other hand, also respond to the infection by increasing

cytokine production, lactic acid release, and glucose mobilization [89] even though this does not confer significant neuroprotection [90].

The role of microRNAs (miR) in disease progression and modulation is currently an area in which a lot of research has been focused. Clues from human postmortem JE cases suggest that miR-155 may be involved in the modulation of neuroinflammatory response during JEV infection via negative regulation of Src homology 2-containing inositol phosphatase-1 (SHIP1) expression [91]. Also, in vitro studies have shown that by inhibition of the anti-inflammatory tumor necrosis factor alpha-induced protein 3, miR-29b regulates JEV-induced microglia activation [92].

3.4 JE from a Clinical Perspective

Infection with JEV may be asymptomatic or manifest as a mild febrile illness, aseptic meningitis [93–95], or classic severe meningomyeloencephalitis. The incubation period of JE varies, and a recent meta-analysis of various published record shows that in JE the median incubation period is 8.4 days (95 % CI: 5.1–9.4) which falls within the 5–15-day incubation period often referenced [96]. The average case fatality rate is approximately 25 %, with 50 % having neuropsychiatric sequelae and 25 % recovering fully. Long-term sequelae in survivors include weakness, ataxia, tremors, athetoid movements, paralysis, memory loss, and abnormal emotional behavior [97]. Based on the clinical observations, the disease can be conveniently classified into three stages:

(a) *A prodromal stage with clinical features common to many other unrelated diseases, which precedes any signs of involvement of the CNS*

The essential features of the prodromal stage are generalized malaise, headache, and fever often associated with nausea. These symptoms are common to various other diseases that are not even related to flaviviral infections, and thus, a clinical diagnosis at this stage is practically impossible. A good example is the characterization of some US military personnel serving during the Korean conflict, to be suffering from war neurosis when they were actually infected with JEV [98]. The onset of this stage may be abrupt (1–6 h), acute (6–24 h), or subacute (2–5 days). In more than 75 % of patients, the onset is subacute. Although spontaneous recovery (the so-called abortive encephalitis) is known following this stage, the disease usually progresses to the acute encephalitis phase [99].

(b) *An acute encephalitic stage marked by CNS signs and hyperthermia*

The acute encephalitis stage is marked by continuous fever, nuchal rigidity, convulsions, altered sensorium progressing in many cases to coma, focal CNS signs, polymorphonuclear leukocytosis in the peripheral blood, and CSF changes marked by pleocytosis with a normal or raised glucose or protein content and the presence of immune complexes [100, 101]. Seizures occur in approximately 85 % of children and 10 % of adults with JE [102]. Continuous unremitting seizures lasting longer than 30 min (status epilepticus) or multiple recurrent seizures are common in JE. Also, subtle motor status epilepticus, in

which the only clinical manifestation might be the twitching of a finger or eyebrow, is common in JE [103]. Approximately 50 % of the patients with JE suffer from a high CSF opening pressure. Brain swelling is a common feature that is observed during autopsy [65], with cerebellar tonsillar and hippocampal uncal herniation observed in some cases [104]. Multiple uncontrolled seizures may be associated with this raised intracranial pressure.

Movement disorders are common in JE, both in the acute encephalitis stages and also in survivors with neuropsychiatric sequelae [105]. The characteristic features include masklike faces, abulia, tremors, and cogwheel rigidity that bear striking similarity to Parkinson's disease so much so that it had even led to the generation of a JEV-induced rat model of Parkinson's disease [106]. Other movement disorders include generalized rigidity, jaw dystonias, opisthotonus, choreoathetosis, orofacial dyskinesias (involuntary tongue protrusions), oromandibular dystonia, myoclonic jerks, and opsoclonus myoclonus [107, 108]. The role of the basal ganglia, particularly the thalamus and the substantia nigra, has for long been considered to be significant in eliciting such responses [109, 110].

Magnetic resonance imaging techniques, such as diffusion-weighted imaging and magnetic resonance spectroscopy, have been utilized by several investigators to correlate the clinical features of JE [111]. MRI reveals prominent changes in the thalamus, basal ganglia, substantia nigra, cerebellum, pons, cerebral cortex, and spinal cord. These MRI lesions are generally hypointense on T1 and hyperintense on T2 and on fluid attenuation inversion recovery (FLAIR) sequence. The thalamic lesions may be of mixed intensity on T1 and T2 in the subacute stage and may suggest hemorrhagic changes. Follow-up MRI after several months reveals shrinkage of acute lesions which are hypointense on T1 and T2 sequences [112]. In a comparative study of CT and MRI, CT scan was abnormal in 55.3 %; MRI was abnormal in all patients and revealed lesions in the thalamus in 94 %, basal ganglia in 35 %, midbrain in 58 %, pons in 26 %, and cerebellum and cerebral cortex in 19 % each [113, 114]. In JE, involvement of the temporal lobe has also been reported in approximately 17 % of the patients, but all of them had thalamic and substantia nigra involvement [115]. Abnormal high-intensity lesions in the bilateral pulvinar and gray matter, with an abnormal appearance mimicking the pulvinar sign, were observed by diffusion-weighted brain magnetic resonance imaging [116]. Single-photon emission computed tomography (SPECT) analysis of JE patients shows thalamic hyperperfusion in the acute stage which is replaced by hypoperfusion in the subacute or chronic stage [117, 118]. EEG recordings during the acute stage were found to be grossly abnormal. The outstanding features are diminution of electrical activity, dysrhythmia, and slowing with periodic lateralized epileptiform discharges (PLEDS). In some patients, intention tremors and ataxia that are indicative of cerebellar involvement are observed. Other focal neurological signs include that of encephalomyelitis, such as cranial nerve palsies, upper motor neuron weakness (in 30–50 % of patients), and flaccid limb weakness, with reduced or absent reflexes, which is often associated with respiratory or bulbar paralysis [119]. The combination of upper and lower motor neuron

damage can lead to bizarre mixtures of clinical signs that can change hourly during the acute stage [120]. JEV can also cause a poliomyelitis-like acute flaccid paralysis, in fully conscious patients [121].

(c) *A late stage marked either by complete recovery or the persistence of mild to severe cognitive dysfunction or motor disabilities for several years or entire life, as a result of irreversible CNS damage*

The late stage of the disease begins when active inflammation is at an end, i.e., when the body temperature is normal and the neurological signs are stationary or tending to improve. When the encephalitic stage is short, recovery occurs rapidly and the patient becomes normal within 2–4 weeks from the onset of illness. However, a prolonged encephalitic stage corresponds to slower recovery or prolonged sequelae of the disease. The neuropsychiatric problems in the survivors (in about 50 % cases) include learning and memory deficits, behavioral abnormalities, and speech disorders. These observations have been the result of several studies over the years [122–128]. However, all these studies have been either in children or young adults, the number of post-JE cases was small, and the different types of observed neuropsychiatric disorders were highly variable. A 14-year follow-up study with 688 adult survivors of the disease has recently characterized the symptoms that are commonly seen at this stage [129, 130]. This study observed that nearly 97 % of all the survivors suffered from neurological deficits during the time of their initial discharge from the hospital. Of the remaining 3 % who were discharged without any apparent deficits, about 20 % of the patients had to be readmitted within a couple of months for bizarre movements of the limbs without any other apparent symptoms associated with the acute phase of JE, such as fever and paralysis. However, these patients had assaultive behaviors, euphoria, and delusions of grandeur with mirror writing. They improved with symptomatic treatment but needed full constant care for 6 months and intermittent help for further 6 months before becoming normal. It was also observed that subjects suffered from seizures; psychological disturbances, such as delusions (both persecution and grandeur, suggestive of diffuse involvement of the frontal and temporal lobes), visual hallucinations, and euphoria; and emotional instability (in the form of spontaneous laughing or crying). Classical features of depression were also noticed. When these subjects were treated with pharmacological agents (phenobarbitone and phenytoin for seizures; chlorpromazine along with diazepam for psychological disturbances; amitriptyline for depression), the rate of alleviation of these disorders was very high. The subjects were also diagnosed with impaired intelligence associated with a general lack of concentration and impaired capacity for reasoning and judgment. Speech disturbances, including dysphasia; dyscalculia; apraxia; agnosia; and constructional apraxia were noted initially but these features gradually diminished between 5 and 14 years of follow-up. The extrapyramidal and pyramidal features present in many of the subjects were reduced due to proper rehabilitation. A new observation that came out of this study was hypothalamic disturbances, which included sleep, appetite, and libido. However, most of these features regressed to normal values over different time periods.

The cellular or molecular basis of the persistence of these changes is not well understood. Since JEV predominantly infects children who are in a dynamic state of brain development, any insult on the CNS may have consequences later in life. Since JEV infection leads to massive neuronal death, effective CNS repair processes which restore the neuronal loss are imperative for complete recovery from JE. In the postnatal/adult CNS, neuronal regeneration is primarily dependent on the pool of neural stem/progenitor cells (NSPCs) and their ability to generate cells of both neuronal and astrocytic lineage. It is hypothesized that JEV infection and the associated inflammation disrupt the NSPC pool in the germinal niches and their efficacy of generating functional neurons, thereby stalling neuronal repair. The lack of functional CNS repair/regeneration possibly culminates in long-term neurological consequences in JE survivors. In animal model and in vitro models of JE, it has been shown that NSPCs are permissive to infection, which leads to their growth retardation. The pathophysiological relevance of these observations was supported by a profound decrement in actively proliferating NSPCs in the subventricular zone (SVZ) of JEV-infected animals. Infection of the NSPCs and suppression of their proliferation may be primarily responsible for dysregulated neurogenesis and the development of cognitive deficits in survivors of JE [131, 132].

3.5 Other Complications Associated with JE

Most of the symptoms associated with JE are also common in various other diseases. Due to the close genetic similarity of flaviviruses, some or most of these clinical characteristics are common to all human flaviviral infections, thereby making differential diagnosis difficult. Other than flaviviral infections, encephalitis due to other viral infections or nonviral causes may also be characterized with some of these features. JE has also been associated with other diseases. Cysticercosis is a risk factor for JE that is attributed to the disruption of the BBB [133, 134]. JEV infection may also predispose to Guillain-Barré syndrome in endemic areas [135]. Inflammation-mediated damage to the spinal cord is not a common feature associated with JE. However, there have been case reports of acute transverse myelitis affecting spinal segments in patients who were found to be seropositive for JEV antigen [136, 137]. Mild encephalopathy with a reversible splenial lesion has also been reported from a single patient [138]. A recent report also characterizes acute disseminated encephalomyelitis in an adult patient 21 days post recovery from JE. The clinical symptoms and abnormal brain lesions on MRI improved gradually after the combination of high-dose intravenous methylprednisolone and oral steroid therapy [139]. Cerebral venous sinus thrombosis, or presence of blood clot in venous (dural) sinuses into which venous blood from the brain flows via the deep cerebral veins, is an uncommon phenomenon with unknown etiology in 20–25 % of patients. This has also been reported from a male patient in China who had also tested positive for JE [140].

4 Therapeutic Approaches to JE

Prophylactic approaches to counter JEV have been in practice for many decades now and have proved quite effective in controlling this disease. A detailed description of the current vaccines that are in use for the prevention of JE can be found in the recent open-access review article by Yun and Lee [141]. However, despite vaccination attempts in several countries by various government as well as nongovernment agencies, cases of JE are still being reported every year. A therapeutic intervention to cure the disease is thus necessary to effectively counter JE. The common approach to hunt for a cure against any pathogenic organism or entity is targeted in two basic avenues: (1) interfering with the organism's entry into the host cell and (2) disrupting the host cell machinery, so as to prevent its replication within the cell. Over the years, innumerous efforts have been made and are still being made to find an effective chemotherapeutic agent to effectively cure JE. A detailed discussion of all such endeavors is beyond the scope of this chapter. But since the cures have been targeted keeping in mind the pathogenesis of the disease, a brief description is merited for a better understanding of the disease as a whole. In the following few pages, we have tried to encompass all known therapeutic endeavors against JE to date. These have been categorized according to the supposed mechanism of action of these agents. As the readers will note, these agents vary from natural compounds to synthetic drugs/chemicals or even oligonucleotides targeted for antisense therapy. Investigators are even in the process of synthesizing derivatives containing the backbone of naturally occurring lupin alkaloid and sparteine, which act as protein secondary structure mimetics, and show that these compounds exhibit antiviral properties [142]. A point to be noted here is that the drugs/compounds mentioned in the following tables are arranged merely in alphabetical order and thus bear no relation to their potency or efficacy in treating JE.

Table 1 depicts some of the drugs/compounds that have been investigated as viral replication inhibitors. Of the following compounds, ribavirin has been tried in a human clinical trial but with little or no promising results [143].

Because CNS inflammation is a hallmark of JE, much effort has been concentrated in modulating inflammatory processes that could limit neuronal loss in the brain. Also, since neuronal death in JE has been proven to be apoptotic in nature, it is quite natural that antiapoptotic agents would also be considered as potential therapeutic agents. Since neuronal death itself acts as a secondary trigger for inflammation in the brain, anti-inflammatory and antiapoptotic drugs/compounds have been included in Table 2.

Of all the compounds mentioned in Table 2, minocycline is currently undergoing a phase II double-blind clinical trial in JE patients. This trail has been already registered to the Clinical Trials Registry of India [registry # CTRI/2010/091/006143]. More details regarding this can be obtained from http://ctri.nic.in/Clinicaltrials/showallp.php?mid1=2529&EncHid=&userName=minocycline. The study is currently in the process of patient recruitment. Out of 250 adults screened, 86 have been enrolled, and out of 445 children screened, 148 have been enrolled for this trial to date.

Table 1 Viral replication inhibitors

Name of compound	Chemical nature	Mechanism of action predicted to be associated with JE
– [144]	microRNA-like polycistrons	Single microRNA-like polycistrons simultaneously expressing effective siRNAs under a single RNA polymerase II promoter to inhibit JEV replication
2-Deoxy-D-glucose and 3-deazauridine [145–147]	Competitors of glucose and uridine	Interference with the synthesis of JEV glycoprotein, DNA, and RNA
2-Methylnaphtho[2,3-*b*] furan-4,9-dione 2-(1-Hydroxyethyl)-analog of naphtho[2,3-*b*] furan-4,9-dione 2-Methyl-5(or 8)-hydroxy-analog naphtho[2,3-*b*] furan-4,9-dione [148]	Furanonaphthoquinone derivatives	Inhibits replication through inhibition of viral RNA and protein synthesis
4-Hydroxypanduratin A [149]	Secondary metabolite of the plant *Boesenbergia pandurata*	Based on computational modeling; predicted to inhibit NS2B/NS3 protease activity that is essential for polyprotein processing and replication
DNAzyme [150]	Single-stranded oligodeoxynucleotides with Mg^{2+}-dependent enzymatic activity	Cleaves the direct repeat sequences within the 3′-noncoding region of JEV RNA, leading to inhibition of virus replication
Monocyte chemoattractant protein 1-induced protein 1 [151]	RNA-binding nuclease	Possesses RNAse activity that targets and degrades viral RNA
Mycophenolic acid [152]	Immunosuppressant drug metabolized from mycophenolate(derived from the fungus *Penicillium stoloniferum*)	Probable replication inhibitor; detailed mechanism lacking
Nitazoxanide [153]	Thiazolide antiprotozoal agent	Speculated to target host functions that are essential for JEV replication
Pentoxifylline [154]	Methylxanthine derivative	Probably by hampering the virus assembly and/or release

(continued)

Table 1 (continued)

Name of compound	Chemical nature	Mechanism of action predicted to be associated with JE
Pokeweed antiviral protein [155]	N-glycosidase ribosomal-inactivating protein isolated from *Phytolacca americana*	Inhibits replication by depurination of JEV genomic RNA
PPMO [156]	Peptide-based morpholino oligomers	Blocks JEV 3′ cyclization sequence, thereby inhibiting replication
Ribavirin [157, 158]	Guanosine analogue	May act by inhibiting inosine monophosphate dehydrogenase, required for de novo guanine synthesis
SCH-16 [159]	*N*-methylisatin-β-thiosemicarbazone derivative	Yet to be defined
shN8010 [160]	Small hairpin RNA (shRNA) against viral NS5	Blocks activity of viral NS5 which is a major component of the viral RNA replicase complex associated with the 3′ noncoding region of genomic RNA in the initiation of viral replication
Suramin [161]	Polysulfonated naphthylurea compound	Inhibit replication by blocking production of viral E and NS3 proteins
Vivo-morpholino [162]	Octagunidiumdendrimer-based morpholino oligonucleotide	Targets 3′ or 5′ untranslated regions of the JEV genome, thereby inhibiting replication

Interferon-α is a glycoprotein cytokine that is produced naturally by immune cells of the body in response to viral infections, including JEV, and has been seen as a promising antiviral candidate [172]. By themselves, interferons are not directly antiviral but induce production of effector proteins in cells, which inhibit various stages of viral replication, assembly, or release [173–176]. Table 3 lists the potential anti-JE actions of interferon-α or interferon inducers.

Contrary to its promising in vitro results, in real life, interferon-α had no significant effect in alleviating JE pathology. Two clinical trials have been conducted using recombinant human interferon alpha to date, but with no beneficial effect in JE. It may be effective at a higher dose or through a different route or in combination with other drugs. However, the resulting cost of treatment may be prohibitive in developing countries within the JE endemic region [181, 182].

The final group of compounds ranges from hormones to extracts of natural compounds to recombinant proteins (Table 4). All of these have been shown to be effective to varying extents in vitro or in vivo, but none is close to reaching clinical trials at the current stage.

Table 2 Anti-inflammatory and/or antiapoptotic substances

Name of compound	Chemical nature	Mechanism of action predicted to be associated with JE
Aspirin Indomethacin Sodium salicylate [163]	Nonsteroidal anti-inflammatory drugs	Cyclooxygenase inhibitors; modulates intracellular MAP kinase pathway following JEV infection
Arctigenin [164]	Phenylpropanoid dibenzylbutyrolactone lignan	Decrease neuronal apoptosis, microglial activation, active caspase activity, and induction of proinflammatory mediators in the brain
Etanercept [165]	TNF-α inhibitor	Reduces neuroinflammation and restores BBB integrity
Fenofibrate [166]	Peroxisome proliferator-activated receptor-α agonist	Helps in detoxification of the potent proinflammatory eicosanoid leukotriene B(4) and inhibits other proinflammatory activity
Minocycline [60, 69, 167–169]	Semisynthetic tetracycline antibiotic	Reduces neuronal apoptosis, microglial activation, active caspase activity, proinflammatory mediator release in brain, ameliorates intracellular oxidative stress, and decreases viral titer; imparts protection to blood–brain barrier
Rosmarinic acid [170]	Polyphenolic compound	Reduces viral replication in mouse brain; ameliorates secondary inflammation resulting from microglial activation
Tilapia hepcidin 1–5 [171]	Antimicrobial peptide from tilapia fish (*Oreochromis mossambicus*)	Reduces neuronal apoptosis, microglial activation, and reduces proinflammatory mediators.

Table 3 Interferon and interferon inducers

Name of compound	Chemical nature	Mechanism of action predicted to be associated with JE
Aloe emodin [177, 178]	Anthraquinone	Upregulates the expression of interferon-stimulated genes such as dsRNA-activated protein kinase and 2′,5′-oligoisoadenylate synthase; activates of nitric oxide production
Carboxymethylacridanone [179]	Acridine derivative	Acts probably by upregulating expression of interferon-stimulated genes
Interferon alpha [180]	Glycoprotein cytokine	Critical part of the innate immune facet; leads to induction of effectors which affect viral survival

Table 4 Other compounds with varying mechanism of actions

Name of compound	Chemical nature	Mechanism of action predicted to be associated with JE
Astragali radix [183–185]	Extract from the root of *Astragalus membranaceus* Bunge; consists of polysaccharides and flavonoids	Protective effect possibly due to nonspecific mechanisms
Bafilomycin A1 [186]	Macrolide antibiotic isolated from *Streptomyces griseus sulphureus* sp.	Inhibits vacuolar-type proton pump; inhibits pH-triggered membrane fusion of endocytosed JEV thereby preventing replication
Baicalein [187]	Flavonoid	Prevents viral adsorption to cells; may be anti-replicative
Cholesterol [188]	Steroid metabolite	Prevents JEV infection possibly by hindering viral entry at fusion and RNA uncoating steps
Curcumin [189]	Natural polyphenolic compound	Inhibits production of infective viral particles by the inhibition of UPS; decreases cellular reactive oxygen species level; restores cellular membrane integrity; decreases pro-apoptotic signaling molecules
Dehydroepiandrosterone [190, 191]	Adrenal-derived steroid hormone	Probably by modulating signaling pathways of extracellular signal-regulated protein kinase
Diethyldithiocarbamate [192]	Organosulfur compound	Possibly by production of nitric oxide via induction of iNOS activity that is meditated by circulating macrophage-derived factor
rEDIII [193, 194]	Domain III of recombinant virus envelope protein	Inhibits viral entry to cells
Filipin III [188]	Part of polyene macrolide antibiotic isolated from *S. filipinensis*	Chelates cholesterol; inhibits replication and viral entry into host
Griffithsin [195]	Antiviral peptide of algal origin	Believed to inhibit entry of the virus into cells at early stages
Heparan sulfate from shrimp (*Penaeus brasiliensis*) head [196]	Proteoglycan	Yet to be defined in detail
Indirubin [197]	Compound from methanolic extract of *Isatis indigotica*	Inhibits viral attachment with cell membrane

(continued)

Table 4 (continued)

Name of compound	Chemical nature	Mechanism of action predicted to be associated with JE
Lactoferrin [198]	Iron-binding glycoprotein	Inhibits JEV entry into host cell by binding directly to the virus particle or to membrane-bound heparan sulfate
Methyl-β-cyclodextrin [188]	Cyclic oligosaccharide	Disrupts lipid raft formation by depleting cholesterol; inhibits replication and viral entry into host
N-nonyl-deoxynojirimycin [199]	Glucosidase inhibitor	Blocks the trimming step of N-linked glycosylation
Nitric oxide [200]	Gaseous hormone	Possibly inhibits viral replication; yet to be defined in detail
NSK [201]	A tripeptide found in loop 3 of domain 3 of JEV E protein	Inhibits viral entry into cells
P3 [202]	Peptide inhibitor of domain III of JEV envelope protein (E)	Intervenes between the interaction of E DIII and its cognate host cell receptor
PI 88 [203]	Sulfated phosphomanno-oligosaccharide	Causes steric hindrance to JEV attachment to host cells; may possess immunomodulatory activity
Sulfated polysaccharide extracts from *Ulva lactuca* [204]	Sulfated polysaccharide extracts from *Ulva lactuca* (algae)	Blocks viral adsorption in cells; may act as anti-inflammatory compound

5 Conclusion

The availability of prophylactic measures against JEV has not been entirely successful in deterring the spread of this disease or affecting susceptible individuals in epidemic zones. Most of this sad fact can be blamed on poor vaccination strategies or lack of general awareness in the populace regarding the severity of this disease. Thus, even with a century of knowledge, there are still parts of the world where frequent epidemics of JE are common. A coordinated strategy needs to be promulgated involving all concerned agencies and individuals dedicated to countering this disease.

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The Biology and Clinical Consequence of Infection with the Human Polyomavirus JCV

G. von Geldern, M.J. Barhams, and E.O. Major

Abstract This chapter gives an overview over the biology and the clinical consequences of infection with the human polyomavirus JCV. Current data as well as those aspects that are not yet fully understood are explained.

JCV virus is a human polyomavirus that leads to an asymptomatic infection in a large percentage of healthy individuals and can lead to a latent infection of the kidneys or the bone marrow. JCV reactivation and lytic infection of oligodendrocytes in the brain lead to a devastating demyelinating disease called progressive multifocal leukoencephalopathy (PML). PML typically occurs in patients with an impaired cellular immune response due to an underlying disease such as human immunodeficiency virus (HIV) infection or a systemic malignancy or due to treatment with immunomodulatory therapies. While supporting the recovery of the patient's immune system (either by giving antiretroviral therapy in HIV-infected patients or by stopping the immunomodulatory therapies leading to PML) is currently the only known effective therapeutic intervention, the response of the immune system can lead to clinical and radiographic worsening known as immune reconstitution inflammatory syndrome (IRIS). The clinical and biological consequences of neuroinflammation in this viral infection are presented in this chapter.

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1 Introduction

In 1972, two different human polyomaviruses were isolated from a lymphoma patient with a CNS demyelinating disease: JC virus (JCV) from the brain of a patient with progressive multifocal leukoencephalopathy (PML) and BK virus (BKV) from a kidney transplant recipient with fulminant polyomavirus-associated nephropathy (PVAN). Both viruses were named from the initials of the patients from whom they were isolated. Although JCV and BKV were shown to be nearly 75 % homologous at both the nucleotide and amino acid level, these new polyomaviruses demonstrated very different characteristics for kinetics of growth, host range, and pathologies [1]. Both JCV and BKV infections are almost exclusively found in individuals with impaired cell-mediated immune responses. Since then, there have been at least four more human polyomaviruses identified including Merkel cell from rare skin carcinomas, Ki and Wu from the respiratory tract, and Trp from trichoplasia skin lesions [2]. All human polyomaviruses, however, are globally ubiquitous, infecting the majority of the population in the early years and maintaining latency in nearly half the population as evidence of viral DNA identification in urinary excretions. This is a common feature of all primate, rodent, and avian polyomaviruses. JCV however is the only human polyomavirus that is predominantly neurotropic and neurovirulent, targeting the myelin-producing cell in the human brain causing a slowly progressing lytic infection of the oligodendrocyte resulting in widespread loss of myelin in the brain. Although many attempts have been made to model PML in rodents and nonhuman primates, there are currently no animal models to study JCV lytic infection. Also, there are no antiviral agents to inhibit JCV infection nor effective treatments for PML. Studies of the biology and pathogenesis of JCV in its human host remain difficult challenges for laboratory and clinical investigations.

2 The Biology of the JC Virus

2.1 Cellular Host Range and Molecular Factors for Productive Infection

Epidemiological studies indicate that seroconversion to positive antibody status occurs early in childhood starting at 4–5 years with increasing prevalence in the population. By the fifth decade, 60–75 % of the population is seropositive although

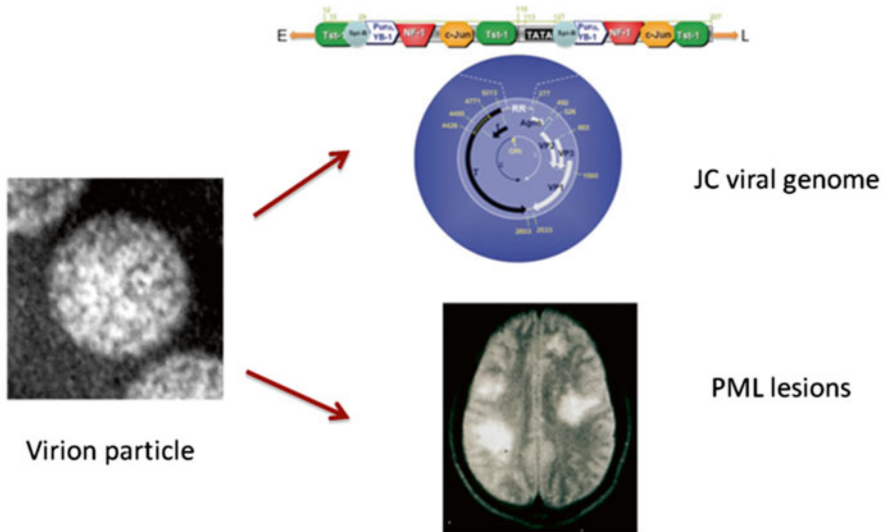

Fig. 1 JC virus characteristics. On the *left*, an electron microscopic picture of a virion particle is shown. The structure of the JC viral genome is depicted on the *right upper part* of this figure with the regions encoding for the early T protein, the noncoding regulatory region (promoter enhancer), and the late capsid proteins VP1, VP2, and VP3. The *lower right corner* of the picture shows lesions caused by JCV as seen on an MRI

antibody levels can rise or decline over time [3]. It is obvious, however, that initial infection that accounts for seroconversion or maintaining high titers of antibody does not occur in the brain. JCV can establish latency in uroepithelial cells and be excreted into the urine at high concentrations without pathogenic consequences unlike BKV. There is no clear understanding of this observation. What is interesting, however, is the genotype of the urine-excreted variant. In Fig. 1, the JCV genome is shown as a closed circular, double-stranded DNA of 5.13 kilobases, packaged into a naked, icosahedral 40-nm virion particle. There are two protein-coding regions for the early proteins, T and t, that have multiple functions for DNA replication and control over cellular metabolism that are synthesized from transcripts on one strand. Capsid proteins VP1, VP2, and VP3 are synthesized from transcripts on the opposite strand. The intergenic region between the 5′ start sequences of the early and late genes is the noncoding regulatory region or NCRR whose nucleotides have the origin for DNA replication, the transcription binding protein sites, and enhancer sequences for transcription efficiency also seen in Fig. 2. In the urine-excreted or archetype variant, the nucleotide sequences show a linear 200-base-pair arrangement. In pathological tissues such as the brain and cerebrospinal fluid, the nucleotide sequences show direct tandem repeats of 98 or fewer nucleotides with frequent deletions and duplication known as the prototype variant. A number of DNA-binding proteins have been identified on the NCRR as shown in Fig. 1 extending from the origin of DNA replication to nucleotide 50 that are essential for virus growth including those for the TATA-binding proteins and NF-1 class X [4].

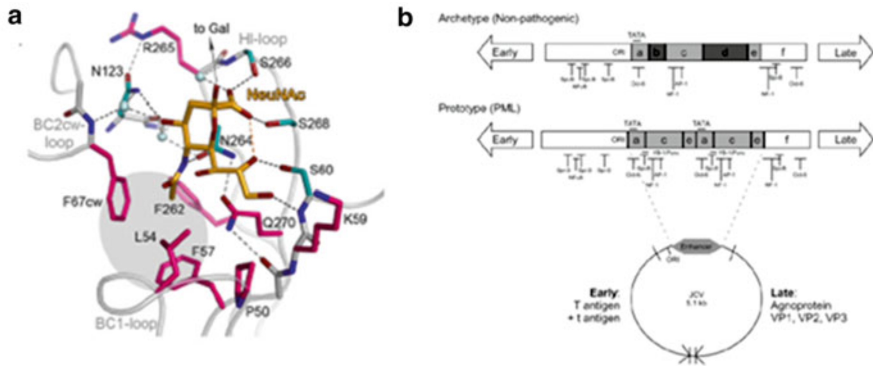

Fig. 2 Viral factors that may favor JCV infection. Part (**a**) shows the amino acid residues for the viral capsid protein VP1, which is responsible for binding to host cells; sequences for VP1 can be altered following latency. Part (**b**) shows two variations of the noncoding regulatory sequences. The nonpathogenic archetype, which is typically found in kidney tissue and contains no tandem repeats, is depicted *above*, whereas the pathogenic prototype, which is found in affected tissues in PML and which contains direct tandem repeat structures, is shown *below*

There has been considerable interest in determining the contributions to cellular host range between the NCRR and the capsid VP1 protein that binds cell receptors. The comparison between these two regions of the viral genome is shown in Fig. 2. The three-dimensional crystallographic structure shows the amino acid domains in color that interact with sialic residues on cell surface membranes for virion attachment [5]. In addition to sialic acids, the key receptor for JCV, the 5HT2A serotonin receptor plays a secondary role in attachments and particle entry [6]. Virions enter the cell cytoplasm and traffic to endosomes using clathrin-coated pits and are then transported to the nuclear membrane as disrupted particles. Viral DNA enters the nucleus that is then transcribed using host DNA-binding factors starting with early region for T proteins. The NCRR plays a key role in viral susceptibility since it contains the transcription binding sites for factors that initiate T protein mRNA and synthesis. The T protein then binds the origin of DNA replication on the 5′ region of the NCRR that interacts with cellular DNA alpha polymerase to form protein complexes for viral DNA synthesis. It is thought that an increase in the amount of viral DNA then allows transcription of mRNA for viral capsid proteins and then virion assembly—all in the nucleus. The current working models suggest that those factors that are necessary for JCV growth in uroepithelial cells bind to the archetype arrangement of the NCRR and those factors for JCV growth in glial cells and some immune system cells bind to prototype arrangement of the NCRR that leads to lytic infection and PML [7].

In cell culture studies and in clinical tissue samples, JCV multiplication is evident in oligodendrocytes and astrocytes in the brain and in CD34+ and CD19+ cells in the bone marrow and peripheral blood, as well as stromal cells in tonsils and B cells in the spleen [8, 9].

2.2 Presence of JCV in Infected Individuals

Without an animal model of JCV lytic infection resulting in PML, tracking virus in tissues requires samples mostly from infected patients, notably those with PML. Nothing is known about the initial site or timing of infection. However, since the majority of the population worldwide demonstrates antibodies to JCV, it is assumed that respiratory inhalation and/or ingestion is the primary route of infection. There are several major techniques used to identify JCV in tissues: quantitative polymerase chain reaction, qPCR, and in situ DNA hybridization that detects the viral genome and immunocytochemistry for viral proteins [10]. For the laboratory confirmatory diagnosis of PML, real-time qPCR assays are done detecting viral DNA in the cerebrospinal fluid of suspected PML patients. One assay currently used at the National Institute of Neurological Disorders and Stroke (NINDS), a validated/certified assay in the Laboratory of Molecular Medicine and Neuroscience (LMMN), is the multiplex qPCR that not only quantitates the level of viral DNA but also distinguishes whether the JCV variant is the more pathogenic prototype from the non-pathogenic archetype genotype [11]. Typically in PML patients, the brain and CSF will show the prototype, while the urine will show the archetype. Plasma or serum of patients may have both but, during the course of active infection leading to PML, will shift from archetype to prototype. In HIV patients, approximately 20 % can be viremic at some time although only 3 % will develop PML. Approximately 2 % of healthy individuals may be viremic at some time point and seem to clear infection. This observation is not surprising since JCV is ubiquitous in all parts of the world. It is surprising, however, that nearly 30 % of the population excretes JCV in urine without pathological consequences.

3 The Pathogenesis of JCV Demyelinating Infection

3.1 Viral Variants and Latency

The principal cells in which JCV DNA has been detected during infection are the uroepithelial cells in the kidney, CD34+ cells in the bone marrow, CD20+ cells in peripheral blood, and oligodendrocytes in the brain. Figure 3 shows nuclear location of replicating DNA using in situ DNA hybridization with a highly specific viral DNA probe and diaminobenzidine as the chromophore (brown) staining [10]. The bone marrow biopsy shown in Fig. 3 was taken 4 years prior to the development of PML in a Wiskott-Aldrich syndrome patient. The NCRR of the variant in the bone marrow was the prototype and nearly identical to what was seen in the B cells and brain taken at the time of PML diagnosis. Observations such as these and in other cases have led to the hypothesis that bone marrow may harbor latent JCV in hematopoietic cells for long periods [12]. If such latently infected cells migrate into the peripheral circulation and differentiate toward a B-cell lineage, then it is possible

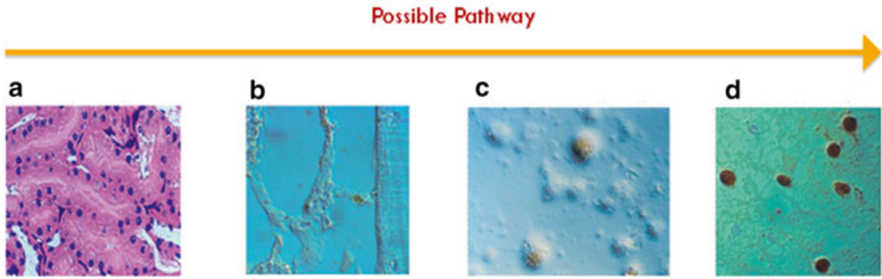

Fig. 3 Possible pathway of JCV in the body. Depicted are tissues shown to contain JC virus. (**a**) Shows the kidney tissue (which usually contains archetype JCV), **b** shows the bone marrow (which usually contains prototype JCV), **c** shows the peripheral blood (which can contain archetype and/or prototype), and **d** shows oligodendrocytes in the brain (which contain prototype)

that JCV can reactivate to slowly multiply as such cells begin the expression of factors that favor viral growth [4]. This pathway is thought to account for the development of PML in multiple sclerosis patients treated with natalizumab, a monoclonal antibody that blocks the alpha 4 cell surface integrin VLA 4 to VCAM, vascular cell adhesion molecule, preventing homing of CD+ 34-positive cells in the bone marrow and extravasation of inflammatory cells into the brain [13]. Certainly, JCV maintains decades-long periods of latency in the kidney with the NCRR archetype variant. If that is the predecessor to the pathogenic prototype, then virions released from the kidney may find their way to lymphoid tissues in which genotypic alterations or rearrangements occur to produce the prototype NCRR. This is also a plausible pathway that may occur in some patients [13].

3.2 Trafficking of Virus from Sites of Latency to the Brain

There is little evidence of how JCV enters the brain from peripheral sites of infection. Two possibilities are possible and not mutually exclusive. Since cell-free virus can be found in the blood, it is possible that JCV can cross the blood-brain barrier and enter the brain parenchyma, directly infecting astrocytes and its primary target, the oligodendrocyte. JCV may also be carried into the brain within a cell such as B cells seen in blood. JCV does not seem to infect monocytes or T lymphocytes that bring other viruses into the brain like HIV-1 and cytomegalovirus (CMV). There have been reports of viral latency in the brain that reactivates due to lack of immune surveillance [14]. However, that observation does not address the low incidence of PML in the many thousands of patients with substantial immune suppression like AIDS, organ transplants, cancer, and rheumatic diseases. Mechanisms of viral entry into the brain remain mostly unknown, requiring better cell culture and clinical studies.

3.3 Predictive Markers for Active JCV Infection

Although PML is considered a rare disease using the NIH criterion for prevalence, of 200,000 cases, the reports of PML have increased substantially over the last 10 years. In fact, PML is now considered a complication of not only HIV-1 infection and an AIDS defining illness but also a risk associated with a number of therapies for other diseases [15]. Consequently assessing that risk becomes a critical issue for patient treatment. Investigations have focused on defining biomarkers that identify active JCV infection in high-risk patients. Three JCV-linked factors for active infection that have well-developed assays have been described including the presence and rise of antiviral antibodies, viremia during the course of a risk treatment, and identification of T-cell responses to JCV multiple antigens [16]. In addition to these markers, molecular factors needed for infection in specific target cells like DNA-binding proteins for viral mRNA transcription may have a useful role, but their detection has not been reduced to a practical assay methodology. Detection of specific antibodies to JCV is a marker for prior exposure. Any increase in the antibody levels would indicate either an active or recent infection. ELISA, enzyme-linked immunosorbent assay, is the most routine technique used to measure antibodies and their levels. There are several such assays in clinical use for JCV that both detect antibody and measure its levels using the viral VP1 capsid protein as the antigen [17, 18]. The VP1 protein makes up the majority of virion structure and receptor for cell attachment. Plasma/serum levels of antibody can be monitored over time in patients to determine not only serostatus but also whether there is a rise in antibody levels. Most frequently, patients about to enter treatment with a drug or biological with a PML risk would have antibody levels determined. Samples that test seronegative however can be confounding since antibody levels can change over time from negative to positive. And in some cases, patients may not develop detectable antibodies but still have been exposed or have an active infection [19, 20]. Consequently, other markers should be used to assess those patients' JCV status.

Recently, CD4 and CD8 T-cell responses have been measured using viral peptides across the entire viral genome. Healthy individuals and MS patients treated with natalizumab showed both T-cell responses to all viral proteins including the nonstructural T proteins and agno and all three capsid proteins. Some of these individuals who had T-cell responses did not have antibody [21]. Assays for T-cell responses require more blood than antibody assays and use flow cytometric analysis and cytokine measurement following viral peptide contact. However, these assays have become routine in many laboratories and so do not present a difficult obstacle for assessment. In combination with serology assays, measurement of cell immune response to JCV provides a more complete analysis of not only exposure but also the ability of the patient to mount an appropriate immune response.

The advantage of measuring immune responses is their relative stability over time. However, they do not reflect direct measurement of JCV infection at times of greatest risk for PML. The advances in qPCR methods that are specific to ultrasensitive detection of viral DNA, ten copies of the genome per ml of sample, have led to the

laboratory confirmation of PML in many cases that may not have been accurately diagnosed. The current multiplex qPCR assay developed at NINDS requires small volumes of blood, CSF, urine, or cells in tissue samples. As a risk-monitoring assay, detection of viral DNA in the plasma or serum in a patient with an underlying disease that is a risk for PML can lead to substantial concern. If the individual remains viremic over weeks or months, particularly with the pathogenic variant, and shows any increase in the amount of viral DNA, then the risk for PML is elevated. At present there are no data that statistically measures that risk, i.e., percent of patients with viremia who develop PML. However, most patients show viremia at the time of PML diagnosis. It is possible to monitor patients then over time using these markers, antibody levels, T-cell responses, and viremia measured from one blood sample. As more data are collected on these patient populations, it may be possible to use a checklist of factors that reflect the risk in which combinations of "checks" can become a quantifiable number.

4 The Clinical Pattern of JCV-Induced PML

4.1 Patients at Risk for PML: Underlying Diseases and Medications

Exposure to JCV occurs in a large proportion of the population [3, 22], but the virus is thought to lead to PML almost exclusively in individuals with an impaired immune system. Some case reports of patients with PML without any apparent immunodeficiency exist [23], but they are the exception, and in most cases, an occult immunodeficiency-like idiopathic CD4+ lymphocytopenia or liver cirrhosis is eventually discovered. However, not all immunocompromised patients develop PML. Some diseases or medications affecting the immune system are more commonly associated with PML than others, and understanding these connections may help us in our understanding of the pathogenesis of PML. The initial description of PML was in a patient with lymphoma [24], and the majority of published cases in the mid-1980s described lymphoproliferative diseases as the underlying disease [25]. The incidence of PML increased significantly with the beginning of the HIV epidemic in the 1990s, and despite antiretroviral therapy, PML is still seen in up to 5 % of HIV-infected individuals [26].

Additionally, since 2004, a number of drugs have been found to be associated with the development of PML. Natalizumab, a monoclonal anti-integrin antibody used for the treatment of MS, is perhaps the most prominent of these immunomodulatory agents found to lead to an increased risk of developing PML [27]. However, other immunomodulatory medications used for the treatment of rheumatological, neurological, or oncological diseases, especially monoclonal antibodies like rituximab, efalizumab, and brentuximab, have been reported to be associated with PML [12, 28]. Patients undergoing bone marrow or solid organ transplant have also been described to develop PML. These patients are often immunocompromised due to

the disease leading to transplant (e.g., kidney or liver failure, leukemia) and are furthermore often on multiple immunosuppressive agents. The exact rate of PML in these patients is difficult to assess. One multicenter study reported a rate of 1 in 1,000 patients undergoing lung or heart transplant [29]. It is challenging to get an accurate assessment of the actual risk associated with each of these underlying diseases or predisposing medications as PML is not a reportable disease, and our knowledge about the incidence and prevalence of PML is limited.

4.2 Diagnosis

An accurate diagnosis of PML can be challenging, especially in patients with other underlying neurological diseases like MS, which like PML is a multifocal demyelinating disease. In the early years of understanding the disease, the diagnosis of PML was usually based on brain biopsy showing typical histological findings (demyelination, multinuclear bizarre astrocytes, oligodendroglial nuclear inclusions; see below). Since JCV was identified as the virus causing PML, laboratory methods have been established that together with clinical and radiological findings can help establish the diagnosis of PML without the need for an invasive brain biopsy. A recent consensus statement from the American Academy of Neurology (AAN) Neuroinfectious Disease Section recommends to base the diagnosis of PML on a triad of compatible clinical features, compatible imaging findings, and a presence of JCV in the CSF by PCR. In cases where JCV can be detected in the CSF but the clinical or imaging findings are not typical, the AAN assigns a diagnosis of probable PML. If only clinical and imaging factors are present but the JCV in the CSF is non-detectable or if the CSF is positive but both clinical and MRI findings are atypical, a diagnosis of possible PML is suggested.

The typical clinical findings seen in PML are discussed in the next section of this chapter. Imaging used for the diagnosis of PML is mainly magnetic resonance imaging (MRI) of the brain. A recent study evaluating the MRIs of 22 patients with natalizumab-associated PML identified typical PML lesions as large (>3 cm), subcortical, T2 or fluid-attenuated inversion recovery (FLAIR) hyperintense, T1 hypointense, and diffusion hyperintense lesions, with a sharp border toward the gray matter and an ill-defined border toward the white matter on T2-weighted images [30]. An example of these typical MRI findings can be seen in Fig. 4. While PML lesions in HIV-infected individuals are usually not contrast enhancing, 41 % of the natalizumab-associated PML cases in this study were found to have contrast enhancement on the first scan at clinical presentation. However, these patients all had clinically diagnosed immune reconstitution inflammatory syndrome (IRIS). This complication is described in more detail in Sect. 5.1 of this chapter. Magnetic resonance spectroscopy of the brain has been suggested by some to add information, but most studies to date have not found this technique helpful [31, 32]. Positron emission tomography can in some cases help to differentiate between lymphoma and PML but is also not commonly used in the diagnosis of PML [32].

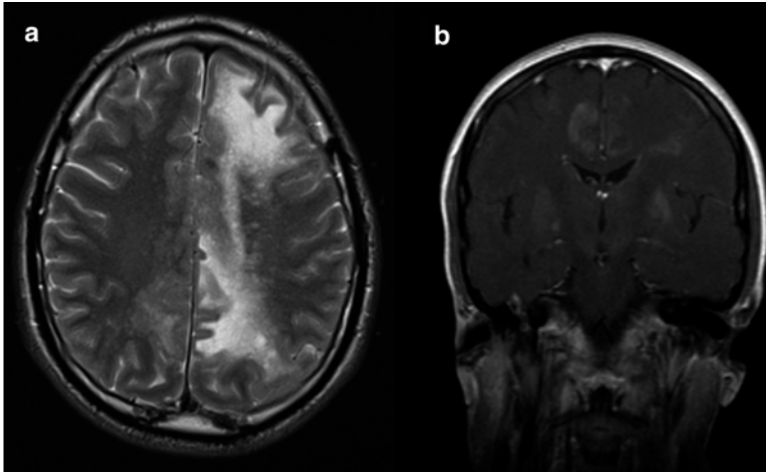

Fig. 4 MRI findings in PML. Panel (**a**) shows an axial T2-weighted image with large multifocal hyperintense lesions with an ill-defined border toward the white matter. Panel (**b**) shows a coronal T1-weighted post-contrast image which demonstrates faint, patchy contrast enhancement in multifocal PML lesions in a patient with IRIS

4.3 Clinical Manifestations

Symptoms in this multifocal disease can be varied, depending on the location of the lesions and the extent of demyelination. As in other brain diseases, patients can present with hemiparesis, ataxia, or visual changes. In contrast to other multifocal demyelinating CNS diseases like MS, optic neuritis and spinal cord involvement are not typically seen [33]. However, there are some rare reports of PML lesions in the spinal cord either by imaging or by histology at autopsy. Unlike MS but similar to acute disseminated encephalomyelitis (ADEM), another demyelinating syndrome more commonly seen in children, changes in alertness, behavior, and cognition are common in PML. In fact, about 50 % of patients with PML exhibit mental status changes at diagnosis [34]. Seizures are also frequently seen in PML and can be the initial presenting feature. About 20 % of patients in multiple different case series describing PML associated with HIV, lymphoproliferative disease, or immunomodulatory medications develop seizures, typically within the first few months, if not at presentation [34, 35]. This is somewhat surprising, since seizures are generally thought to arise from the cortical gray matter but PML patients with seizures are often found to have lesions immediately adjacent to the cortex.

4.4 Histopathology

While the development of JCV PCR detection in the CSF has replaced brain biopsy as the most important diagnostic tool in PML care, evaluation of brain tissue is still

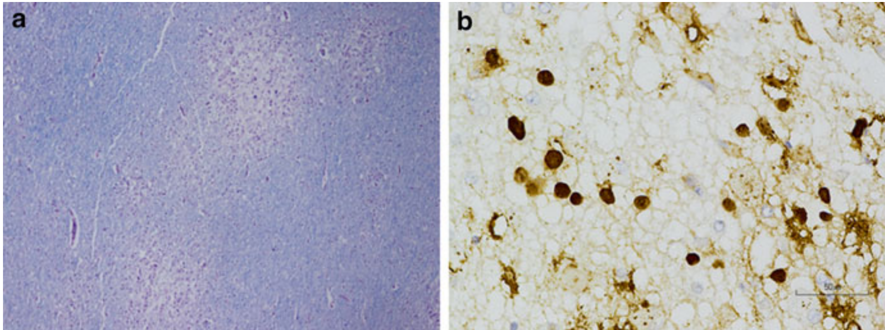

Fig. 5 Histopathology in PML. Panel (**a**) shows multifocal demyelination of the brain (hematoxylin-eosin staining). Panel (**b**) shows evidence of JCV detected by immunohistochemistry

done in cases of diagnostic uncertainty, especially when recurrence of lymphoma is a differential consideration or in cases of IRIS. Analysis of brain tissue can also increase our pathophysiological understanding of the disease. Biopsy or autopsy material of patients with PML is characterized by the combination of multifocal demyelination, bizarre astrocytes with hyperchromatic nuclei, and nuclear inclusions in oligodendrocytes in the white matter. When demyelination is extensive, necrosis can also be seen [36]. Lymphocyte infiltrates are not typically seen in PML lesions, unless there is presence of immune reconstitution (see the following section). Immunostaining can demonstrate presence of JCV capsid antigen, primarily in the white matter and typically in oligodendrocytes and to a lesser degree in astrocytes. Detection of JCV DNA in infected tissue with in situ hybridization or in situ PCR may be more sensitive and specific than immunohistochemistry [36]. In addition to causing the infection of oligodendrocytes characteristic for PML, JCV has also been reported by some to infect neurons, especially the granule cell neurons in the cerebellum. Histopathologically, granule cell neuropathy is characterized by hypochromatic granule cells with enlarged nuclei [37]. Significant gliosis is seen throughout the cerebellar cortex with relative sparing of the Purkinje cell and molecular layers [38]. Examples of typical demyelination and immunohistochemistry of autopsy brain tissue of a patient with PML are depicted in Fig. 5.

5 Treatment and Prognosis of PML

5.1 Inflammation and IRIS

In some patients with PML, rapidly worsening neurological symptoms, fever, and seizures develop. This clinical worsening is thought to be due to an improvement of immune function called immune reconstitution inflammatory syndrome (IRIS). IRIS occurs in 10–20 % of HIV patients that are started on antiretrovirals and in most patients with natalizumab (up to 90 %). HIV patients are at an increased risk

of developing IRIS if they are antiretroviral naïve and have a CD4 count of less than 50 cells/mm^3 [39]. IRIS typically develops about 3–12 weeks after antiretrovirals or plasma exchange to remove natalizumab are initiated, but in some cases, IRIS can develop up to 6 months after the initiation of antiretroviral therapy [42]. On MRI, there may be enhancement detected due to the local inflammation and breakdown of the blood-brain barrier. However, only in about 50 % of clinically or biopsy-diagnosed IRIS have contrast enhancement on MRI [39]. Edema or mass effect may also be seen on imaging. In the most severe cases, the inflammation and subsequent edema can lead to cerebral herniation and death. Analysis of brain biopsy samples of PML patients with and without IRIS shows an increase in cytotoxic CD8 T cells in patients with IRIS. This is associated with a better control of JCV dissemination but at the cost of oligodendrocyte cell death and demyelination [40, 41].

While there are no randomized trials assessing the best management of IRIS, most physicians use corticosteroids to dampen the immune response and avoid deleterious cerebral edema. A retrospective analysis of 54 patients with PML-IRIS in the setting of HIV infection showed that corticosteroids, especially if administered early and for a prolonged period of time, may improve survival [42].

5.2 Immune Responsiveness: T and B Cells

The immune system encompasses humoral, mainly B cell driven, as well as cellular, mainly T cell driven, immunity. The role of these components of the immune system in the development of and the recovery from PML is not fully understood, but there are many clues that can help answer this question. T-cell-mediated immune response appears to be a significant risk factor for developing PML as a deficit in CD4+ T cells is a prominent component of HIV infection. Rituximab, a drug that depletes B cells but not T cells, however, has also been found to be associated with PML risk, albeit at a lower rate than HIV infection [12]. A recent study showed that natalizumab-treated MS patients with PML have absent or aberrant JCV-specific T-cell responses compared with non-PML MS patients, indicating that changes in T-cell-mediated control of JCV replication may contribute to the risk of developing PML [21].

While brain biopsy or autopsy samples of PML without IRIS generally do not reveal many lymphocytes, in patients with IRIS, there are usually infiltrates with predominately cytotoxic CD8 T cells present. Plasma cells or macrophages can also be seen, but B cells or immunoglobulin deposits are uncommon, suggesting a mainly cellular immune response to PML at least in the case of IRIS [40, 41].

5.3 Treatment Targets: Failures and Future Directions

Several agents have been proposed as treatments for PML, but no specific anti-JCV therapy has been proven to have clinical efficacy to date. On the basis of in vitro experiments demonstrating inhibition of JCV replication as well as anecdotal case

reports, intravenous and intrathecal cytarabine were tested in a clinical trial but neither form of application was found to be beneficial [43, 44]. Although cidofovir is not effective against JCV in cell culture, case reports and retrospective case series implicated efficacy in both HIV-positive and HIV-negative patients with PML. However, subsequent studies demonstrated no survival benefit and no improvement in residual disability at 12 months [45, 46]. Since JCV infection of glial cells is at least partially mediated through the serotonergic receptor 5-hydroxytryptamine receptor 2A (5HT2A) and several 5HT2A receptor antagonists blocked JCV infection of glial cells in vivo [6], the serotonin receptor blocker mirtazapine has been used off-label in a number of cases. No rigorous placebo-controlled trials have been undertaken, but analysis of the existing case series does not show any statistically significant clinical benefit [47]. A screen of chemical compounds indicated that the antimalarial drug mefloquine can inhibit JCV replication in vitro [48]. However, despite anecdotal reports of the beneficial effect of mefloquine, a multicenter clinical trial could not demonstrate an effect of mefloquine on CSF JCV titers, clinical, or MRI findings [49]. None of these agents is therefore recommended for the therapy of PML.

Since no specific therapy for PML has been identified, the main approach to treatment currently consists of a reversal of the immune suppression interfering with the normal host response to JCV. Treatment strategies depend on the patient's underlying predisposing condition. In HIV-infected individuals, antiretroviral therapy (ART) is the most important aspect of PML management. In patients who are not on ART, this should be started immediately. For patients on ART but with detectable HIV viral load, antiretroviral resistance should be investigated, and their ART regimen should be optimized to accomplish viral suppression. More problematic are patients who develop PML despite successful viral suppression on ART. In these patients, intensification of their antiretroviral regimen with special attention to the CNS penetrance of their ART should be considered, though the effectiveness of this approach requires further study. While no study directly comparing the outcome of patients receiving ART and those not receiving ART in the setting of PML has been done, the comparison of the clinical outcomes of patients receiving ART and historic controls prior to the availability of ART shows a dramatic improvement in survival from 10 to 50 % when antiretrovirals were given [50]. In patients who develop PML due to treatment with immunomodulatory medications like natalizumab, removal of the immunomodulatory drug with plasmapheresis or immunoabsorption is generally recommended [34]. In patients with other underlying immune deficits (like idiopathic CD4+ lymphocytopenia or hematologic malignancy), however, restoring the immune system in a timely manner can be challenging or impossible. Immunomodulation with interferon alpha has been implicated after a retrospective analysis suggested improved mortality in HIV-infected patients with PML, though a subsequent study did not support a benefit of this treatment. Anecdotal reports suggested a benefit of interleukin-2 therapy, though no controlled trials have substantiated this claim. In summary, only initiation of antiretroviral therapy in HIV-infected individuals and discontinuation or removal of immunomodulatory or immunosuppressive medications can currently be recommended given the existing evidence.

As discussed above, immune reconstitution achieved by initiation of ART or removal of immunomodulatory medications is often associated with the development of IRIS. Corticosteroids are currently the mainstay of management of clinically significant IRIS, though further data are needed to establish the optimal dose and duration of this therapy.

5.4 PML Prognosis and JCV Persistence

The prognosis of PML is generally thought to be poor, though survival depends on the underlying condition. Before antiretrovirals were available, the mortality in HIV-infected individuals was about 90 %, whereas the outcome is significantly improved when viral load suppression is achieved with antiretrovirals [50]. A retrospective study assessing 87 patients with PML, mainly associated with HIV, showed no survival benefit for the 27 individuals who developed IRIS [39]. In patients who develop PML due to immunomodulatory therapy, mortality can be close to 90 % as in the reported cases of rituximab [12] or as low as 22 % as reported for natalizumab. This may be in part due to the fact that most PML cases associated with rituximab occur in patients with lymphoma and therefore worse general heath at baseline compared to MS patients who receive natalizumab. However, outside of survival, it is important to note that many natalizumab-treated patients who survive PML have significant residual disability [34].

Since we do not currently have specific treatments for PML and our main approach is to promote restoration of the immune system (while managing IRIS if it occurs), what happens with JCV in the brain is an interesting question. A small retrospective study analyzed serial CSF samples of a cohort with natalizumab-associated PML and found persistence of JCV in the CSF for 3 years or longer in more than 50 % of patients [51].

5.5 Epilogue

As summarized in this chapter, PML is a devastating demyelinating disease of the brain that typically occurs in individuals with an impaired immune system. The natural history of this disease and the role different risk factors play in the development and in the course of this disease are not sufficiently understood. As several new monoclonal antibodies and other immunomodulatory drugs are currently being developed and new medications have recently been implicated as risk factors for developing PML, the epidemiology of PML may be changing. Since PML is not a reportable disease, there are no publically available data to better study this disease. An additional challenge to the development of a unified approach to the diagnosis and management of PML is that patients with PML can be seen by physicians from different specialties like neurology, hematology-oncology, dermatology,

gastroenterology, transplant medicine, or rheumatology. To improve our understanding of the epidemiology and underlying pathophysiology of PML, a group of experts from different institutions (Mayo Clinic, Rochester, MN; Cleveland Clinic, Cleveland, OH; Massachusetts General Hospital, Cambridge, MS; Center for Disease Control and Surveillance, Atlanta, GA; Washington University, St. Louis, MO; and NINDS, NIH, Bethesda, MD) has formed a steering committee and initiated and implemented a web-based disease registry for PML (https://pmlregistry.ninds.nih.gov). Health-care providers from around the world can enter anonymized clinical, radiographic, laboratory, and demographic data as well as treatment strategies and outcomes on their patients with PML, regardless of the underlying etiology. The data in this registry is stored on a secure server and administered by a team at the NIH. Researchers can request access to the data by contacting the steering committee, and a summary of the epidemiological data will be posted annually on the registry website. Additionally, the website serves as an informational source for providers as well as for patients and their families.

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Role of Retrovirus-Induced Transactivator Proteins in Neuroinflammatory Disease

Gregory Antell, Michael R. Nonnemacher, Vanessa Pirrone, and Brian Wigdahl

Abstract Human immunodeficiency virus type I (HIV-1) and human T-cell leukemia virus type 1 (HTLV-1) lead to acquired immunodeficiency syndrome (AIDS) and adult T-cell leukemia, respectively, and these viruses have also been identified as the etiologic agents of HIV-associated neurocognitive disorders (HAND) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Pathogenic processes associated with these diseases are due to HIV-1 and HTLV-1 infecting not only cells of the host immune system but also cells of the central nervous system (CNS). For each of these viruses (HIV-1 and HTLV-1), the major viral regulatory proteins, Tat and Tax, respectively, have been shown to be implicated as extracellular neurotoxic proteins. This chapter will provide an overview of the structure and function of Tat and Tax and describe their role in viral replication focusing on their interaction with their viral promoters. Information will then be presented that describes known modifications of these important regulatory and neurotoxic proteins and the current understanding with respect to how these proteins are secreted into the extracellular environment. The specific roles that Tat and Tax play as extracellular proteins especially their role in neuropathogenesis are discussed with a focus on comparing their functional similarities and differences. Finally, the review will discuss the immune response in the CNS with respect to these two essential viral transactivator proteins.

Keywords HIV-1 • HTLV-1 • Tat • Tax • Neuropathogenesis • HAND • HAM/TSP

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1 Introduction

Human immunodeficiency virus type I (HIV-1) and human T-cell leukemia virus type I (HTLV-1) have both been shown to infect not only cells of the host immune system but also cells of the central nervous system (CNS) [1, 2]. As members of the retrovirus family, HIV-1 and HTLV-1 contain RNA genomes and hijack the host cell transcriptional machinery in order to synthesize viral proteins and replicate within human host cell targets. Infection with HIV-1 and HTLV-1 can lead to acquired immunodeficiency syndrome (AIDS) and adult T-cell leukemia, respectively, and these viruses have also been identified as the etiologic agents of HIV-associated neurocognitive disorders (HAND) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). While HIV-1 and HTLV-1 CNS infection do not cause acutely fatal diseases, the resulting neurological problems persist as chronic conditions that cause a wide range of neurological abnormalities and remain relevant medical concerns [3–5]. With no successful vaccine existing for HIV-1 or HTLV-1 and antiretroviral drugs having a reduced efficacy on targets within the CNS, investigation into the causes and consequences of retroviral-induced neurodegeneration remains a prominent research focus [6–8].

1.1 HIV-1-Associated Neurological Disorders

Since the adoption of highly active antiretroviral therapy (HAART), the symptomology of HIV-1 disease has been increasingly transformed from a devastating attack on the peripheral immune system to a chronic disease that continues to involve pathogenesis in the CNS [3, 5]. As a result, most AIDS-defining illnesses have seen a dramatic decrease in both severity and frequency, in addition to reduced viral loads and increased $CD4^+$ T-cell counts in HIV-1-infected individuals [9, 10]. In contrast to this trend, HAND have increased in prevalence although the most severe form of HAND, HIV-1-associated dementia (HAD), has become much less common [3, 11]. The other manifestations of HAND consist of asymptomatic neurocognitive impairment and HIV-associated mild neurocognitive disorder [12]. These conditions are distinguished on the basis of the severity of cognitive dysfunction, with symptoms including memory loss, impaired coordination, and reduced adherence to medication regimens [13]. HAND are also often accompanied by HIV encephalitis (HIVE), a brain pathology caused by immune activation of microglia and macrophages in response to HIV-1 infection and associated with poor prognosis [14]. In addition to activating an antiviral immune response within the CNS, the production of HIV-1 proteins also leads to neurotoxicity through direct and indirect mechanisms. Of particular interest is the HIV-1 transactivator protein Tat, which in addition to driving viral transcription also functions as an extracellular neurotoxic protein, as discussed below [15].

1.2 HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis

HAM/TSP is a chronic and progressive inflammatory disease of the CNS [16]. The disease is primarily a demyelinating disease of the spinal cord caused by the overstimulation and dysfunction of the immune compartment [17]. Especially prominent in the neuropathogenesis of HAM/TSP is the cytotoxic T-lymphocyte (CTL) response to the HTLV-1 transactivator protein, Tax, and increased expression of inflammatory cytokines and chemokines [18–21]. HAM/TSP does not present in all HTLV-1-infected patients, and the pathogenesis of the disease remains poorly understood [4]. A wide variety of factors likely contribute to whether an HTLV-1-infected individual will develop HAM/TSP including the mode of infection, viral strain, and human leukocyte antigen (HLA) subtype [22–27]. Clinically, HAM/TSP may result in a variety of symptoms including urinary incontinence, lower back pain, sexual dysfunction, and weakness of the lower limbs (paraparesis), for which it is named [28, 29]. These symptoms are consistent with the majority of neuronal damage being found in the corticospinal tract [30]. The disease typically progresses through two stages, an initial inflammatory disorder and a chronic long-term degenerative stage [31]. The acute phase is characterized by infiltrating lymphocytes and the release of proinflammatory cytokines, while chronic disease is marked by the accumulation of $CD8^+$ T cells in the cerebrospinal fluid (CSF) and peripheral blood and at lesions within the spinal cord [32].

1.3 Significance of HIV-1 Tat and HTLV-1 Tax in Neurodegenerative Disease

The molecular and cellular mechanisms responsible for neurodegenerative disease during HIV-1 and HTLV-1 infections are complex and multifaceted [17]. In particular, the viral transactivator proteins Tat and Tax play significant roles due to their requirement in viral replication and known toxic properties to a wide variety of CNS cells [33–36]. Notably, Tat can be continually produced by HIV-1-infected cells located in the CNS despite the widespread use of HAART, demonstrating that neurologic vulnerability to this protein persists in the HAART era [15]. It has also been demonstrated that HIV-1 Tat can contribute to HAND by modifying amyloid precursor protein processing and increasing amyloid beta production [37]. Because Tat and Tax are essential for viral replication, in addition to demonstrating neurotoxic properties, both proteins remain central to the development of effective HIV-1 and HTLV-1 therapies. This review will focus on the multidimensional roles and requirements of Tat and Tax in pathogenesis in the CNS, with a particular focus on their functional similarities and differences.

2 Structure of HIV-1 Tat and HTLV-1 Tax Proteins

2.1 *Tat Structure and Functional Domains*

HIV-1 Tat is a predominantly nuclear, multifunctional protein that is essential for productive viral replication and processive transcription from the HIV-1 promoter, termed the long terminal repeat (LTR). The protein typically consists of 101 amino acids encoded by two exons, the first encoding residues 1–72 and the second encoding residues 73–101 [38, 39]. Notably, while 86-amino-acid laboratory strains of Tat (LAI, HXB2, NL4-3) are most commonly used for in vitro experimentation, this form represents a truncated protein relative to the majority of clinical isolates that maintain a 101-amino-acid full-length structure [40, 41].

Tat consists of six major functional domains (Fig. 1) as determined by mutational analysis, with the first five being encoded by exon 1 [42]. Located at the N-terminus of the protein are the acidic domain (residues 1–21), the cysteine-rich domain (residues 22–37), and the core domain (residues 38–48). Together, these three domains function as the minimal region of Tat required for transactivation of the LTR. While the acidic domain is functionally robust with respect to single-residue mutations, changes in positions 25–40 are generally deleterious to Tat-mediated transactivation, particularly the conserved cysteine residues at positions 22, 25, 27, and 37 [15, 43]. Of importance to posttranslational modifications of Tat in this region is the lysine residue at position 28, which functions in the stabilization of the Tat–cyclinT1–TAR

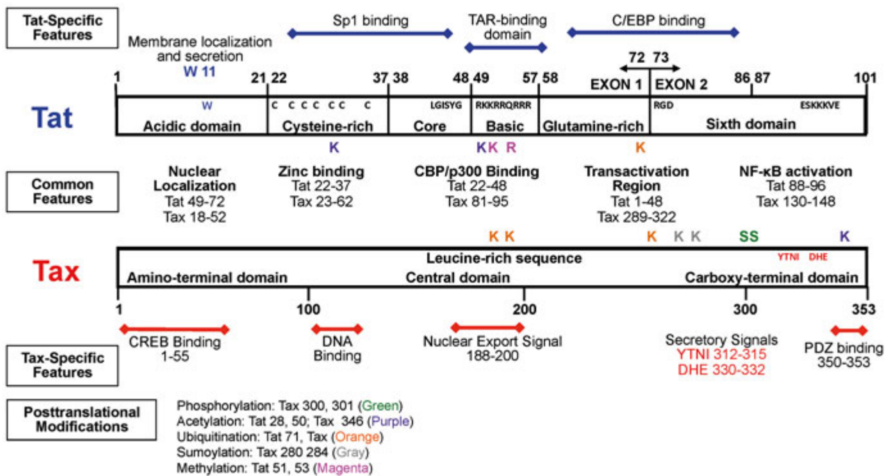

Fig. 1 Functional comparison of HIV-1 Tat and HTLV-1 Tax protein primary structures. The major functional and protein–protein interaction domains of HIV-1 Tat (*blue*) and HTLV-1 Tax (*red*) are highlighted, with the common features of the two proteins displayed in the *middle*. Important amino acid residues and sequences are designated, as well as sites of posttranslational modifications

(transactivation response element) complex in the early stages of transactivation [44]. Furthermore, position 31 of Tat mediates neurotoxicity, as the cysteine residue at this position is involved in binding of neuronal *N*-methyl-D-ASPARTATE (NMDA) glutamate receptors [45]. Importantly, the cysteine region has been shown to play a role in synaptodendritic injury [46]. The fourth domain (residues 49–57) contains the well-conserved arginine-rich motif $_{49}$RKKRRQRRR$_{57}$ that functions as the TAR-binding domain [47, 48]. This domain has also been referred to as the basic domain and has been shown to be required for the uptake of extracellular Tat (residues 48–60) as well as nuclear localization [38, 49]. The fifth domain (residues 58–72) is a glutamine-rich region that demonstrates the greatest amount of genetic variability and functions in both nuclear localization and Tat-mediated apoptosis of T cells [43].

The sixth domain (residues 73–101) of Tat has been shown to be the only domain encoded by the second exon and has gained attention for a variety of functions [50]. Most relevant to this review is the enhancement of HIV-1 replication in cells of the monocyte–macrophage lineage [51]. Additionally, the sixth domain contains an RGD motif and a highly conserved ESKKKVE motif, which are thought to function in integrin binding and optimal in vivo replication, respectively [52, 53]. Tat exon II also contains conserved glutamic acid and lysine residues that are believed to function in the activation of nuclear factor-κB (NF-κB) in T cells [54]. Finally, it has been proposed that Tat exon II plays a role in the dysregulation of the blood–brain barrier (BBB) resulting in increased levels of inflammatory cytokines and toxic viral proteins in the CNS [41].

Although the sequence of Tat is relatively well conserved, as an HIV-1 protein it still has demonstrated a significant amount of genetic variation that impacts its function and role in HIV-1 neuropathogenesis [43]. Tat is also a flexible and inherently disordered protein, allowing it to spatially adapt to a wide variety of binding partners [55]. The crystal structure of Tat complexed with positive transcription elongation factor b (P-TEFb) demonstrates extensive surface contact at the protein interface made possible by the plasticity of Tat and induced conformational changes to P-TEFb [56]. Structural changes as a result of Tat contact are likely present in other Tat-binding partners as well, possibly providing an assortment of potential drug targets that can mitigate Tat-induced pathogenesis.

2.2 Tax Structure and Functional Domains

Tax is an HTLV-1 protein consisting of 353 amino acids that has been shown to function predominantly as a transactivator protein but also interfaces with a variety of cellular proteins, pathways, and processes [57, 58]. In parallel with functional analyses of Tat, Tax has also been studied via mutational analysis demonstrating that most mutations do not significantly alter Tax function [59]. The major functional regions of HTLV-1 Tax (Fig. 1) are more loosely annotated than Tat and are generally referred to as the amino-terminal, central, and carboxy-terminal domains. To date, no crystal structure of HTLV-1 Tax or its complexes has been reported.

The amino-terminus of Tax is cysteine- and histidine-rich, resulting in the formation of overlapping zinc-finger motifs essential for transactivation of the HTLV-1 LTR [60]. Also functioning in transactivation in this region are domains facilitating the interaction with the transcription factor cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), the coactivators CREB-binding protein (CBP), and p300 and for contact with DNA [61–66]. The amino-terminal domain of Tax has largely been shown to be responsible for cellular localization as well and has been shown to contain a unique nuclear localization signal (NLS) (residues 18–52) required for Tax to locate to the nucleus [67, 68]. Studies have also shown the central domain of Tax to contain a leucine-rich sequence region that can also function as the nuclear export signal (NES) (residues 188–202), allowing the protein to travel between the nucleus and cytoplasm [69]. Importantly, the Tax NES is concealed in the context of the full-length protein, suggesting a mechanism by which posttranslational modifications or interaction with other proteins may be required for nuclear export [69, 70]. The carboxy-terminus of Tax has been shown to host additional domains required for transcriptional activation (residues 289–322) [71]. This includes domains responsible for the interaction with CBP and p300/CBP-associated factor (p/CAF) [61, 72]. The secretory signals of Tax are also present in the carboxy-terminal domain, and these signals are critical to the demonstrated functional role of Tax as an extracellular and neurotoxic protein [17, 73]. Finally, proper in vivo function of Tax has been shown to be dependent upon dimerization of the protein for processes such as the assembly of the transcriptional machinery at the HTLV-1 LTR and nuclear transport [74, 75]. Dimer formation has been shown to be largely dependent upon the zinc-binding domain in the amino-terminus in addition to three distinct sequences located throughout the central domain [76].

3 Viral Replication and Transactivation of the HIV-1 and HTLV-1 Promoters

Chronic retroviral infection and disease is dependent on continuous or intermittent viral replication and/or production of viral proteins that can induce pathologic consequences. In the case of HTLV-1, some of the pathologic consequences may involve an oncogenic state induced by the activity of Tax and other proteins produced during the course of viral replication in the T-cell population. Accordingly, the mechanisms of viral promoter transactivation remain important in the understanding of retroviral pathogenesis. Both Tat and Tax are categorized as transactivator proteins and facilitate the binding of cellular transcription factors to their respective LTRs in order to fuel processive and productive viral replication (Fig. 2). Productive HIV-1 and HTLV-1 infection can also occur beyond the primary target of $CD4^+$ T cells. The susceptible cell populations extend to $CD34^+$ bone marrow progenitor cells, dendritic cells (DCs), astrocytes, microglia, and cells of the monocyte–macrophage lineage [32, 77].

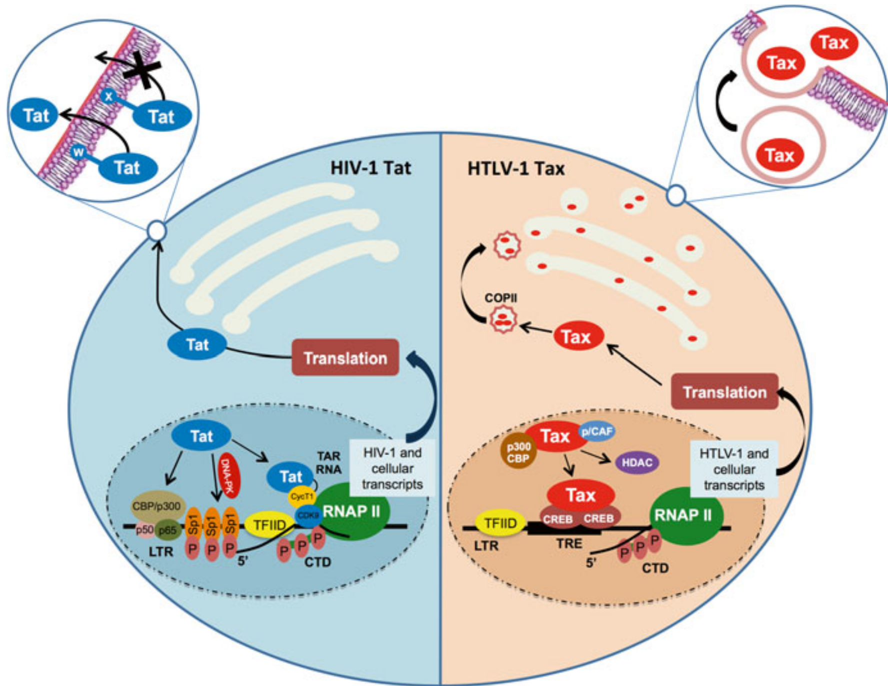

Fig. 2 HIV-1 Tat- and HTLV-1 Tax-mediated transcription and secretory pathways. Tat mediates processive transcription by binding to the "UCU" bulge of the nascent RNA TAR element and recruiting multiple transcription factors and cofactors. Tat associates with P-TEFb, a protein complex consisting of cyclinT1 and CDK9 responsible for the hyperphosphorylation of the carboxy-terminal domain tail of RNA Pol II (RNAP). Tat also mediates recruitment of coactivators CBP/p300 and the kinase DNA-PK, which interface with cellular transcription factors. Tat secretion occurs via an unconventional mechanism that utilizes neither endosomes nor the ER/Golgi apparatus. Critical to Tat secretion is the tryptophan (W) at position 11, which functions in cellular membrane insertion and results in reduced secretion when mutated (X). Tax-mediated transcription occurs via Tax binding to the TRE-1 domain of the LTR and the recruitment of CREB to the CRE-like element of TRE-1. Tax also recruits the chromatin-remodeling factors p300/CBP, P/CAF, and histone deacetylase (HDAC), which are essential for transcriptional regulation. Tax has been demonstrated to localize within the ER and Golgi apparatus. Tax secretion is controlled by two putative signaling sequences in the carboxy-terminal domain (CTD), DHE and YTNI, the first of which mediates concentration of Tax into COPII vesicles for transport from the ER to the Golgi. *TFIID* transcription factor IID

3.1 Tat-Mediated Transactivation of the HIV-1 LTR

HIV-1 transcription is controlled principally by Tat and consists of an early, Tat-independent phase and a late, Tat-dependent phase [78, 79]. Initially, viral transcription produces predominantly short, aborted RNA transcripts of 30–50 nucleotides owing to the inefficiency of RNA polymerase II (RNA Pol II) [80]. However, in spite of the overall inefficient elongation, longer transcripts are also produced at low abundance and encode for early regulatory proteins such as Tat [81]. As the concentration

of intracellular Tat rises beyond threshold levels, it fuels a positive feedback mechanism in which the protein has been shown to be capable of effectively transactivating the HIV-1 LTR and producing full-length transcripts. Therefore, stochastic fluctuations in Tat gene expression can be thought of as a molecular switch that works on the level of elongation and is responsible for full-length HIV-1 mRNA transcripts [82, 83].

Tat functions by binding to the "UCU" bulge of the HIV-1 TAR element, a cis-acting RNA enhancer and stem–loop structure located at the 5′ end of all viral transcripts [33, 47, 84–86]. The Tat–TAR interaction is critically important with respect to tethering Tat and allowing for recruitment of the host transcriptional machinery. However, it is important to note that in astrocytes Tat has been shown to be capable of transactivating the LTR in the absence of the TAR region [87]. Specifically, Tat associates with P-TEFb, a protein kinase complex consisting of cyclinT1 and cyclin-dependent kinase 9 (CDK9) [88, 89]. A conformational change to P-TEFb as a result of Tat binding has been shown to be responsible for the activation of CDK9 and results in the hyperphosphorylation of the carboxy-terminal domain of RNA Pol II and the promotion of transcription elongation [56, 88–91]. Transcription from the HIV-1 LTR can be further regulated through interactions with host transcription factors that can enhance or repress this process as well as by genetic variation in the HIV-1 LTR [92–94].

3.2 Tax-Mediated Transactivation of the HTLV-1 LTR

Congruent with the role of Tat in HIV-1, Tax interfaces with the host transcriptional machinery and cellular transcription factors to promote viral gene expression and productive viral replication [35, 95]. Essential to transcriptional transactivation is the binding of Tax to the Tax-responsive element 1 (TRE-1), which has been shown to consist of three 21-base pair TREs located in the U3 region of the LTR [96, 97]. Each of the repeating 21-base pair elements is in turn composed of three domains, termed A, B, and C. The B domain has been shown to be important to transactivation because it contains a conserved core sequence (TGACGTCA) and flanking 5′ and 3′ G/C-rich sequences that closely resemble a cAMP response element (CRE) [98]. Tax recruits the transcription factors CREB and serum response factor to the CRE-like element of the HTLV-1 LTR, resulting in facilitated transcription [97, 99]. The association of Tax with homodimeric CREB results in the formation of a stabilizing ternary complex at the binding domains of TRE-1 [98].

Tax has been shown to independently recruit the chromatin-remodeling factors p300/CREB-binding protein (p300/CBP) and p300/CBP-associated factor (P/CAF) to the CREB/DNA complex [62]. These coactivators bind to two specific Tax regions located at the amino- and carboxy-termini of the protein, respectively, and are essential in transcription initiation [98]. While CREB is activated via the cAMP-dependent protein kinase A signaling pathway, Tax also regulates transcription by activating the NF-κB and ATF/CREB cellular signaling pathways, demonstrating that both the direct and indirect interaction of Tax with cellular transcription factors may influence transactivation of the viral LTR and regulation of cellular transcription

[100–102]. Recent studies have demonstrated that Tax reduces histone protein transcript levels in addition to mediating the downregulation of microRNAs (miRNAs) that function in chromatin remodeling [103]. Combined, these studies emphasize the sophisticated coordination of HTLV-1 Tax, miRNAs, and chromatin remodeling in the regulation of retroviral transcription and replication.

4 Posttranslational Modifications

Following translation, Tat and Tax are subjected to a variety of posttranslational modifications (PTMs) that may alter the tertiary structure and activity of the protein. The importance of these alterations in mediating viral replication highlights the importance of PTM-associated enzymes in the HIV-1 Tat and HTLV-1 Tax interactomes [104]. Such modifications have been demonstrated to be necessary for optimal transcriptional activity of both Tat and Tax and by extension are relevant to the persistence of neuroinflammation.

4.1 HIV-1 Tat Posttranslational Modifications

Acetylation and deacetylation of Tat is a reversible process performed by the acetyltransferase activities of p300 and P/CAF, important HIV-1 transcriptional coactivators, and the deacetylase activity of class III deacetylase sirtuin 1 (SIRT1) [105–107]. These modifications have been found to occur in the arginine-rich and cysteine-rich domains of Tat, specifically at lysine positions 28 and 50 [105, 108]. Importantly, mutations of lysine at these positions or inhibition of SIRT1 reduce transactivation ability and inhibit HIV-1 replication [107, 109]. Furthermore, inhibition of SIRT1 has also resulted in a reduction in Tat transactivation. The protein–protein interactions of Tat differ according to whether Tat is acetylated at lysine position 50 as well. As previously discussed, nonacetylated Tat binds with the cyclinT1 component of P-TEFb via the cysteine-rich domain, and this complex cooperatively binds to TAR RNA using the arginine-rich domain of Tat. However, once acetylated, Tat dissociates from TAR RNA and cyclinT1 and binds instead with RNA Pol II and P/CAF [105, 108, 110, 111]. Accordingly, the dynamic acetylation of Tat appears to be essential for Tat to function in both the early and late transactivation stages, and the manipulation of this process has been considered for the development of new therapeutic strategies [112, 113].

Ubiquitination and methylation have also been observed as important posttranslational modifications of HIV-1 Tat capable of altering transactivation. Of particular importance to the transactivation ability of Tat is lysine 71, a highly conserved residue that functions as the principal ubiquitination target site and may contribute to cyclinT1 binding in the early stages of HIV-1 transcription rather than proteasomal degradation [109]. Methylation of Tat proceeds through the catalytic activity of protein arginine methyltransferases (PRMTs) such as PRMT6 in the arginine-rich region, in addition

to lysine position 51 by methyltransferases Set7/9 (KMT7) [114–116]. Arginine methylation results in reduced transactivation, likely as a consequence of disrupted binding with P/CAF. The extent of methylation plays a role in determining whether Tat will be recycled or tagged for degradation, as the reversibility of this modification has been shown to depend on whether an arginine residue is mono- or di-methylated [104].

4.2 HTLV-1 Tax Posttranslational Modifications

Tax protein also undergoes various posttranslational modifications that can influence disease progression and protein functionality. The principal modifications observed include sumoylation, ubiquitination, acetylation, and phosphorylation [70]. Five lysine residues within the central domain of Tax, designated K4–K8, are targets for polyubiquitination. Similar to Tat, this modification does not trigger proteasomal degradation and is likely necessary for activation of the NF-κB pathway. Alternatively, lysine residues at positions 280 and 284 of Tax can be monoubiquitinated, leading to nuclear export [117]. These residues also function as polysumoylation sites, and polysumoylated Tax likely works in concert with ubiquitinated Tax to activate the NF-κB pathway [118, 119]. Acetylation of Tax occurs via p300 acetyltransferase activity at lysine position 346 within the C-terminal domain of Tax. This modification has also been demonstrated to promote activation of the NF-κB pathway [120]. Tax is also a phosphoprotein, with serine residues 300 and 301 within the transactivation domain being the primary phosphorylation targets [121, 122]. Phosphorylation of Tax at these positions is critical for Tax nuclear localization and transcriptional activity. Notably, Tax phosphorylation appears to be a prerequisite for Tax ubiquitination, polysumoylation, and acetylation [70].

5 Cellular Localization and Secretion

Tat and Tax are multifunctional proteins that can localize to multiple cell compartments. While their role as transactivating proteins requires localization to the cell nucleus, both proteins can also be found in the cytoplasm. Most important to neuroinflammation, Tat and Tax are also secreted into the extracellular environment where they interact with multiple CNS cell types and activate a variety of cellular and immune responses.

5.1 HIV-1 Tat Cellular Localization

The role of exogenous Tat is well established, and the protein can enter a variety of cell types present in the CNS [123, 124]. Both clathrin and caveolar pathways have been proposed for extracellular Tat uptake, consistent with the ability of Tat to bind

to the endocytic receptors CXCR4, heparan sulfate proteoglycans, and lipoprotein receptor-related protein—the primary receptor involved in internalization [125–130]. Importantly, the endocytic pathway utilized by Tat is likely cell-type dependent, as caveolae are absent in T cells [131].

It is well established that Tat is required for HIV-1 transcription and therefore is capable of reaching the nucleus. The arginine-rich basic domain of Tat is responsible for nuclear import as the mutation or deletion of this domain inhibits transport to the nucleus as well as HIV-1 transcription [49, 132, 133]. As previously reviewed, the majority of mechanistic studies of nuclear import have attached the Tat basic domain to β-galactosidase, glutathione S-transferase, or green fluorescent protein, demonstrating that the basic domain is not only necessary but also sufficient for nuclear import [55]. However, nuclear import studies utilizing the full-length Tat protein have yet to be performed, limiting the extent to which these results can be applied to the actions of full-length Tat. This shortcoming is critical, as binding partners of Tat, such as NF-κB-α inhibitor (IκBα) and human I-mfa domain-containing protein, can interfere with nuclear import by promoting nuclear export and cytoplasmic sequestration, respectively [134, 135].

5.2 HIV-1 Tat Secretion

Tat has been shown to be released by infected monocytes, glial cells, and astrocytes in addition to infected lymphocytes [136–139]. Overall, Tat secretion is a productive process, with approximately two-thirds of cellular Tat being exported over the life span of an HIV-1-infected T cell [140]. Secreted Tat is biologically active and capable of transcellular activation of the HIV-1 LTR in addition to its extensive effects as an extracellular protein [140, 141]. Additionally, release of Tat by infected cells occurs optimally in low-serum conditions, such as the brain environment [139]. The secretion of Tat occurs via an unconventional mechanism that utilizes neither endosomes nor the endoplasmic reticulum (ER)/Golgi apparatus. Tat, however, does bind tightly to plasma membrane-embedded PI[4,5]P_2 in the cell membrane. Most critical to this process is the tryptophan residue at Tat position 11, as it functions in membrane insertion. Tat mutants without this residue remain predominantly in the cytoplasm and are weakly secreted [140]. The exact mechanism by which Tat localizes to the cell membrane is unknown, but localization of Tat likely is dependent upon cell type [55, 140].

5.3 HTLV-1 Tax Cellular Localization

HTLV-1 Tax localization to the nucleus is well known because of its transactivation activity [35, 95]. However, Tax is also localized in the cytoplasm of infected cells and may shuttle between the nuclear and cytosolic compartments [142]. The shuttling of Tax between the nucleus and cytoplasm is predominantly controlled by the

nuclear localization signal (NLS) found in the amino-terminus of the protein and the leucine-rich nuclear export signal (NES) found at the carboxy-terminus, respectively [67, 69]. The balance between nuclear localization and export hinges on the masking of the NES by protein–protein interactions or posttranslational modifications to Tax, allowing the NLS to function as the dominant cellular localization signal [121, 143].

5.4 HTLV-1 Tax Secretion

Previously, Tax has been shown to associate with the ER and Golgi apparatus, with cytoplasmic transport progressing via secretory vesicles shuttled by kinesin motor proteins along microtubules [144]. In contrast to HIV-1 Tat, which lacks a formal secretory pathway, secretion of Tax is largely controlled by two putative secretory signals located in the carboxy-terminal domain of Tax, DHE and YTNI, that are responsible for the interaction of Tax with cellular secretory proteins [73]. Specifically, DHE functions as a DXE signal required for the concentration of Tax into coat protein complex II (COPII) vesicles, which are responsible for mediating transport of proteins from the ER to the Golgi [145]. Tax has also been demonstrated to interact with multiple other proteins associated with secretion [73, 146]. Among its other roles, secreted Tax interacts with calreticulin (CRT) and is proposed to play a role in the axonal degeneration associated with HAM/TSP [147].

6 Presence and Diversity of Tat and Tax in the CNS

HIV-1 Tat is pervasive in the brain and has been detected in patients with HIVE [126, 148–150]. The protein has also been linked to neurological impairment by the observation that Tat mRNA is detectable in patients with HIVE but absent in those without dementia [150]. Specifically, Tat has been observed in the cytoplasm of microglial cells and perivascular macrophages, which are likely HIV-1-infected cells. Importantly Tat is also found in the nuclei of neurons and oligodendrocytes, further demonstrating the ability of Tat to affect and enter additional cells of the CNS [126, 148]. Furthermore, sequence analysis of Tat has demonstrated compartmentalization in brain versus lymphoid tissue [151]. Brain-derived sequences demonstrate higher nonsynonymous/synonymous and transversion mutation rates in HAND patients and display phylogenetic clustering within clinical groups [152]. These observations are critical, as brain-derived Tat from HAD patients has a decreased ability to transactivate the LTR and may exert pathogenic effects as a result of Tat mutations [153]. However, a recent study has shown that despite the heterogeneity of Tat in the brain, the transactivation function is conserved in Tat relative to the non-CNS compartment [154]. Overall, genetic compartmentalization of HIV-1 Tat between CNS and lymphoid tissue likely reflects altered requirements for HIV-1 replication in cells of monocytic lineage and brain-specific immune surveillance.

Extracellular Tax may be found to accumulate in the peripheral blood and CSF of HAM/TSP patients as well [155]. Tax expression levels have been shown to be higher in CSF cells than in peripheral blood mononuclear cells among HTLV-1 patients overall, and HTLV-1 mRNA levels correlate with disease severity in HAM/TSP patients, suggesting a role for exogenous Tax in HAM/TSP pathogenesis [156, 157]. Multiple subgroups of Tax have been identified, with one group, taxA, found to have a higher incidence among HAM/TSP patients [158, 159]. However, these results have been disputed by other research, demonstrating the need for greater sampling in order to validate the ability to predict disease progression on the basis of Tax sequence [160].

7 Neuroinflammatory Effects of Tat and Tax on CNS Cellular Targets

Extracellular Tat and Tax proteins have been thought to play a large role in the pathogenesis of both HAND and HAM/TSP, as these proteins can cause both direct and indirect damage to cells of the CNS [15, 17]. The effects of Tat and Tax on cells of the CNS include altered gene transcription, direct neurotoxicity, induction of neuroinflammatory cytokine secretion, increased immune cell trafficking, and disruption of the BBB (Table 1).

7.1 Effects of Extracellular HIV-1 Tat

Tat has been shown to cause the loss of neurons both in vitro and in vivo, with the greatest neurotoxicity in the brain occurring in the striatum, dentate gyrus, and the CA3 region of the hippocampus [161–164]. This observation is consistent with the role of NMDA receptors in mediating Tat excitotoxicity in neurons, as these brain regions are rich in NMDA receptors [165]. Importantly, reduced toxicity of subtype C Tat has been attributed to a cysteine-to-serine mutation at position 31, although this position does not affect binding to NMDA receptors [45]. Accordingly, it has been proposed that the cysteine residue is required for NMDA activation, with other regions functioning in receptor binding [45]. Additionally, Tat-induced phosphorylation of the NMDA receptor and dopamine D1 receptor pathways has also been implicated in Tat neurotoxicity, with Tat upregulating proapoptotic signaling pathways mediated via D1/NMDA receptor interaction [166, 167]. Tat also causes excitotoxicity in neurons through the interaction with both NMDA receptors and glutamate receptors, with the latter being implicated in the process of neuronal death [17]. Studies using a Tat-transgenic mice model have shown that the constitutive expression of Tat in brain generates a latent excitatory state that may exacerbate the development of HAND [168]. In response to Tat, intracellular calcium levels in neurons dramatically rise as a result of release by inositol 1,4,5-triphosphate (IP3)

Table 1 Impact of transactivation proteins HIV-1 Tat and HTLV-1 Tax on resident cells of the CNS

CNS cell type	HIV-1 Tat	HTLV-1 Tax
Neurons	• Dopamine transmission and D1 receptors are implicated in Tat neurotoxicity [166] • NMDA receptor levels correlate with Tat-induced apoptosis [165] • Elevation of intracellular calcium, mitochondrial calcium uptake, and generation of reactive oxygen species in response to Tat [167, 170]	• Induction of TNF-α in cells exposed to Tax [36] • Axonal degeneration accompanies demyelination due to axonal oligodendrocyte damage [207, 213]
Astrocytes	• Dysregulation of glutamate homeostasis [180] • TAR-independent transactivation [87] • Induction of nitric oxide synthase [182] • Production of CCL2/MCP-1 for monocyte recruitment [184]	• Dysregulation of glutamate uptake and catabolism [203] • Tax RNA detected in astrocytes [200] • Induction of proinflammatory cytokines IL-1α, IL-1β, TNF-α, TNF-β, and IL-6 in astrogliomas [212] • Sensitization of astrocytomas to apoptosis [212]
Microglial cells	• Increased secretion of chemokines including CCL2 [173] • Induces increase in intracellular calcium [177] • Induces a migratory phenotype via CCL2/MCP-1 [185]	• Secretion of proinflammatory cytokines TNF-α, IL-1β, and IL-6 [196] • Upregulation of proinflammatory cytokine profile [197]
Dendritic cells	• Tat induces chemokine expression in dendritic cells, resulting in T-cell and macrophage recruitment [176] • Tat enhances maturation, function, and antigen presentation [187]	• Internalize extracellular Tax and mediate the priming of the anti-Tax CTL response [256] • Activation in response to Tax, resulting in constant antigen presentation and T-cell proliferation [257]
Monocyte-macrophages	• TNF-α production via NF-κB pathway in response to Tat [172]	• Tax RNA expression detected, suggesting role as a possible reservoir [201]

and the subsequent influx of extracellular calcium via a glutamate receptor-mediated mechanism [169]. This increased concentration results in the mitochondrial uptake of Tat, the generation of reactive oxygen species, and eventually Tat-induced toxicity and apoptosis [34, 170]. Synaptodendritic damage is also observed in HAND and cognitive decline, with HIV-1 Tat having been demonstrated to increase the frequency of inhibitory synaptic connections in rat hippocampal neurons [171].

The toxic effect of Tat is not limited to neurons, as astrocytes, DCs, macrophages, and microglia also produce proinflammatory mediators, reactive oxygen and nitrogen species, and excitatory amino acids that contribute to neuropathogenesis in response to Tat [172–177]. Of great importance is the secretion of tumor necrosis

factor-α (TNF-α) by these cells in response to Tat, owing to its neurotoxicity [178, 179]. One of the primary functions of astrocytes is to regulate extracellular glutamate levels in the brain, and TNF-α secretion by monocytic cells has been shown to disrupt glutamate metabolism, resulting in dysregulated glutamate homeostasis and neuronal damage [180]. Tat also activates astrocytes and induces the production of chemokines and nitric oxide; however, astrocytes do not apoptose unless cocultured with neurons, suggesting that Tat-induced apoptosis requires a signal from NMDAR-expressing neurons [181–183]. Extracellular Tat has been shown to induce production of monocyte chemoattractant factor-1 (MCP-1)/CCL-2 in human fetal astrocytes and microglial cells [184, 185]. The MCP-1/CCL-2 functions in the recruitment of monocytic cells to sites of inflammation in the CNS and the trafficking of monocytes across the BBB. This mechanism is consistent with findings that individuals with HIVE and HAD have elevated levels of CCL2 in CSF [186]. Tax also has been shown to promote the maturation of DCs, resulting in enhanced antigen presentation [187]. In astrocytes, CCL5 expression is upregulated by Tat, through a pathway that involves AP-1, C/EBPα, and C/EBPβ [188]. Finally, HIV-1 Tat displays important properties related to HIV-1 coreceptor usage and cell tropism. The ability of Tat to bind the CXCR4 chemokine receptor suggests that it may function as a virion antagonist pressuring CXCR4-utilizing HIV-1 strains to evolve towards CCR5 tropism [189]. As a result, Tat production may influence increased targeting of cells of the monocyte–macrophage lineage in the CNS. Furthermore, Tat can induce CXCR4 expression on both lymphocytes and monocyte/macrophages and CCR5 expression on monocyte/macrophages [190], which can result in increased viral replication.

7.2 Effects of Extracellular HTLV-1 Tax

Principally, the accumulation of extracellular Tax may result in the cytokine-mediated destruction of neuronal tissue via both autocrine and paracrine mechanisms [17]. Proinflammatory cytokines found to exist at elevated concentrations in the CNS of HAM/TSP patients include interferon (IFN)-γ, TNF-α, interleukin (IL)-1 and IL-6, and granulocyte–macrophage colony-stimulating factor (GM-CSF) [191–194]. In human neuronal cells exposed to Tax, TNF-α has been produced at a concentration similar to that of HTLV-1-infected T cells [36, 195]. Within the CNS, cytokine secretion is not limited to neurons as adult microglial cells also demonstrate a proinflammatory response and produce TNF-α, IL-1β, and IL-6 in response to Tax [196, 197]. Importantly, HTLV-1-infected microglial cells cannot produce IL-1β, stressing the importance of a paracrine mechanism in a Tax-induced inflammatory response [196]. HTLV-1 Tax RNA has been detected in astrocytes as well as monocytes, suggesting that these cells may also be a source of extracellular Tax and could potentially serve as a viral reservoir [198–201]. The HTLV-1 Tax oncoprotein stimulates reactive oxygen species (ROS) production through multiple mechanisms and interactions. ROS production is normally inhibited by antistress host factors such as ubiquitin-specific protease 10 (USP10); however, this activity is attenuated by Tax [202]. Tax also has been found to dysregulate glutamate uptake and

catabolism in astrocytes, potentially leading to neuronal and oligodendrocyte death [203]. As a consequence of the various inflammatory responses caused by Tax, the BBB may also be altered, resulting in increased lymphocyte trafficking into the CNS [204, 205]. Tax is also an important factor in the dysregulation of autophagy in HTLV-1-infected T cells, as it leads to the recruitment of autophagic molecular complexes containing Beclin1 and Bif-1 to lipid raft microdomains [206].

Demyelination of neurons is observed among HAM/TSP patients and can be directly attributed to extracellular Tax protein [36, 207]. Myelin functions in the insulation of axons and is responsible for the speed and accuracy of neuronal action potential impulses [208]. Oligodendrocytes, being the myelinating cell type of the CNS, are sensitive to TNF-α but also secrete the proinflammatory cytokines IL-1β, TNF-α, TNF-β, and IL-6 in response to HTLV-1 Tax transduction [207, 209–212]. Overall, it is unclear whether axonal oligodendrocyte damage occurs prior to axonal degeneration ("outside-in theory") or if the opposite scenario occurs ("inside-out theory") [32, 213]. Additionally, the expression of Tax causes the activation and proliferation of T cells, with increased ratios of T helper 1 (Th1) effector cells to regulatory T cells (Tregs) observed in PBMCs of HLTV-1-infected patients with HAM/TSP [214]. CD39 and CD25 co-expression can be used to identify regulatory ($CD39^+CD25^+$) and effector ($CD39^+CD25^-$) T-cell subsets, with CD39 upregulation being suggested as a surrogate diagnostic marker of HAM/TSP progression potentially attributable to Tax expression [215].

8 The Adaptive Immune Response in the CNS

8.1 Immune Surveillance in the Central Nervous System

The CNS is shielded from the free diffusion of circulating molecules, cells, and pathogens by the BBB, which has been reviewed in detail previously [41, 216]. Importantly, the BBB can be disrupted as a result of HIV-1 and HTLV-1 infection and proteins, including Tat and Tax. Both circulating lymphocytes and microglia, the resident macrophages of the CNS, contribute to the immune surveillance of the CNS in order to maintain optimal function and protection. Consistent with the critical role of the BBB, cellular immune surveillance can vary according to CNS region, with the highest concentration of cells located near areas with reduced tight junctions of the BBB [217]. Peripheral immune cells, such as perivascular macrophages and meningeal DCs, are strategically located along the BBB and function in antigen presentation and surveying the periphery for foreign antigens [218]. Within the CNS, microglial cells are the principal mediators of homeostasis and the primary defenders of the CNS parenchyma against infection and injury [218, 219]. Because of the minimal overlap and motility of microglia, the entire extracellular environment of the CNS is continuously sampled even when these microglial cells exist in a presumed resting state [220]. Despite these inherent immune protections of the

brain, HIV-1 and HTLV-1 infection of the CNS remains extremely common [1, 16]. The unique microenvironment and immune surveillance within the brain allows viruses to enjoy immune privilege owing to the relative absence of adaptive immune cells, such as B and T cells, and inherent mechanisms that limit neuroinflammation in order to protect nonrenewable neuronal pathways [77, 221]. Overall, the number of T cells infiltrating the healthy CNS is small relative to peripheral organs, owing to reduced interaction with endothelial adhesion molecules and leukocyte rolling in the brain [222].

8.2 HIV-1 Tat Antibody Response

A Tat-targeting antibody response is found in nearly 50 % of seropositive HIV-1 patients [223]. However, this antibody response does not appear to prevent disease progression even though anti-Tat antibodies have been shown to inhibit viral replication in vitro [224, 225]. Uninfected individuals have natural IgM antibodies against the Tat epitopes located in the cysteine-rich and basic domains that contribute to HIV-1 pathogenesis resistance [226]. Genetic variation likely plays an important role in an anti-Tat antibody response, as subtype C Tat variants demonstrate the ability to avoid an antibody response via mutation while maintaining transactivation ability [223]. Researchers recently reported that anti-Tat antibody titers are higher in individuals without HAND than in those who display neurocognitive impairment, even among those with higher viral loads and lower $CD4^+$ T-cell counts [227]. Tat immune complexes have been shown to interact with NMDA receptors and prevent excitotoxicity in a novel host defense mechanism, an effect that does not occur in the presence of either Tat or antibody alone [228]. This observation, in conjunction with the high anti-Tat antibody levels detected among patients with long-term nonprogressing disease as compared with patients with rapidly progressing disease, has fueled continued interest in developing a Tat vaccine for the prevention of HAND [229–233].

8.3 HIV-1 Tat Cytotoxic T-Lymphocyte Response

The natural time course of HIV-1 infection consists of an initial acute stage in which viremia peaks, followed by a sharp decline in viral load resulting from the natural antiviral CTL response [234, 235]. CTLs recognize and destroy cells that present foreign epitopes in the context of class I major histocompatibility complex (MHC-I) molecules on the cell surface. These epitopes are created by the cleavage of viral proteins by the proteasome, which are then delivered to MHC-I molecules on the cell membrane by the transporter associated with antigen processing (TAP) [236]. The diverse population of quasispecies that emerge as a result of HIV-1 replication provides an abundant substrate for the selective pressures that favor the most genetically

fit variants. The CTL component of the adaptive immune system is particularly prominent in shaping viral evolution, as mutations are selected for that decrease MHC-I binding and CTL recognition [237, 238]. In principle, viruses could rapidly eliminate all of their CTL epitopes. However, this does not occur because the need to evade the immune response is counterbalanced by the need to maintain functionality and the ability to replicate [239, 240]. These competing influences indicate that toggling mutations between more functional sequences that display epitopes and less functional sequences that can evade the host immune response occur in HIV-1 [241].

While the majority of analyses on the HIV-1-specific CTL response initially focused on structural proteins, researchers are now exploring early regulatory genes such as Tat and Rev in more detail. Significantly, Tat is frequently targeted by CTLs, representing a selective pressure after the onset of the asymptomatic period of infection [242, 243]. The HIV-1 CTL epitope repertoire across multiple HLAs has been analyzed on the basis of their "size of immune repertoire" (SIR) score [244]. This study demonstrated that HIV-1 removes $CD8^+$ CTL epitopes as an evasion strategy and that regulatory proteins such as Tat have low SIR scores in comparison with late virion-associated genes [244]. The evolution of HIV-1 Tat over time may be due either to escape mutations from the host immune response or to mutations that confer enhanced viral replication ability. One study suggests that the primary determinant is the former, with Tat mutants that emerge over time displaying no significant difference in transactivation ability [242]. The CTL response within the brain has been shown to be distinct from that of the peripheral blood in simian immunodeficiency virus (SIV) studies [245]. SIV studies have also demonstrated that the persistence of a CTL response in the brain is associated with increased levels of IL-15 and the absence of IL-2 [246]. Accordingly, the anti-Tat CTL response within the brain may contribute to a unique cytokine environment.

8.4 HTLV-1 Tax Antibody Response

The presence of an anti-Tax antibody response has been well established among individuals infected with HTLV-1 [247]. The percentage of individuals expressing anti-Tax antibody as well as the level of anti-Tax antibody titers have been demonstrated to be significantly greater in HAM/TSP patients compared with asymptomatic HTLV-1 carriers [247, 248]. In contrast, anti-Tax levels have been demonstrated to be low for HTLV-1 patients with a diagnosis of adult T-cell leukemia, stressing the role of the Tax-specific antibody response in HAM/TSP pathogenesis [249, 250]. In addition to conferring an increased risk of developing HAM/TSP, the presence of anti-Tax antibody has been shown to correlate with increased proviral loads and the potential for the virus to replicate [251]. Notably, the resistance to apoptosis conferred by HTLV-1 Tax production is reversed by the presence of anti-Tax antibodies [252]. Together, these observations demonstrate that a robust anti-Tax antibody response may be a contributing factor in neuropathogenesis.

8.5 HTLV-1 Tax CTL Response

HTLV-1 Tax and HBZ protein have been associated with HTLV-1 disease progression due to their roles in inducing cellular proliferation and the CTL-mediated immune response. Accordingly, Tax mRNA expression levels have been identified as the best marker to estimate risk for the development of HAM/TSP [253]. Tax exists as an extracellular protein within the CNS, and as a result it can be internalized and made available for immune recognition by antigen-presenting cells. Accordingly, DCs play a major role in directing the anti-Tax cellular immune response, and their rapid maturation is associated with HAM/TSP stimulation of $CD4^+$ and $CD8^+$ T cells [254, 255]. Recently DCs have been shown to successfully mediate the priming of the anti-Tax CTL response both in vitro and in vivo [256]. Tax plays a central role in this process, as DCs exposed to Tax undergo activation, resulting in T-cell proliferation [257]. CTLs specific for HTLV-1 Tax are the primary proliferating cell type observed in the CSF of HAM/TSP patients [155, 258]. In particular, the Tax_{11-19} epitope is recognized and targeted by infiltrating CTLs, and the continual activation of these cells suggests that viral replication and production of HTLV-1 proteins persist in the CNS [259]. The expanding population of activated $CD8^+$ T cells is believed to contribute to the CNS damage via the lysis of Tax-presenting cells and the ensuing release of proinflammatory molecules. Additionally, infiltrating CTLs are an important source of proinflammatory mediators, including IFN-γ, TNF-α, IL-16, matrix metalloproteinase-9, macrophage inflammatory protein-1α, and macrophage inflammatory protein-1β [260]. Molecular mimicry contributes to neuronal damage in HAM/TSP as well, as the CTL response directed against Tax also demonstrates cross-reactivity to cells displaying the heterogeneous ribonuclear protein-A1 antigen in the CNS [261]. Although a high frequency of HTLV-1-specific CTLs correlates with risk of HTLV-1-associated inflammation, host and viral immunogenetics suggest that a strong CTL response is associated with a low risk of HAM/TSP. As a result, the avidity of the CTL response is essential to controlling HTLV-1 neuropathology, as efficient control of HTLV-1 infection depends on the lytic efficiency of HTLV-1-targeting CTLs [262].

9 Conclusion

HIV-1 Tat and HTLV-1 Tax are analogous proteins in terms of both virologic function and the ability to stimulate a neuroinflammatory response as extracellular proteins. However, these proteins are also distinct with respect to their role in disease progression. Tax is the dominant force in HAM/TSP, whereas Tat-mediated neuroinflammation comprises only one aspect of HIV-1 neuroinflammation. The multifaceted and complex interactomes of Tat and Tax have been studied in detail, elucidating a vast network of viral and cellular protein interactions that control nearly every aspect of Tat- and Tax-induced neuropathogenesis, including

transcriptional regulation, secretion, posttranslational processing, neurotoxicity, and immune recognition [43, 57]. As previously discussed, particular Tat and Tax mutations can significantly alter protein–protein interactions, with the effects potentially resonating throughout these intricate protein networks and contributing to neuropathogenesis. Given the low fidelity of HIV-1 and HTLV-1 reverse transcriptase, genetic variation is a pervasive attribute of retroviral proteins that influences not only drug resistance but also protein functionality. Accordingly, the selection of escape mutations within Tat and Tax epitopes likely affects both viral evolution and the extent of the neuroinflammatory response. Future research must be cognizant of the differences between the anti-Tat and anti-Tax immune responses on either side of the blood–brain barrier, as this will likely shape the persistence and selection of particular HIV-1 quasispecies within the brain compartment. Accordingly, Tat and Tax variants that are selected for on the basis of optimal survival within the cells of the CNS may demonstrate a unique set of characteristics that contribute to the progression of HAND and HAM/TSP.

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Immunopathogenesis of Bacterial Meningitis

Uwe Koedel, Matthias Klein, and Hans-Walter Pfister

Abstract Despite effective antimicrobial therapy, case-fatality rates and neurologic sequelae of bacterial meningitis remain unacceptably high. Adverse outcomes are related primarily to neurologic complications occurring secondary to meningitis. These complications are mainly a consequence of a hyper-inflammatory reaction to bacterial infection of the subarachnoid space. The harmful inflammatory response is initiated by the recognition of bacterial products through pattern recognition receptors such as toll-like receptors. Their activation leads to a MyD88-dependent production of multiple pro-inflammatory factors like cytokines of the interleukin-1 family or terminal complement products. Subsequently, huge numbers of neutrophils are recruited to the site of infection where they release their antimicrobial arsenal, e.g., oxidants. This can cause collateral damage to brain tissue, resulting in the liberation of endogenous danger molecules. Their presence is also recognized by host pattern recognition receptors and, in consequence, mediates an aggravation and propagation of the hyper-inflammatory response. Based on this knowledge, the most promising targets for adjunctive therapy of bacterial meningitis seem to be limiting the release of bacterial products and interfering with the generation of key pro-inflammatory host factors.

Keywords Pneumococcal meningitis • Toll-like receptors • Interleukin • Anaphylatoxin • High-mobility group box 1 protein • Pneumolysin • Daptomycin

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Abbreviations

C	Complement
CSF	Cerebrospinal fluid
DAMP	Danger-associated molecular pattern
HMGB1	High-mobility group box 1 protein
IL	Interleukin
LTA	Lipoteichoic acid
MAC	Membrane attack complex
MMP	Matrix metalloproteinase
MyD88	Myeloid differentiation primary response gene 88 protein
NF	Nuclear factor
NLR	NOD-like receptor
NLRP3	The NLR family, pyrin domain-containing protein 3
NOD	Nucleotide-binding oligomerization domain
PG	Peptidoglycan
PLY	Pneumolysin
PRR	Pattern recognition receptor
RAGE	Receptor for advanced glycosylation end products
TLR	Toll-like receptor
TNF	Tumor necrosis factor

1 Introduction

Bacterial meningitis remains a serious threat to global health. Every year, meningococcal meningitis epidemics threaten millions of people in the African meningitis belt. In this area, close to 1,000,000 cases were reported in the last 20 years. Of these cases, approximately 100,000 died, with another 100,000–200,000 developing neurological sequelae [1]. Aside from epidemics, at least 1.2 million cases of endemic bacterial meningitis are estimated to occur worldwide each year with 135,000 deaths [2]. This makes bacterial meningitis one of the top ten infectious causes of death on Earth. Three species, *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *S. agalactiae*, are responsible for most cases of bacterial meningitis. Among these bacteria, only *N. meningitidis* is able to generate epidemics. *S. agalactiae* is the predominant pathogen among newborns, *N. meningitidis* among children 2–18 years old, and *S. pneumoniae* among adults [3, 4]. Pneumococcal meningitis has the worst prognosis: even with the best medical care and the use of modern antibiotics (plus adjuvant dexamethasone therapy), still about 15 % of the patients with pneumococcal meningitis die of the disease and up to one-third of survivors remain with neurologic deficits [4–7]. Unfortunate courses of the disease are mainly due to intracranial complications occurring secondary to meningitis, notably cerebrovascular alterations such as vasculitis, vasospasm, or venous thrombosis as well as hydrocephalus

and brain edema [8–10]. These alterations result in hypoperfusion and increased intracranial pressure, frequently leading to cerebral ischemia and/or herniation [11, 12]. Nearly 60 years ago, the hypothesis that these complications occur predominantly as a consequence of a hyper-inflammatory reaction within the central nervous system was formulated [13]. This hypothesis became the rationale for treating patients suffering from bacterial meningitis with immunosuppressive corticosteroids. Nowadays, dexamethasone is recommended for adjunctive therapy in selected patients, namely, in adults who suffer from pneumococcal meningitis and have not yet received antimicrobial treatment. Adjunctive dexamethasone is, however, far from giving complete protection. It only halves mortality and has only marginal effects on neurologic sequelae. Furthermore, a positive effect of dexamethasone was not found in studies performed in low-income countries (for review, see [14, 15]). Thus, there still is the urgent need for additional treatment strategies which can further reduce the adverse outcome of the disease. It is likely that the key will lie in more pathophysiologically targeted approaches. The scope of this article is to summarize the current knowledge on the pathophysiology of bacterial meningitis, using the example of pneumococcal meningitis which is the experimentally best characterized subtype, and to provide an outlook on promising therapeutic approaches.

2 Pathophysiology of Pneumococcal Meningitis

2.1 Survival of Pathogens in the CSF

Pneumococcal meningitis typically develops when bacteria enter the subarachnoid space from the blood compartment (hematogenous meningitis; predominant route in neonates and children) or through continuous spread of infection from a nearby focus (the mostly used route in adults). The subarachnoid space is the space between the arachnoid mater and the pia mater, which contains cerebrospinal fluid (CSF). From an immunological point of view, the subarachnoid space is a special compartment of the body. The subarachnoid space lacks a fully organized drainage by lymphatic vessels [16]. Moreover, soluble pattern recognition receptors (PRRs) like complement factors that perceive the presence of bacteria and mediate their uptake by phagocytes are virtually absent [17, 18]. Additionally, highly specialized blood–CSF barriers seclude the subarachnoid space from the blood circulation and impede the entry of most blood components like soluble PRRs into the CSF [19]. Even in the presence of bacterial meningitis, which is regularly associated with damage to blood–CSF barriers, concentrations of soluble PRRs remain far below those found in serum [20]. In contrast to this humoral deficit, functionally active macrophages, dendritic cells, and mast cells are present in tissues lining the CSF, namely, the choroid plexus, the perivascular spaces, and the leptomeninges [21, 22]. These cells are potential candidates for sensing the invasion of bacteria into the CSF through their cellular PRRs [21, 22]. The PRRs are expressed on the surface (like toll-like

receptor (TLR) 2), within endosomes (like TLR9), and in the cytoplasm nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs) of these cells. Activation of PRRs can initiate an inflammatory response by activating specific transcription factors (like nuclear factor (NF)-κB) and subsequently stimulating the synthesis and release of a variety of cytokines. However, the reactivity of the immune cells is probably restricted by diverse immunosuppressive factors that are constitutively expressed in the CSF, like members of the transforming growth factor family, cystatin C, or tumor necrosis factor (TNF)-related apoptosis-inducing ligand [23–26]. As a consequence, when bacteria reach the subarachnoid space, they can multiply easily, reaching similar high titers (up to 10^9 colony-forming units (CFU)/ml) as under bacterial culture conditions [27]. Bacteria like *S. pneumoniae* undergo autolysis when they are injured by a hostile environment or attain the stationary phase of growth. Hence, pneumococcal degradation products are liberated into the extracellular milieu. Their recognition by PRRs is the starting shot for the host inflammatory reaction. All in all, the CSF space exhibits a defective humoral (but not cellular) immunity which allows bacteria to prosper. This leads to the generation of large quantities of bacterial products and, as a result, a massive inflammatory reaction in the subarachnoid space.

2.2 Initiation of the Immune Response

In landmark experiments in the 1980s, the major pneumococcal cell wall components peptidoglycan (PG) and lipoteichoic acid (LTA) were indicated to be the key activators of the host immune response during meningitis (Fig. 1) [28, 29]. In a rabbit model, intracisternal injection of pneumococcal PG or LTA was sufficient to induce meningeal inflammation and to cause clinical symptoms of meningitis [30]. Accordingly, in patients with pneumococcal meningitis, mortality and morbidity from the disease were significantly associated with high LTA concentrations in the CSF [31]. In the last 15 years, the mechanisms underlying immune activation by pneumococcal cell wall components have been clarified to a great extent. First insight came from overexpression assays in cell lines: when the pattern recognition receptor TLR2 was ectopically expressed in fibroblast cell lines, the cells became responsive to pneumococcal degradation products or live *S. pneumoniae* [32, 33]. In further experimental series, the pneumococcal cell wall components PG and LTA were identified as the key ligands for TLR2 [34]. Moreover, TLR4, also located at the cell surface, was reported to interact with the pneumococcal toxin pneumolysin (PLY) [35, 36], but this finding was questioned later by other groups [37, 38]. In addition, TLR9 was described to sense the presence of genomic DNA from *S. pneumoniae* [39]. More recently, TLR13 was implicated in the recognition of pneumococcal RNA [40]. Investigations on isolated macrophages of gene-deficient mice demonstrated that single deficiencies of TLR2, TLR4, or TLR9 had no significant impact on pneumococci-induced macrophage activation. The combined loss of TLR2, TLR4, and TLR9, however, resulted in a marked reduction in cytokine

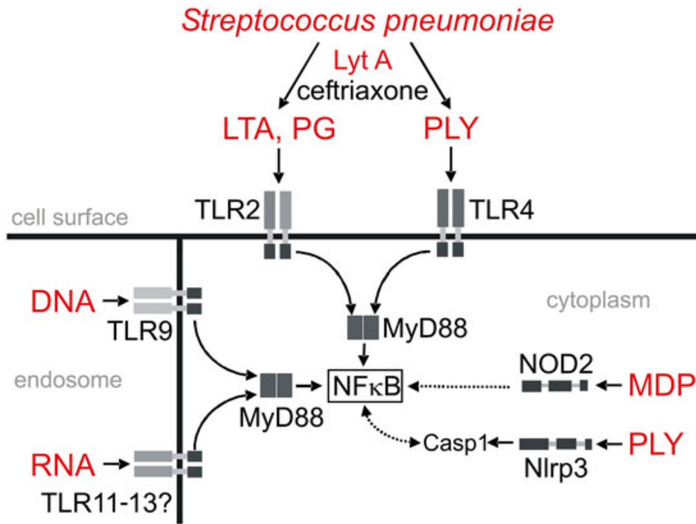

Fig. 1 A simplified model for the recognition of *Streptococcus pneumoniae* by pattern recognition receptors. See text for details. *Lyt A N*-acetylmuramoyl-L-alanine amidase (autolysin), *LTA* lipoteichoic acid, *PG* peptidoglycan, *PLY* pneumolysin, *MDP* muramyl dipeptide, *TLR* toll-like receptor, *Nod2* nucleotide-binding oligomerization domain (Nod)-like receptor 2, *Nlrp3* Nod-like receptor family, pyrin domain-containing protein 3, *NF* nuclear factor

production by macrophages upon exposure to *S. pneumoniae* [41]. It is also noteworthy that macrophages become nearly unresponsive to Gram-positive bacteria when they have defects in endosomal TLR signalling in addition to the lack of TLR2 and TLR4 [40]. Besides endosomal TLRs, NLRs likely contribute to the immune activation in response to internalized pneumococci. This is supported by the following observations: (1) Opitz et al. [42] reported that viable *S. pneumoniae* are capable of invading human fibroblasts. (2) Genetic complementation studies in human fibroblasts revealed that NF-κB activation induced by *S. pneumoniae* depends on the NLR Nod2. (3) By using primary cells from gene-deficient mice, NOD2 was found necessary for mounting a maximal inflammatory responses of microglial cells and astrocytes to live *S. pneumoniae* [43]. (4) Apart from NOD2, the NLR family, pyrin domain-containing protein 3 (NLRP3) was implicated as a sensor for pneumolysin and was capable of mediating interleukin (IL)-1β production by macrophages following challenge with pneumolysin or viable *S. pneumoniae* that express pneumolysin [37, 38, 44]. Collectively, these in vitro findings suggest that *S. pneumoniae* is sensed by immunocompetent cells through TLRs and NLRs in a cooperative manner. Thereby, extracellular bacterial components are sensed in a synergistic fashion by TLR2 and TLR4, while internalized bacterial fragments are recognized by endosomal TLRs and NOD2.

Substantial in vivo evidence for the involvement of TLRs in pneumococcal meningitis came from studies in mice lacking functional MyD88 which is kind of a bottleneck in the signalling cascades of all TLRs except TLR3. In an adult mouse

model of pneumococcal meningitis, MyD88-deficient mice exhibited a defective host immune response inside the CSF, as evidenced by a substantial abrogation of the expression of pro-inflammatory cytokines (e.g., IL-1β), chemokines, and complement factors in the brain and, hence, an insufficient neutrophil infiltration [45]. By utilizing mice with single or combined deficiencies of cell surface and endosomal TLRs, our group was able to demonstrate that TLR2, TLR4, and TLR11, TLR12 or TLR13 (but not TLR3, TLR7, and TLR9) are crucial for mounting an immune response in the CSF in pneumococcal meningitis [41] (unpublished data). This is deduced from the following constellation of findings: first, infected TLR2-TLR4-double-deficient mice showed a 50 % reduction in CSF leukocyte counts and a selective cytokine production, whereas the single deficiency of neither TLR2 nor TLR4 had any substantial impact on meningeal inflammation. Moreover, additional TLR9 or TLR3-TLR7-TLR9 deficiency did not result in a significant further attenuation of the inflammatory reaction as observed in TLR2-TLR4-double-deficient mice [41] (unpublished data). In addition, infected Unc93b1 mutant mice that lack endosomal TLR signalling (TLR3, TLR7, TLR9, TLR11, TLR12, TLR13) exhibited an inflammatory phenotype comparable to that of TLR2-TLR4-double-deficient mice. Finally, the combined loss of endosomal TLR signalling, TLR2, and TLR4 was accompanied by a reduction of CSF pleocytosis by about 75 % (unpublished data). This reduction is quite similar to that observed in MyD88-deficient mice [45]. The fact that MyD88 deficiency was paralleled by a strong but incomplete inhibition of the host immune response argues for the presence of additional PRRs in the recognition of *S. pneumoniae* in vivo. Genetic association studies showed an increased risk for pneumococcal infections in humans with complement (C) deficiencies [46]. Moreover, studies in animal models demonstrated the importance of an intact C system for a successful host defense against systemic pneumococcal infections like pneumonia and sepsis [47]. Accordingly, in a mouse model of pneumococcal meningitis, mice lacking the complement factors C1q or C3 displayed an enhanced bacterial outgrowth in the brain, which was associated with an attenuated innate immune response [48]. As mentioned above, complement concentrations are rather low in the CSF under normal conditions but increased substantially during the course of meningitis. The increase in C concentrations, however, occurs in a MyD88-dependent manner, arguing against a role of C factors as initial sensors of pneumococcal infection. Other potential sensors for pneumococcal infection of the subarachnoid space include NOD2 and NLRP3, as indicated by in vitro data [43]. This concept is strengthened by findings in mouse models where (1) increases in brain levels of the inflammatory cytokine TNFα and the chemokine CCL3 that were observed after intracerebral *S. pneumoniae* inoculation were virtually absent in NOD2-deficient mice and (2) the infiltration of leukocytes into the subarachnoid space following intracisternal pneumococcal infection was significantly lower in NLRP3-deficient mice than in wild-type mice [43, 44].

All in all, the presence of *S. pneumoniae* in the subarachnoid space seems to be initially recognized by TLR2, TLR4, a currently unidentified endosomal TLR (possibly TLR13) as well as other PRRs like NOD2 and NLRP3. Their engagement leads to the activation of transcription factors like NF-κB [49] and, as a conse-

quence, the production of pro-inflammatory cytokines and complement factors [45, 48]. These host mediators, in turn, seem to be critical for the amplification of inflammation in pneumococcal meningitis, as described in the following section.

2.3 Amplification and Propagation of the Immune Response

2.3.1 Role of IL-1 Family Cytokines in Pneumococcal Meningitis

Among the cytokines that have been implicated in the amplification and perpetuation of meningeal inflammation, IL-1 family cytokines are prominent (Fig. 2) (for detailed information about this cytokine family, see [50]). Elevated concentrations of IL-1β and IL-18 were observed in CSF samples withdrawn from patients with bacterial meningitis on hospital admission. High CSF IL-1β (but not IL-18) levels were found to be significantly associated with high CSF leukocyte numbers and an adverse clinical outcome [51, 52]. In animal models, intracisternal injection of recombinant IL-1β was sufficient for inducing meningitis [53, 54], and antibodies directed against IL-1β attenuated meningeal inflammation after intracisternal pneumococcal infection [55]. In line with the latter finding, mice lacking the IL-1 receptor type 1 exhibited less profound inflammatory infiltrates in the leptomeninges and lower brain cytokines levels than wild-type mice in a mouse model of hematogenous pneumococcal meningitis [56]. In this model, IL-18-deficient mice also showed a suppressed inflammatory response, as evidenced by a less profound inflammatory infiltrate around the meninges as well as lower brain cytokine and chemokine concentrations [57]. Accordingly, using a mouse model in which *S. pneumoniae* is instilled directly into the CSF, we observed that (1) IL-1 receptor

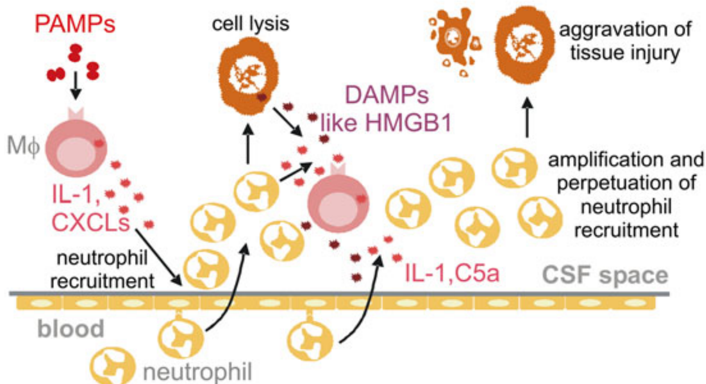

Fig. 2 Schematic diagram of the key steps in the pathophysiological cascade of pneumococcal meningitis. See text for details. *CSF* cerebrospinal fluid space, *PAMPs* pathogen-associated molecular patterns, *DAMPs* danger-associated molecular patterns, *HMGB1* high-mobility group box 1 protein, *IL* interleukin, *C5a* complement component C5a, *CXCLs* chemokines

blockade (by anakinra) significantly attenuated meningeal inflammation and (2) IL-18 neutralization (using recombinant IL-18 binding protein) in addition to IL-1 receptor blockade resulted in a further reduction of CSF pleocytosis [44]. Moreover, mice lacking caspase-1 which is crucial for the generation of active IL-1β and IL-18 showed a strongly diminished inflammatory host response, and treatment of rats with a broad spectrum caspase inhibitor resulted in a marked attenuation of meningeal inflammation [58]. Similarly, Braun et al. reported that this inhibitor also attenuated leukocyte influx into the CSF in rabbits with pneumococcal meningitis [59].

Combined, these data provide substantial evidence for IL-1 family cytokines as key regulators of inflammation. On the one hand, they may boost TLR-induced inflammation by forming a positive feedback loop involving IL-1 receptors and MyD88, the adapter molecule shared by IL-1β, IL-18, and most TLRs. On the other hand, IL-1 family cytokines may contribute to the perpetuation of inflammation. This hypothesis is deduced from our observation that treatment with IL-1β-neutralizing antibodies when started at 21 h after pneumococcal infection (in combination with the antibiotic ceftriaxone) is still effective in reducing meningeal inflammation. In contrast, co-application of ceftriaxone with antibodies directed against both TLR2 and TLR4 had no impact on the host immune response, arguing against a major role of these signalling receptors in established pneumococcal meningitis [60].

2.3.2 Role of the Anaphylatoxin C5a in Pneumococcal Meningitis

Apart from complement opsonins (like C1q or C3b), the complement activation cascade yields soluble factors known as anaphylatoxins (like C3a and C5a) and ends in the formation of the terminal complement complex (C5b-9) [61, 62]. The anaphylatoxins are generally considered pro-inflammatory polypeptides. Their effector functions include chemotaxis and activation of granulocytes, mast cells, and macrophages [63]. C5b-9 causes cytolysis through the formation of the membrane attack complex (MAC), and sub-lytic MAC and soluble C5b-9 also possess a multitude of non-cytolytic immune functions [64]. Markedly elevated C5a and C5b-9 concentrations were detected in the CSF of meningitis patients and in brain lysates of infected mice that correlated with CSF leukocyte counts [60]. In rabbits, intracisternal injection of C5a caused a rapid influx of leukocytes into the CSF [65]. Similarly, intracerebroventricular application of C5b-9 resulted in the production of cytokines (like IL-1β), chemokines, and subsequent accumulation of neutrophils in the CSF [66]. Moreover, the chemotactic activity of CSF samples obtained from rabbits with pneumococcal meningitis could be inhibited by treatment with antibodies to native human C5 [67]. In experiments using mouse strains with selected complement deficiencies, C5a was singled out to be crucial for the propagation of the inflammatory response in pneumococcal meningitis. The deficiency of the receptor for C5a (but not the receptor for C3a or of C6) was associated with a profound reduction of brain cytokine/chemokine expression and in CSF pleocytosis [60]. In addition, treatment of mice with neutralizing antibodies directed against C5,

irrespective if started prior or 24 h after infection, dampened the host immune response, suggesting that C5a acts both as an early and late mediator of inflammation in pneumococcal meningitis.

All in all, as a consequence of release of IL-1 family cytokines and C5a, large numbers of neutrophils are recruited into the subarachnoid space. The recruitment of neutrophils to sites of infection is required for an effective host defense against invading pathogens. However, their defense mechanisms that destroy or digest pathogens can also be deleterious to host tissue.

2.4 Maintenance of the Immune Response

Stressed or injured cells can release alarm signals (so-called danger-associated molecular patterns, DAMPs) that can orchestrate inflammation [68, 69]. Well-known DAMPs include heat shock proteins, S100 proteins, and high-mobility group box 1 protein (HMGB1) [70]. HMGB1 is a ubiquitously expressed, highly conserved nuclear protein with multiple intracellular functions including stabilizing nucleosome structure and facilitating DNA bending [71]. It can be actively secreted by macrophages upon stimulation with PAMPs or pro-inflammatory cytokines (through a nonconventional pathway which requires inflammasome assembly and caspase-1 activation [72]) or passively released from dying cells following nuclear and cell membrane disintegration (for review, see [71, 73, 74]). Extracellular HMGB1 behaves much like a cytokine. HMGB1, by itself and/or by forming complexes with exogenous or endogenous pro-inflammatory molecules, can induce and enhance the production of cytokines and chemokines. In addition, HMGB1 can promote chemotaxis and accumulation of granulocytes at inflammatory sites (for review, see [71, 73, 74]). Recently, two case studies reported that HMGB1 levels were significantly elevated in CSF samples from children with bacterial meningitis as compared to those from children with no or aseptic meningitis [75, 76]. Correspondingly, we detected large quantities of HMGB1 in the CSF of adult patients with pneumococcal meningitis as well as in mice subjected to pneumococcal meningitis [77]. In the mouse model, we further observed a substantial rise in CSF HMGB1 between 24 and 45 h after infection, pointing at a possible role of this protein in advanced rather than in early stages of the disease. Accordingly, in the mouse model, treatment with the HMGB1 antagonists, ethyl pyruvate or Box A protein, had no effect on the development of meningitis but led to better resolution of inflammation during antibiotic therapy. Additional experiments using gene-deficient mice and murine neutrophils provided evidence that HMGB1 acts as a chemoattractant for neutrophils in a RAGE (receptor for advanced glycosylation end products)-dependent fashion. Moreover, by using macrophages, we observed that the release of HMGB1 from these cells upon challenge with *S. pneumoniae* is passive in nature. All in all, these data suggest that HMGB1, presumably released from dying cells, acts as a propagator of inflammation in pneumococcal meningitis. This may provide an explanation for the empiric observation that inflammation can persist over days even though

antibiotic therapy sterilizes the CSF quickly and is paralleled by a fast reduction in CSF pneumococcal fragments (within hours) [78–82]. This delay in the resolution of inflammation may be the consequence of a vicious cycle in which inflammation-induced cell injury leads to the release of endogenous DAMPs that drive the inflammatory response, causing further damage.

3 Mechanisms of Brain Injury in Pneumococcal Meningitis

Accumulation of neutrophils at sites of infection is required for an effective host defense. However, activated neutrophils secrete a large arsenal of cytotoxic agents which can also damage host cells. Over 50 years ago, Johnson and colleagues [13] were the first to hypothesize that, in pneumococcal meningitis, the inflammatory response does more harm than good. The validity of this hypothesis was established by studies in animal models of the disease. First strong evidence for a harmful role of neutrophils in bacterial meningitis came from studies with antibodies against adhesion-promoting receptors of neutrophils. In a rabbit model, intravenous injection of anti-CD18 antibodies was reported to effectively block the development of pleocytosis in the CSF of animals challenged intracisternally with living *S. pneumoniae* or pneumococcal cell wall components [30]. This effect was associated with protection from blood–brain barrier injury. Therapy with anti-CD18 antibodies also prevented development of brain edema and death in animals infected with a lethal dose of *S. pneumoniae* [30]. Similarly, in a mouse model, parenteral treatment with anti-CD18 antibodies effectively inhibited leukocyte recruitment to the CSF and attenuated hippocampal injury 24 h after instillation of pneumococcal cell wall components into the lumbar spinal channel [26]. These findings were strengthened by results of mouse studies in which neutrophils were depleted by cell-specific antibodies [26, 60, 83]. The elimination of neutrophils resulted in a dramatic reduction of meningeal inflammation, as indicated by markedly lower CSF leukocyte numbers and brain cytokine concentrations. This was paralleled by significant reductions in intracranial pressure, blood–brain barrier breaching, and intracerebral bleeding (due to vasculitis) [44, 60, 83]. Combined, these studies implicated neutrophils to be major effector cells of brain injury in pneumococcal meningitis.

Among the effector molecules in the neutrophils' arsenal are strong oxidants like peroxynitrite and proteolytic enzymes such as matrix metalloproteinase (MMP). Oxygen radicals can exert a vast variety of cytotoxic effects, e.g., through lipid peroxidation, DNA strand breakage, or mitochondrial damage. Oxidative alterations to vital macromolecules such as membrane phospholipids, DNA, or proteins were detected in brain samples obtained from both patients who died from meningitis and in animal models of meningitis (for review, see [84]). In humans with bacterial meningitis, high-grade oxidative stress as indicated by high CSF levels of biomarkers of oxidative stress such as nitrotyrosine was significantly associated with an adverse outcome of the disease [85]. Accordingly, studies in rodent models provided substantial evidence that antioxidant therapy can be protective against

meningitis-associated brain injury (for review, see [84]). Besides oxygen radicals, proteolytic enzymes like MMP are released from activated neutrophils. Abnormal production and activation of these proteases can result in blood–brain barrier breaching and neuronal cell death [86]. High concentrations of MMP-9 were found in CSF samples of patients and animals with bacterial meningitis (for review, see [87]). Thereby, CSF MMP-9 concentrations were significantly higher in patients who developed neurologic sequelae than those who fully recovered. Moreover, experimental studies conducted in animal models of pneumococcal meningitis showed that MMP inhibitors (like GM6001 and BB-1101) are capable of reducing brain damage, neurologic sequelae, and mortality from pneumococcal meningitis.

Taken together, meningitis-associated brain damage is predominantly due to the massive accumulation of neutrophils inside the central nervous system whose antimicrobial weapons, namely, oxidants and MMPs, cause collateral damage to host cells.

4 Potential Targets for Therapy

New ideas for adjunctive therapy have emerged from studies on the mechanisms underlying meningitis-associated brain pathology. The principle behind novel treatment strategies is to reduce CNS inflammation by interfering at critical steps of the inflammation cascade which compromise (1) release of inflammatory bacterial products (PAMPs), (2) recognition of these PAMPs, (3) amplification and perpetuation of the immune response, as well as, (4) generation and release of cytotoxic agents (see also reviews [15, 88]). The following section will highlight two promising approaches for adjunctive therapy of pneumococcal meningitis, namely, the coadministration of non-bacteriolytic antibiotics as well as neutralizing antibodies directed against C5.

4.1 Limiting the Release of Inflammatory Bacterial Products

During conventional treatment of pneumococcal meningitis with β-lactam antibiotics, large amounts of pneumococcal cell wall degradation products are liberated into the CSF. As a consequence, the inflammatory host reaction is boosted, potentially causing additional harm to host tissues. Therefore, non-bacteriolytic antibiotics like daptomycin may represent a promising option for meningitis therapy. Daptomycin appears to insert into the cell membrane of Gram-positive cells, leading to pore formation and cellular depolarization, resulting in an arrest of DNA, RNA, and protein synthesis, and subsequently in non-lytic cell death [89]. In a rabbit model of pneumococcal meningitis, daptomycin monotherapy was superior to ceftriaxone monotherapy and was highly efficacious in sterilizing the CSF [82]. Administration of dexamethasone prior to daptomycin affected the antibacterial activity of

daptomycin only marginally, either as monotherapy or combined with ceftriaxone, although the penetration of daptomycin into inflamed meninges was significantly reduced by two-thirds [90, 91]. In an infant rat model of pneumococcal meningitis, daptomycin monotherapy was demonstrated to clear pneumococci more rapidly from the CSF than ceftriaxone, to attenuate CSF inflammation, and to prevent the development of cortical injury [92, 93]. Since daptomycin (due to a lack of efficacy in pneumococcal pneumonia) is not a candidate for monotherapy of pneumococcal meningitis, supplementary studies assessed whether combining daptomycin with ceftriaxone is superior to ceftriaxone monotherapy. In an infant rat model, the combination therapy was accompanied with reduced inflammation, less brain damage, and improved hearing capacity [94]. The neuroprotective efficacy of this therapeutic approach was recently confirmed by our group in an adult mouse model (unpublished data). Open questions like the comparison of the antibiotic co-treatment with co-therapies consisting of dexamethasone and ceftriaxone (current standard therapy) as well as of daptomycin, dexamethasone, and ceftriaxone underline the need of further experimental investigations before clinical trials can be attempted. Moreover, human data on the CSF penetration of daptomycin are scarce. A recent study reported that mean concentrations of daptomycin in the CSF after a single intravenous dose (10 mg/kg) were significantly lower in patients than that previously reported in animal studies [95]. In order to better characterize the CSF penetration of this drug, additional pharmacokinetic studies evaluating multiple and/or higher dosages of daptomycin are necessary in humans, especially in those suffering from pneumococcal meningitis.

4.2 Neutralizing Endogenous C5a Activity

The anaphylatoxin C5a was identified to be a key player in the inflammatory cascade of pneumococcal meningitis. Both genetic deficiency of the receptor of C5a and pharmacologic neutralization of C5 resulted in a marked reduction of meningeal inflammation but also of meningitis-associated neuropathologic alterations like blood–brain barrier disruption or cerebral hemorrhages [60]. Interestingly, in adults with bacterial meningitis (including pneumococcal meningitis), high CSF C5a levels were associated with death and an unfavorable outcome [60]. Therefore, C5a was hypothesized to be a promising target for adjunctive therapy in pneumococcal meningitis. In line with this hypothesis, adjuvant treatment with a monoclonal antibody directed against C5 was completely protective against death due to pneumococcal meningitis in an adult mouse model. Moreover, this treatment strategy was effective in dampening meningitis-induced neuropathologic alterations. Its efficacy was clearly superior to that of adjuvant dexamethasone [60]. Since anti-C5 antibodies are already licensed for clinical use (e.g., in patients with paroxysmal nocturnal hemoglobinuria), adjuvant therapy with anti-C5 antibodies may be a promising therapeutic approach for patients with bacterial meningitis. However, this treatment

approach still needs to be evaluated in meningitis models that measure neurologic (long-term) sequelae and/or use other meningitis pathogens (especially *Neisseria meningitidis* which can be killed by the membrane attack complex whose formation is blocked by anti-C5 antibodies). Moreover, data on its efficacy in combination with dexamethasone are lacking.

5 Conclusion

During the past two decades, great progress has been made in our understanding on the immunopathogenesis of pneumococcal meningitis. Mechanisms of immune activation, amplification, and perpetuation just as well as causes of meningitis-associated brain damage have been largely unveiled. This knowledge provides the basis for the development of novel strategies for treatment of this disease. Two novel therapeutic approaches have been recently evaluated in animal models of pneumococcal meningitis, namely, killing bacteria without lysing them and blocking the pro-inflammatory activity of C5a in combination with antibiotic therapy. Early results from experimental studies are very encouraging. However, there is still a difficult way to go until the ultimate goal of helping patients with meningitis is reached. First of all, animal studies are needed to assess the efficacy of these strategies when applied together with steroids—a prerequisite for clinical trials as steroids are part of the standard therapy for bacterial meningitis. Moreover, it has to be investigated whether and how novel therapeutic strategies affect the outcome of meningitis due to pathogens other than pneumococci as well as in special patient groups. This appears necessary in consideration of the observation that steroids are ineffective in less developed countries or in patients suffering from meningitis due to pathogens other than *S. pneumoniae*. Additionally, different pharmacologic issues, like the CSF penetration of daptomycin in humans, have to be clarified. It has also to be checked whether a successful treatment of bacterial meningitis requires the simultaneous targeting of multiple steps of the pathophysiologic cascade, like killing bacteria softly and blocking critical steps in the inflammatory cascade. Even when a therapeutic approach has been proven highly beneficial in animal models, its translation into the clinical practice will be challenging since the recruitment of sufficient number of patients requires a multicenter study design. Moreover, patient cohorts are usually relatively heterogeneous (with regard to the causative agent or the degree and type of comorbidities). However, the promising data from animal models, coupled with the still unfavorable prognosis in humans, should be incentive enough for researchers and physicians to follow this path all the way to the end.

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Cerebral Malaria

Gregory S. Park and Chandy C. John

Abstract Cerebral malaria (CM), defined as the presence of *P. falciparum* asexual stages on peripheral blood smear in a person with coma and no other cause for encephalopathy, is estimated to affect more than 800,000 people a year and has a 15–20 % mortality rate. CM predominantly affects children <5 years of age in Africa, but in Southeast Asia it is more common in adolescents and adults. Approximately 25 % of African children with CM develop long-term cognitive impairment. The pathogenesis of cerebral malaria appears to involve several components. The primary factor in pathogenesis of CM is thought to be sequestration, a blockage of microcirculatory vessels in the brain by parasitized red blood cells, along with lesser numbers of leukocytes and platelets. Other factors that appear to be involved in pathogenesis include systemic and central nervous system (CNS) production of proinflammatory cytokines and chemokines, including tumor necrosis factor, interferon-γ, and RANTES; release of free heme during hemolysis; endothelial activation leading to blood–brain barrier breakdown; CNS nitric oxide production; and genetic polymorphisms (e.g., sickle cell trait) that alter these responses or protect in other ways from severe disease. Murine models of cerebral malaria have provided new insights into the disease, but the difference in the parasite species and the host response has limited translation of findings from murine models into human CM studies. Nonhuman primate models are closer to human

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disease, but are limited by cost and ethical concerns. Therapies currently being studied for adjunctive therapy in CM include arginine (a donor of nitric oxide), inhaled nitric oxide, and recombinant erythropoietin. The potential benefits and harm of each therapy require close study, as many areas of CM pathogenesis remain unclear. Further studies are required, particularly in human disease, to better understand pathogenesis so that effective adjunctive therapy for this illness can be developed.

Keywords Cerebral malaria • *Plasmodium falciparum* • Human • Animal • Murine • Experimental • Model • Inflammation • Sequestration

Abbreviations

ANG1	Angiopoietin-1
ANG2	Angiopoietin-2
BBB	Blood–brain barrier
CM	Cerebral malaria
CNS	Central nervous system
CSF	Cerebral spinal fluid
EC	Endothelial cell
ECM	Experimental cerebral malaria
EDHF	Endothelium-derived hyperpolarization factor
EPO	Erythropoietin
ET-1	Endothelin-1
G6PD	Glucose-6-phosphate dehydrogenase
GPI	Glycosylphosphatidylinositol
HO	Heme oxygenase
ICAM-1	Intercellular adhesion molecule-1
iNOS	Inducible nitric oxide synthase
LT	Lymphotoxin
MT	Metallothionein
NK	Natural killer
NO	Nitric oxide
P	Plasmodium
PfEMP-1	*P. falciparum* erythrocyte membrane protein-1
pRBC	Parasitized red blood cell
SNP	Single nucleotide polymorphisms
TLR	Toll-like receptor
VCAM-1	Vascular cell-adhesion molecule-1
VWF	von Willebrand factor
WHO	World Health Organization

1 Introduction

Five *Plasmodium* parasites have been implicated in causing malaria in humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. Of these, it is estimated that 90 % of cases involve infections with *P. falciparum* [1]. There were an estimated 1.24 million deaths [2] and 219 million cases of malaria in 2010 [1], and 58 % of deaths were in children less than 5 years old [2].

Cerebral malaria (CM) is among the deadliest forms of severe malaria, with mortality of 15–20 % [3]. CM is clinically defined as coma with the presence of asexual forms of *P. falciparum* on peripheral blood smears and no other cause of encephalopathy [4, 5]. *P. falciparum* infections are confined to the intravascular space of the brain, and there is no cerebral spinal fluid (CSF) leukocytosis in children with CM, though proinflammatory cytokines are present [6]. In this respect, CM differs from viral or bacterial CNS infections [4]. Cognitive impairments and neurological deficits afflict children who survive CM. Neurological consequences of CM include visual and hearing impairments, hemiparesis, ataxia, and speech and language impairments, which occur in 25–40 % of children at the time of discharge, but resolve in all but ~4 % of children by 6-month follow-up. Twenty-five percent of children who survive CM survivors have evidence of long-term cognitive impairments and neurological deficits, including spasticity, behavior problems, and epilepsy [7–10]. In adults that recover from CM, <5 % have neurological deficits, and the deficits are different from those observed in children. CM primarily affects children in sub-Saharan Africa but typically affects adolescents and adults in Southeast Asia. The clinical features of CM (other than coma) may differ between children and adults, and those clinical signs that are shared typically vary in frequency [7, 11]. The pathogenesis of CM is not completely understood, but is thought to involve the intersection of the immune response to *P. falciparum* replication and the sequestration of infected and uninfected erythrocytes in the microvasculature [12]. The mechanisms that are involved in the pathogenesis of CM are broadly categorized into mechanical obstruction, cytokine responses, inflammation, and hemostasis.

Cerebral capillary sequestration and retinopathy are associated with fatal CM and are a hallmark of the brain pathology of children who die of CM [12]. Sequestration of parasitized red blood cells (pRBCs) occurs in vital organs, including the brain (gray and white matter), kidney, heart, lung, and intestine [12, 13]. Additionally, autopsy studies of the brains from patients diagnosed with CM have shown lymphocyte margination of capillary endothelial cells (ECs) and lymphocytes and monocytes extravasating into the interstitium [14]. Children in sub-Saharan Africa with CM sequester platelets and leukocytes in addition to pRBCs [15], while in adults in Southeast Asia, pRBCs in the brain microvasculature are linked to coma and axonal damage [16, 17]. Several factors contribute to sequestration. First and foremost is the cytoadherence of pRBCs to ECs through *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) interactions with host receptors such as intercellular adhesion molecule-1 (ICAM-1), vascular cell-adhesion molecule-1 (VCAM-1), CD36, CD31, NCAM, and gC1qR/HABP1/p32 [18–20].

Binding of pRBCs and/or proinflammatory cytokine stimulation to ECs results in their activation and the release of a host of molecules that can lead to a dysregulation of hemostasis and a procoagulant state [21, 22]. In addition, uninfected RBCs can adhere to pRBCs to form rosettes [23], pRBCs can bind platelets to form clumps [24, 25], and upon infection there is a decrease in pRBC deformability due to alterations in the cytoplasmic skeleton, changes in membrane fluidity, and a more spherical morphology [26, 27]. Regardless of the many likely contributors, the principal trigger of sequestration is still not clear.

Activation of the cerebral endothelium is likely a critical factor in the pathophysiology of CM. After activation, ECs release ultra-large von Willebrand factor (VWF) multimers, angiopoietin-2 (ANG2), and increase the cell surface expression of ICAM-1, VCAM-1, P-selectin, and E-selectin. VWF provides an additive mechanism of sequestration by mediating the binding of pRBCs to ECs via platelets and CD36 [19, 28]. Angiopoietin-1 (ANG1) is the agonistic ligand of TIE2, and ANG2 is the antagonistic ligand of TIE2. Quiescent endothelial cells constitutively express ANG1 and TIE2. Upon release from EC activation, ANG2 antagonizes the ANG1-TIE2 interaction resulting in a facilitation of the responsiveness of ECs to cytokines, particularly TNF [29]. ANG2 stores in ECs vary but are significant in the microvessels of the brain [30], and elevated levels of ANG2 have been associated with severe malaria. Endothelial cells can also influence the cerebrovascular tone through the release of endothelin-1 (ET-1), prostaglandin $F_2\alpha$, thromboxane A_2, nitric oxide (NO), endothelium-derived hyperpolarization factor (EDHF), prostacyclin, and prostaglandin E_2 [31]. Thus, activation of ECs results in the release of factors that likely contribute to sequestration, cytokine release, inflammation, hypoxia, and dysregulated hemostasis. Activation of ECs by pRBC binding may also induce EC apoptosis [32].

2 Animal Models of Cerebral Malaria

There are several experimental CM (ECM) animal models used to study human CM, but the majority of studies are in either rodents or nonhuman primates. A problem central to all animal models of malaria is that no model shows the exact pathology of human disease, and pathology is dependent on parasite strain, host, inoculation dose, and timing of the infection. Of note, *P. falciparum* is not infective to most animals used in CM models, and other *Plasmodium* strains differ from *P. falciparum* in many ways, including a lack of knob formation [33, 34], which is critical to development of sequestration. Additionally, many animal models use inbred animals, and the clinical signs observed in any specific model do not reflect the spectrum of disease outcomes in a diverse genetic human population. Development of an animal model for CM is also complicated by variability in the cerebral pathophysiology and complications of human CM in adults and children [35–37]. For example, in classical human CM, brain pathology demonstrates capillary pRBC sequestration, cerebral ring hemorrhages, and cerebral edema. However, sequestration has been observed in individuals without defined CM [38], and some children fit the WHO

definition of CM but do not have sequestration [12]. Further, cerebral edema is not present in some children and adults with CM [37, 39], and elevated lumbar puncture opening pressures, which are present in 80 % of children with CM, are much less common in adults [40]. It has been suggested that what some consider a weakness in animal models may actually be a strength as the diversity in animal models may in some ways reflect the diversity of infection in humans [41].

Specific murine models of CM demonstrate a pathology of sequestration and hemorrhage that is similar in some respects to that of human CM, though no murine model to date completely mimics the changes seen with sequestration in human CM. In murine ECM, the species of parasites most often used are *Plasmodium chabaudi*, *Plasmodium berghei*, *Plasmodium yoelii*, and *Plasmodium vinkei vinkei*. *P. chabaudi* can cause lethal and nonlethal infections depending on the strain of mouse, but lethality is due to hemolysis. The *P. yoelii* strain 17XL has been studied in CF1 mice, and the *P. berghei* strain K173 has been studied in C57BL/Rij mice [42]. The pathological features in the *P. yoelii* 17XL model are sequestered pRBCs in the brain microvasculature and neurological issues, associated with high levels of parasitemia. The *P. yoelii* 17XLNL (nonlethal) strain has been further used in vaccine research to investigate immune responses [43]. *P. berghei* K173 can also show some signs of murine ECM, including early levels of IFN-γ, but disease is dependent on the mouse strain, how parasites are introduced, and the inoculation dose [44, 45].

The most common murine ECM model uses the *P. berghei* ANKA strain in several strains of mice, mostly C57BL/6 (6J or 6N) or CBA mice. The *P. berghei* ANKA strain murine ECM model has been extensively studied for over 20 years [46]. The disease course of *P. berghei* ANKA in C57BL/6 mice from a range of inoculum doses is described as a rapid progression toward death after an encephalopathy accompanied by seizures and respiratory distress. There is a reduction in mobility, with ataxia seen at 6 days postinoculation, followed by paralysis, convulsions, coma, and death within 7–10 days postinoculation in the presence of low parasitemia and any survivors dying in the second week postinoculation from hyperparasitemia and severe anemia [36, 47, 48]. There is also an increase in EC expression of ICAM-1, VCAM-1, and P-selectin in murine ECM suggesting activation of the endothelial cells similar to that seen in human CM [43]. Sequestration involves a mix of leukocytes and few pRBCs. The leukocyte population consists of a mix of macrophages and dendritic cells, T cells, B cells, neutrophils, and natural killer (NK) cells [49]. *P. berghei* ANKA infection also results in a disruption of the blood–brain barrier (BBB) [50, 51].

Many of the observed human CM systemic immune responses have been demonstrated in murine ECM using *P. berghei* ANKA. The use of the murine ECM model has allowed for the testing of specific mechanisms behind the observed immune responses. For example, the importance of cytokines and the strength of the proinflammatory response have been investigated through depleting key cell populations involved in early trigger events or through disrupting downstream cytokine signaling. Overall, the murine ECM model implicates Th1 responses but not Th2 responses. Specifically, NK cells and CD8+ T cells are essential components to murine ECM, and CD8+ T cells are the principle effector cells [49]. NK cells, IFN-γ

and IFN-γR signaling, are required for the migration and sequestration of CD8+ T cells in the brains of mice [52, 53], and migration is also influenced by NK T cells [54]. Movement of the CD8+ T cells to the brain happens in a malarial antigen-specific manner, and sequestered CD8+ T cells damage cerebral ECs and disrupt the BBB [55]. CD4+ T cells also contribute to murine ECM, with CD4+ αβ T cell populations higher in mice with ECM. However, depletion studies show that depending on the mouse strain, CD4+ T cells may be involved in either or both the induction or effector phase of ECM [55]. B cells do not appear to be involved in murine ECM as B cell-deficient mice show ECM and B cell antigen presentation is not required [56]. Finally, γδ T cell numbers are higher in mice with ECM [49], but their role in ECM may not be necessary or may involve inducing ECM. For, mice are protected if γδ T cells are depleted at 0 or 3 day postinoculation, but ECM can manifest if depletion of γδT cells is delayed to 5 days postinoculation [57].

The role of cytokines, chemokines, and chemokine receptors in *P. berghei* ANKA ECM seems to revolve around the effects of IFN-γ. ECM can be prevented in *P. berghei* ANKA-infected mice by neutralizing IFN-γ [58], and IFN-γ is necessary for the trafficking to and sequestration of leukocytes in the brain. Cytokines and chemokines secreted by sequestered monocytes include TNF, IP-10, MCP-1, and RANTES, but levels can vary depending on the mouse strain infected [59]. Sequestered neutrophils also secrete cytokines and chemokines such as IL-12_p40, IL-18, IFN-γ, TNF, MIP-1α, MIG, and IP-10.

Studies investigating pathogenesis have shown that TNF, lymphotoxin (LT)-α, and TNFRII are also important in murine ECM [60–62]. Neuropathologic studies of murine ECM have shown neuronal and endothelial apoptosis, with cerebral hemorrhages and no topographical pattern [63], that were associated with cognitive impairments [64]. Studies have further shown that *P. berghei* infections result in a breakdown of the BBB and are associated with brain edema and enlarged perivascular spaces [65–70]. It is likely that ECs are activated by proinflammatory cytokines such as TNF, LT-α, or IFN-γ. However, breakdown of the BBB on the "blood" or EC side is most likely a result of the killing of ECs by CD8+ T cells that have migrated to the brain [55]. On the brain side, activation of microglia and astrocytes may further worsen BBB breakdown. Murine ECM studies show increases in glial fibrillary acidic protein astrocytes, microglia, and activated caspase 3+ apoptotic astrocytes [71]. Activated glia secrete proinflammatory cytokines, NO, platelet-activating factor, quinolinic acid, and matrix metalloproteinases (MMPs). MMPs are known to degrade tight junction proteins and basal lamina proteins leading to BBB leakage [72]. Observed increases in intracellular metallothionein (MT)-1 and MT-II in astrocytes and microglia may reflect the need for protection against oxidative stress and apoptosis, and these cysteine-rich proteins may also reduce edema and modulate proinflammatory cytokine expression [63].

Despite the many similarities between human CM and murine ECM, important differences remain [46]. The most important difference is that in human CM, sequestration is dominated by pRBC, with additional contributions from leukocytes and platelets, while in murine ECM, leukocytes and platelets dominate the pathology, with varying and often low frequencies of pRBC [73–76]. For example, some studies with luciferase-expressing *P. berghei* ANKA do not demonstrate

sequestration in the brain, whereas they do show a degree of sequestration in the brain [36, 76]. Other murine ECM studies have shown that sequestration of pRBCs is regulated by CD8+ T cells and IFN-γ [77]. Indeed, murine ECM is T cell dependent, as ECM does not happen in nude mice, αβ-TCR knockouts, and mice depleted of CD4+ T cells [78]. In humans with CM, however, the role of T cells has not been fully characterized, but T cells have not been clearly implicated as a primary agent in CM. Further, autopsy immunohistochemistry of brain from CM children do not show leukocyte numbers similar to those seen in murine ECM. Thus, murine ECM pathology is dominated by a pattern of inflammation, something not seen nearly to the same extent in pathological studies of human CM.

Primate models are also used in studies of CM. Primate models of CM have used *Plasmodium knowlesi*, *Plasmodium coatneyi*, *Plasmodium fragile*, and *Plasmodium cynomolgi* or human species and strains that have been adapted to a specific simian species. Both New World and Old World monkeys have been used in primate models, such as rhesus macaques (*Macaca mulatta*), squirrel monkeys (*Saimiri sciureus*), and owl monkeys (*Aotus spp.*). *P. knowlesi* infections in rhesus monkeys can be severe and result in a breakdown of the BBB and cerebral edema. Though traditionally thought of as a primate *Plasmodium*, *P. knowlesi* can infect humans and can cause severe disease, and it may serve as a model for adult CM in Southeast Asia [79, 80]. *P. coatneyi* infections in rhesus monkeys can result in coma, with binding of pRBCs on ECs, sequestration of pRBCs in the brain, and increased expression of CD36, ICAM-1, and thrombospondin [81–83]. In addition, the *P. coatneyi* model shows a decrease in cerebral microcirculation [84]. However, earlier studies of *P. coatneyi* infections did not produce sufficient clinical data for a full evaluation of the model. Other studies have begun to address this and have shown pathology similar to what is observed in human CM, including multiorgan sequestration, proinflammatory cytokine and chemokine responses (IFN-γ, IL-6, TNF, MIP-1β, MCP-1), increased fibrinogen levels, and increased coagulopathy [85, 86]. Another potential CM model is *P. falciparum* infections of Saimiri monkeys. Studies with this model have shown sequestration of pRBCs in the brain [87], and brain EC lines from this model showed increases in ICAM-1 and CD36 upon stimulation with TNF [88]. Primate models may present a more relevant model to study CM, but they are difficult to conduct, due to cost and ethical concerns, so study findings from these models have been limited when compared to murine models.

3 The Systemic Immune Response in Human Cerebral Malaria

While it may be a specific parasite genotype that substantially influences the host immune response [89], severe disease is associated with a Th1 cytokine response, and protection is associated with Th2-type responses through both antibody actions and cell-mediated immunity. Typically, protective immunity comes with repeated exposures [90], with some protection from severe disease from as little as one or

two exposures [91]. Cerebral malaria often manifests in children in endemic regions of Africa between the ages of 6 months and 5 years, whereas CM in Southeast Asia typically presents in adolescents and young adults [92, 93]. The reasons for these differences are unclear, but may partly relate to differences in transmission intensity in the areas.

Study of pathophysiology in human CM at the brain level have been limited to date to autopsy studies and to a lesser extent studies of cerebrospinal fluid. Even autopsy studies carry the limitation of studying brain tissue only in individuals who died of CM. The pathological processes in these individuals may not be identical to those in individuals who survive CM. Results from autopsy and cerebrospinal fluid studies are summarized in the next section ("The Central Nervous System Response in Human CM"). Functional imaging studies such as functional magnetic resonance imaging or positron emission tomography of the brain are generally cost prohibitive for the low-income countries in which CM typically occurs.

A number of studies have assessed how systemic changes in samples from peripheral blood (plasma, serum, mononuclear cells) may relate to the pathogenesis of CM in humans. It has been proposed that a previous exposure may prime the Th1 response for an extreme proinflammatory immune response that results in CM [93]. To this end, TNF has been extensively examined in CM. TNF initiates inflammation [94, 95], activates ECs, and induces the release of other cytokines, including IL-6, IL-10, IFN-γ, and IL-1β. TNF release is thought to follow early IFN-γ release, which is induced by IL-12 and IL-18 stimulation from macrophages or dendritic cells [96].

In CM, peripheral blood CD4+ T cells are reduced [97], but there is an increase in CD4+ TCR Vβ21.3 cells [98]. However, CD4+ T cells have not been clearly implicated in the pathogenesis of CM [36]. Major sources of IFN-γ in individuals with CM include γδ TCR+ T cells and NK cells [99, 100]. In CM, a strong inflammatory Th1 response is not balanced by regulatory T cell ($CD4^{+}CD25^{hi}FOXP3^{+}CD127^{-/low}$) function. $CD25^{-}FOXP3^{-}CD45RO^{+}$ effector T cells are the major source of IL-10 [101].

Inflammation during CM may be due to host or parasite factors, including host genetic factors that regulate the inflammatory response [102, 103]. During the course of blood-stage replication, host and parasite products are released into the plasma after schizont rupture and can initiate innate immune responses by interacting with various host receptors on a variety of cells. The glycosylphosphatidylinositol (GPI) of *P. falciparum*, similar to endotoxin, binds to toll-like receptor (TLR) 2 and TLR4 on dendritic cells, macrophages, ECs, and adipocytes and induces the secretion of proinflammatory Th1 cytokines (TNF, IL-1, and IL-12). GPI also binds CD1d and Vα-Vβ8 T-cell receptor on natural killer cells to induce Th1 or Th2 cytokine production. Additionally, schizogeny releases hemozoin, hemoglobin, and erythrocyte arginase. Both hemozoin and hemoglobin can stimulate a cytokine cascade, including TNF and IL-12, and the Th2 cytokine IL-10 [104, 105]. Hemozoin can also suppress leukocyte proliferation [106] and affect monocyte/macrophage phagocytosis and MHC class II expression [106, 107], potentially inhibiting the immune system's ability to deal with parasite replication. Free hemoglobin in the blood is typically bound by haptoglobin to form a complex recognized by CD163 and apolipoprotein

A-1 on monocytes or macrophages [108, 109]. The complex is then internalized by these cells, often in the liver and spleen, and broken down by heme oxygenase (HO). If not bound by haptoglobin, hemoglobin scavenges NO and is oxidized into methemoglobin and nitrate. Erythrocyte arginase can use up stores of L-arginine, which is the source of NO, thus potentially increasing hemoglobin levels. If not scavenged by physiological protective mechanisms, hemoglobin and heme can trigger proinflammatory responses, such as the increases in peripheral blood TNF, IL-1, and IL-6 levels observed in children with CM [6, 110], and have pro-oxidant effects. Cytoadhesion of pRBCs and EC activation can also recruit monocytes resulting in the production of TNF [111] and can activate NF-kB signal transduction to increase proinflammatory chemokines and cytokines [112]. Thus, through mechanisms such as GPI, hemozoin, heme, hemoglobin, or adhesion activation, a proinflammatory cascade and innate immune response begins or is enhanced, and the resulting inflammation may be a strong prognosticator of disease severity.

Protection against severe disease, including CM, also relates in part to host genetic factors that may or may not involve the immune system. Numerous studies have documented host promoter and gene polymorphisms associated with protection against severe malaria. Such polymorphisms have included promoters and/or genes including red cell components, TLRs, immune system factors and/or receptors, reactive oxygen species regulators, EC surface receptors, and hemostasis elements (reviewed in [113]). Inherited genetic disorders of red cell components that can confer resistance or susceptibility include sickle cell trait, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and thalassemia. Sickle cell trait provides the strongest protection against severe malaria, as has been reviewed in multiple studies. The genetic mutations in disorders that affect red cell surface antigens, enzymes, hemoglobin, or the red cell cytoskeleton may inhibit parasite replication. For example, G6PD deficiency can result in hemolytic anemia and vulnerability to oxidative stress due to reduced levels of nicotinamide adenine dinucleotide phosphate. However, G6PD deficiency may also confer protection from malaria by reducing parasite growth through an increase in surface markers that target the pRBC for phagocytosis [113, 114]. Heme oxygenase (HO) is involved in the breakdown of free heme into biliverdin, iron, and carbon monoxide. HO is generally considered cytoprotective, The promoter of *HMOX1* has a (GT)n repeat polymorphism that can affect expression. Higher numbers of the (GT)n repeat result in lower expression, and lower numbers of the repeat have higher expression of HO-1. Conflicting results have associated severe malaria to both low [115, 116] and higher [117] numbers of (GT)n repeats. Polymorphisms in TLRs have been identified that alter TLR expression (TLR2, TLR9) or the ligand-binding site (TLR4) and have been associated with altered cytokine responses in children with CM [118] and with increased risk for severe malaria [119]. A number of gene and promoter single nucleotide polymorphisms (SNPs) for the genes regulating cytokines such as LT-α, TNF, IFN-γ, IL-12, IL-4, IL-1, and IL-10 have also been associated with protection or susceptibility to symptomatic and sometimes severe malaria (reviewed in [113]). Thus, host genetic factors appear to play an important role in susceptibility to CM.

4 The Central Nervous System Response in Human Cerebral Malaria

Assessment of the CNS response in human CM has largely been limited to autopsy and cerebrospinal fluid findings, though recently in vitro models of the BBB have provided some additional understanding of potential mechanisms in CM. The pathways through which vascular and CNS responses to *P. falciparum* may lead to CM are outlined in Fig. 1. Mechanisms by which CM may result in neurological sequelae

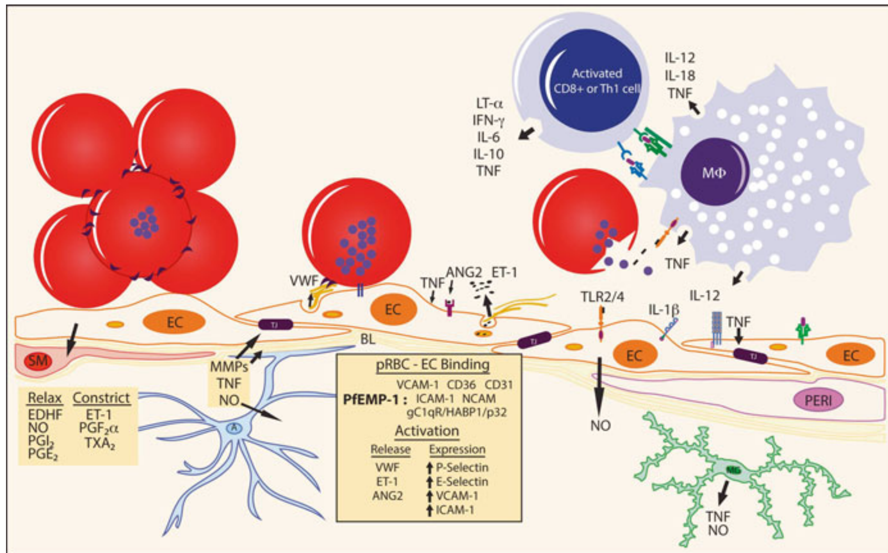

Fig. 1 Mechanisms of cerebral malaria pathogenesis. Several factors likely contribute to the pathogenesis of CM including mechanical obstruction, cytokine responses, inflammation, and a dysregulation of hemostasis. Ultimately, sequestration of parasitized RBCs (pRBCs) is associated with human CM. Uninfected and pRBCs can adhere with platelets to form rosettes, which may obstruct the cerebral microvasculature. The binding of pRBCs through PfEMP-1 to multiple endothelial cell (EC) receptors may also result in sequestration or in EC activation, which may exacerbate sequestration through the EC binding of pRBCs via platelets, CD36, and ultra-large von Willebrand factor (VWF) and through increased expression of EC receptors (ICAM-1, VCAM-1). EC activation also results in the release of angiopoeitin-2 (ANG2), endothelin-1 (ET-1), and other factors that affect vascular tone via signaling to smooth muscle (SM). In addition, ANG2 can increase EC responsiveness to TNF (among other cytokines). Inflammatory cytokine responses to toll-like receptor (TLR) 2 or TLR4 binding of parasite glycosylphosphatidylinositol (GPI) on macrophages (MΦ) or ECs may initiate or compound EC activation. MHC class I or class II activation of CD8+ or Th1 cells (respectively) may also initiate or compound inflammatory cytokine responses. Schizont rupture also releases hemozoin and hemoglobin, which can contribute further to the cytokine cascade. TNF and EC binding of pRBCs can activate ECs, potentially increasing the permeability of the blood–brain barrier, opening tight junctions (TJ), and inducing apoptosis. Factors released from stimulated glial cells can have both protective and harmful effects on the brain side. Astrocytes (A) can produce metalloproteinases (MMPs), which may degrade tight junction and basal lamina (BL) proteins. Microglia (MG), astrocytes, and pericytes (PERI) may produce TNF and NO, which can have both neuroprotective and neurotoxic effects

include the movement of cytokines and other molecules into the brain through a break in the BBB, or through active or passive transport through the BBB, or production of these factors from a signal transduction event in the brain. Early autopsy studies did not find evidence of breakdown of the BBB in CM [120], but more recent studies have documented that a degree of breakdown in the BBB does occur in human CM [121–123], though the breakdown is less severe than that seen in bacterial or viral CNS infections [124]. Other studies have shown that levels of several proinflammatory cytokines and chemokines, including TNF, are elevated in the CSF, though CSF levels do not correlate with serum levels [6]. These studies demonstrate that there remains some compartmentalization of cytokine expression between the blood and brain in CM, suggesting that cytokine levels in the brain may be due to a combination of CNS production and systemic production that crosses the BBB.

The mechanisms underlying a breakdown of the BBB may include a physical process in which occluded microvessels create a local hypertension that leads to a break in the tight junctions of the ECs and subsequent hemorrhage. Another potential mechanism is cell signaling that disrupts EC homeostasis or leads directly to EC death. A number of factors in severe malaria can be detrimental to ECs or to maintaining BBB function or tone, such as cytokines, parasite products, red blood cell components, and reactive oxygen species. TNF and IFN-γ are elevated in human CM [94, 95, 125] and can activate ECs and increase the permeability of the BBB [12, 126–128]. Decreased levels of TGF-β, an anti-inflammatory Th2 cytokine that has neuroprotective effects, have been associated with human CM [129]. However, TGF-β1 also can be released from platelets and induce apoptosis in ECs [130]. Another mechanism that may contribute to the breakdown of the BBB is binding of pRBCs to ECs. In vitro models show a transfer of parasite membrane and antigens to ECs that results in EC activation and in the opening of intercellular junctions [131], and other in vitro studies have shown that pRBC binding can induce apoptosis [32, 132, 133]. Finally, immunohistochemistry studies of human CM autopsy tissues show a decrease in the tight junction proteins ZO-1 and occludin and the focal adhesion protein vinculin in areas of pRBC sequestration [122].

ECs are linked together by tight junctions and anchored to the extracellular matrix and, along with pericytes, astrocytes, and neurons, make up the BBB. An interesting component to the BBB that is often overlooked is the role of the pericyte in human CM. Recent publications have implicated pericytes as the regulator of BBB through regulated gene expression of ECs and through interactions with astrocyte end-feet [134]. Pericytes are distributed along the wall of the precapillary arterioles, capillaries, and postcapillary venules at higher ratios to endothelial cells in the brain than in other organs and at higher ratios in the retina than the brain [135]. The functional role of pericytes also extends to phagocytosis, immunomodulation, regulation of the microcirculatory blood flow, and blood coagulation [135, 136]. Pericytes express ICAM-1 and VCAM-1, cytokines and chemokines, and NO, and they can process and present antigens to T cells [135, 137]. The functional overlap between pericytes and the mechanisms important in human CM warrant further investigation into their role.

In children with CM, a compromised BBB is associated with ring hemorrhages, and ring hemorrhages are associated with axonal and myelin damage [123].

Autopsy studies confirmed the results of previous studies investigating CSF levels of tau and S100B, which had suggested that axonal injury was contributing to the neuropathogenesis of CM [138]. Adults with CM also show ring hemorrhages with axonal injury and myelin damage by immunohistochemistry. Further, the severity of axonal injury distinguished between adult CM patients and those with severe malaria without CNS manifestations, but other factors, such as demyelination, microglial cell or astrocyte responses, and the presence of leukocytes, did not distinguish between individuals who died of severe malaria with vs. without CNS clinical manifestations [17]. Two other features often observed in children and adults with CM are severe metabolic acidosis and hypoglycemia. Metabolic acidosis and hypoglycemia are also associated with neurological symptoms in non-CM severe malaria and are contributors to axonal degeneration [139]. In addition, hypoglycemia and hypoxia have the potential to contribute to axonal injury to CM [140, 141], and autopsy studies suggest a correlation between one or both factors and brain injury in CM [138, 142].

Cytokine responses are thought to be responsible for some of the observed cerebral pathology and neurological sequelae seen in CM [6]. TNF from glial cells can maintain synaptic strength at excitatory synapses [143], but it can also inhibit glutamate transporters and lead to microglial cell death [144]. Higher levels of CSF TNF were associated with neurological and neurocognitive sequelae 6 months after an episode of CM in Ugandan children [6], suggesting that TNF plays primarily a neurotoxic role in CM. CNS NO production may also be important in CM. In the CNS, NO can be neuroprotective, but overexpressed NO can be neurotoxic and may contribute to a breakdown of the BBB [145]. Data from human CM studies on CNS NO is limited and contradictory. A Zambian study of children with CM showed higher CSF NO levels in children who died vs. survivors [146], while a study of Ghanaian children showed no difference in CSF NO levels in children who died vs. survivors [147]. In addition, an autopsy study showed elevated inducible nitric oxide synthase (iNOS) in the brain blood vessels of children who died of CM. The authors speculated that NO might contribute to mortality or severe disease via increased vascular dilatation leading to cerebral pressure and eventual hemorrhage in the brain microvasculature [148]. However, NO can diffuse through cell membranes, and it is a known vasodilator and can also inhibit platelet and leukocyte adhesion to ECs [145, 149, 150]. NO also assists in the regulation of neuronal cell proliferation and differentiation and can affect neurotransmission [151, 152]. Additionally, NO production may have a role in learning and memory [153]. These findings suggest that NO production could also be neuroprotective, in amounts below a threshold level of toxicity. A recent study of Ugandan children documented elevated CSF NO levels in children with CM as compared to control children, and there was a trend toward long-term neuroprotection in children with CM with elevated levels of CSF NO (C. John, unpublished data). At present, it is unclear whether NO treatment will be helpful or harmful to the CNS in severe malaria, though there is ample evidence that it could be helpful systemically in severe malaria [154–156]. A current trial of inhaled NO for severe malaria, including CM, may help to answer this question [157].

5 Potential Treatments and Interventions

Treatments given prior to development of neurological signs prevent murine ECM, but treatment prior to development of neurological signs is not possible in human CM, in which presence of coma is part of the disease definition. Thus, treatments must be able to reverse CM to be useful in humans. To date, no therapies based on murine ECM or in vitro cell culture studies have translated to successful adjunctive therapies for treating human CM [158]. A number of possibilities are being considered, and NO and erythropoietin (EPO) have moved into early clinical trials.

Murine ECM and human CM studies suggest that low bioavailability of NO contributes to disease, and the administration of exogenous NO (via L-arginine) is protective in mice and improves endothelial dysfunction in humans [154, 155, 159]. As noted earlier, NO can be neuroprotective, and NO administration could theoretically mitigate the effects of many mechanisms implicated in human CM. NO could reduce sequestration by several mechanisms: (1) by vasodilation, which could reduce mechanical obstruction due to reduction in deformability or morphologic changes in pRBC; (2) through inhibition of platelet aggregation and adhesion [160] and inhibition of factor XIII [161], all of which would reduce the procoagulant state; (3) by providing more substrate to be scavenged by free hemoglobin [109] and reducing hemoglobin inhibition of ADAMTS13 activity [162]; and (4) by reduction of the expression of VCAM-1, ICAM-1, and endothelial leukocyte adhesion molecule 1 [108]. Reduction of free hemoglobin by NO could also decrease proinflammatory cytokine responses. Given the potential danger of excessive NO, clinical trials with varying amounts and methods of NO administration are required to determine if and how NO can be used as adjunctive therapy in CM. Trials of L-arginine and inhaled NO are currently ongoing.

EPO is a cytoprotective agent in the vascular system and neuroprotective agent in the brain, showing protection in experimental models for a wide range of pathological conditions. However, EPO appears to have a threshold effect from which it moves from protecting to damaging via apoptosis. EPO is also a mediator of ischemic preconditioning, and EPO receptor is upregulated during an ischemic event. In addition, EPO receptor is further upregulated by the presence of EPO during hypoxia. Thus, the threshold at which EPO becomes damaging shifts lower during a hypoxic insult because an increase in EPO receptor delivers more EPO to the brain [163]. Due to the combination of anemia, sequestration, and activation of the endothelium anemia typically seen in human CM, it is possible that a cytoprotective treatment that induces NO production and is traditionally used for anemia may assist in BBB stability and EC protection. In murine ECM, mice were protected from *P. berghei* ANKA infection with EPO [164, 165], and higher levels of EPO have been associated with protection from death and neurological sequelae in children with CM [166]. However, some human CM studies have not shown associations of EPO levels with clinical manifestations [167], and there have been a number of studies in other disease processes showing increases in mortality in a dose-dependent manner with EPO administration [163]. Thus, studies have provided

evidence that EPO could be beneficial in CM, but there is also considerable potential for neurotoxicity and systemic toxicity in the proinflammatory and procoagulant milieu of CM. For example, a large study of individuals with stroke treated with EPO documented increased mortality with EPO treatment [168]. An early clinical trial of EPO treatment in children with CM showed no increase in mortality or short-term adverse events with EPO treatment [169], but the sample size was small, follow-up was short, and the ability to detect adverse events limited. In light of the harm seen with EPO treatment in stroke and cardiac trials also showing no benefit and potential harm [170], EPO therapy for CM should be approached with caution. Newer EPO analogs which target neuroprotection but do not stimulate erythropoiesis may have more promise in CM, and other neurological diseases are in the early stages of phase I trials at present [171].

6 Future Directions

The mechanisms behind the neuropathophysiology of CM are likely multifactorial. Sequestration in the cerebral microvasculature appears to be important in the pathophysiology of human CM, whether as a consequence of or a co-initiator of inflammation. Additionally, brain endothelial cell activation is likely a critical component to the neuropathology of human CM. It seems clear now that there are focal breaks in the BBB in human CM and that these play a role in the pathogenesis of CM, but the extent of that role is not yet defined.

Additional studies on the roles of endothelial activation, NO, proinflammatory cytokines, cerebral edema, and other factors noted previously are needed in human CM because the paucity of clear data in this area has left a gap in the ability to rationally develop interventions to prevent or ameliorate the effects of human CM. Animal studies have provided insight into some areas of CM pathogenesis, and improvement in animal models could eventually provide a better way to do early testing of adjunctive therapies, but the translation of interventions that were successful in ECM to human CM has been disappointing. In vitro models using human cell lines may provide an additional way to assess the pathophysiology of human CM. Studies of the long-term neurological and cognitive consequences of CM are critical, because it is important to consider how these could be prevented in survivors of CM, and no interventions can be considered without knowledge of the pathways that lead to neurocognitive sequelae. It is hoped that with advances in all of these areas, interventions to decrease short- and long-term complications of CM will be developed. Finally, the ultimate prevention of CM lies in reduction and eventual elimination of malaria from an area, a goal that is a steppingstone toward the larger goal of malaria eradication. It is imperative that we continue to work on ways to reduce morbidity and mortality from CM and other forms of severe malaria, as they continue to affect millions of children every year, but success in the greater goal of malaria control and elimination will lead to fewer cases of complicated malaria and therefore less need for adjunctive therapy. Both goals, better therapy for

severe malaria and better prevention of malaria, must be addressed with continued strong support from funding agencies and ministries of health if we are to decrease the burden of severe malaria and CM morbidity.

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Part IV
Drugs of Abuse and NeuroAIDS

HIV and Cocaine Interplay in HIV-Associated Neurocognitive Disorders

Honghong Yao, Crystal Bethel-Brown, and Shilpa J. Buch

Abstract While antiretrovirals are becoming the gold standard in HIV care and are effective in suppressing viremia, the relative inability of these drugs to penetrate the blood-brain barrier, the latency of HIV in the tissues, and the increased life span of individuals on therapy often lead to complications of HIV in the central nervous system (CNS) termed as HIV-associated neurocognitive disorders (HAND). Among the individuals inflicted with HAND, almost 30 % have a history of substance abuse. Among the commonly abused drugs, cocaine is the most widely used and has emerged as a key contributor to the seroprevalence and progression of HIV infection. Both epidemiological and laboratory-based studies demonstrate that cocaine promotes HIV replication and has multifaceted deleterious effects on the various cells of the CNS resulting in a disrupted blood-brain barrier, enhanced glial activation, and neurotoxicity. Effects of cocaine alone or in combination with HIV proteins lead to augmented neuropathogenesis. This review summarizes current understanding of the diverse effects of cocaine on the various cells of the CNS and how the drug synergizes with HIV and HIV proteins to exacerbate neurotoxicity.

Keywords HIV • AIDS • Cocaine • Glial cell • HIV-1-associated neurocognitive disorders • CNS • Non-opioid receptor

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1 Introduction

HIV infection remains a critical health and socioeconomic problem worldwide. Currently there are over 34 million individuals worldwide living with HIV with almost 1.4 million that have died due to HIV-related complications as reported by WHO in 2011. While the majority of infections and related mortality are found in sub-Saharan Africa, HIV infection continues to be a burgeoning problem in other parts of the world as well. It is estimated that in the United States, there are over one million persons living with HIV with approximately 50,000 new infections each year. The advent of effective antiretroviral therapy (ART) in 1996 has considerably improved the lives of those infected with HIV, with the control of systemic virus replication leading to increased longevity. Paradoxically, however, increased longevity coupled with ART toxicity, low-level chronic activation, and limited penetration of the drugs into the CNS results in premature aging of the infected individuals with the brain as a sanctuary for persistent latent virus. Often the undesired effect of long-term usage of ART or ART interruption is associated with increased prevalence of HIV-associated cognitive disorders (HAND) affecting almost 30 % of infected individuals. The spectrum of HAND can range from asymptomatic neurocognitive impairment to minor cognitive motor disorders and in extreme cases HIV-associated dementia (less than 7 % of patients).

HIV infection is associated with various comorbidities including but not limited to substance abuse. In fact, intravenous drug using (IVDU) individuals form a significant proportion of the at risk of HIV population primarily due to needle sharing. Use of illicit drugs and HIV-1 infection are two intertwined global health epidemics. It has been estimated that in the United States HIV-1 infection is one of the leading causes of death among 25–44-year-olds, with IVDU accounting for almost one-third of all newly diagnosed cases. According to the National Youth Risk Behavior Survey in 2007, at least 3.3 % American teenagers (aged 14–17) had some form of cocaine abuse history during their lifetime. Previous reports have identified that use of crack cocaine is a risk factor for acquisition of HIV infection and is also associated independently with exacerbated progression to AIDS [1–3]. While it is well recognized that cocaine and other recreational drugs affect the addiction pathways in the brain leading to dependence, it is becoming increasingly clear that these drugs alone or in conjunction with HIV and HIV proteins can also exert physiological and molecular effects that are distinct from the addiction pathways. Cocaine abuse can thus be envisioned to contribute to progression of clinical AIDS via its interplay with HIV and HIV proteins.

Both HIV and cocaine target the brain while also impairing the functions of macrophages and $CD4^+$ lymphocytes [4–8]. Cocaine is also known to enhance HIV-1 expression in these cells [9–13] and is known to cause impairment of immune functions [9, 14–16]. It has been postulated that cocaine could aid as a cofactor in the pathogenesis of HIV infection and in the susceptibility and progression of HAND [1–3]. Of the commonly abused drugs, cocaine abuse (by multiple routes) has been linked more commonly to increased incidence of HIV seroprevalence and progression

to AIDS [17–21]. Both epidemiological and laboratory-based studies demonstrate that cocaine promotes HIV replication and has multiple deleterious effects on various cells of the CNS resulting in a disrupted blood-brain barrier, enhanced glial activation, and neurotoxicity. This chapter summarizes current understanding of the interplay of HIV infection and cocaine in modulating enhanced pathogenesis of HAND.

2 Cocaine Potentiates HIV-1 Replication

Since cocaine abuse exacerbates HIV infection, it was hypothesized that cocaine could play a direct role in HIV replication. Elegant studies by Peterson et al. demonstrated that in cocultures of peripheral blood mononuclear cells (PBMC) from healthy donors infected with a clinical isolate of HIV-1, cocaine stimulated replication of the virus [22, 23]. These authors demonstrated that HIV replication, measured by the release of HIV p24 antigen in cell culture fluids, was significantly upregulated in activated cells compared to cells that were not activated but exposed to cocaine [22]. In this study a critical role of the pleiotropic cytokine transforming growth factor (TGF)-β was implicated in cocaine-mediated induction of virus replication. Based on the role of immune activation in pathogenesis of HIV infection [24–26], these cell-based studies suggested clinical relevance of cocaine in augmenting disease pathogenesis.

Since cellular activation rather than virus replication is critical for HIV-associated pathogenesis, the authors also sought to understand the role of cell activation in cocaine-mediated upregulation of virus replication. In this study PBMCs stimulated with or without cytomegalovirus (CMV) were pretreated with cocaine followed by coculturing in the presence of HIV-infected PBMCs, and supernatants were monitored for HIV p24 antigen as an index of virus replication [23]. These findings demonstrated that although cocaine by itself failed to trigger HIV-1 replication, it was able to enhance virus replication in the presence of other activation signals such as CMV [27–30].

Since macrophages promote HIV replication, effect of cocaine on potentiation of virus replication in these cells was also investigated. Cocaine markedly enhanced virus production in simian human immunodeficiency virus (SHIV)-infected monocyte-derived macrophages (MDMs). Interestingly, even in U1 cells, a chronically infected promonocytic cell line harboring a latent virus genome, cocaine was able to enhance virus replication in these cells, thereby underscoring the role of cocaine in pushing the virus out of latency. This property of cocaine could have serious ramifications in HIV-infected individuals on ART as they harbor reservoirs of latent virus in various tissues [22, 23, 31]. In addition to enhancing virus replication, cocaine was also able to induce upregulation of the macrophage activation marker, human leukocyte antigen (HLA)-DR, in MDMs. Taken together, cocaine usage in an HIV-infected individual could lead to increased virus replication in both the productive as well as latently infected cells while also upregulating cellular activation culminating ultimately into perpetuation of an inflammatory cascade within the CNS [31].

The interaction of cocaine with HIV observed in cell culture studies has also been validated in the rodent model system using a hybrid mouse model (huPBL-SCID mouse) infected with HIV-1 in the presence or absence of cocaine. Systemic cocaine administration in this model correlated with upregulation of HIV-1 replication in human peripheral blood leukocytes (PBL), downregulation of $CD4^+$ cells, and increased circulating virus load [12]. The mechanism of cocaine-mediated upregulation of virus involved upregulation of CCR5 expression in the peritoneal cells of HIV-infected, cocaine-treated huPBL-SCID mice with a subsequent increase in the numbers of virally infected cells.

Cocaine is known to interact with σ-1R [32, 33], a class of non-opioid receptors that are localized specifically at the endoplasmic reticulum (ER)/mitochondrial interface commonly termed as the mitochondrion-associated ER membrane or the MAM. σ-1R are known to function as molecular chaperones that are critical for maintaining the activity of the IP3 receptor [32]. It has been shown that following stimulation with stimuli such as cocaine, σ-1R can translocate to the plasma membrane [34, 35]. Owing to this translocation, σ-1R can in turn regulate the expression and functions of other diverse receptors or ion channels present on the cell membrane [35–37]. In the huPBL-SCID model using the pharmacological blocker of σ-1R, Roth et al. [38] demonstrated that cocaine mediated its effects on virus replication via the σ-1R.

2.1 HIV Replication in Microglia: Role of σ-1R

Cocaine-mediated potentiation on HIV replication has also been investigated in another myeloid cell type in the CNS, namely, microglia which are the resident macrophages of the brain and the target cells for virus replication in the brain [39]. Microglia play a pivotal role in defense against toxic stimuli as well as in various pathogenic states including HIV-1 infection of the CNS. Consistent with the role of cocaine in promoting systemic virus replication in the PBMCs and macrophages, cocaine also augmented virus replication in microglial cells infected with HIV [9, 11, 12, 23]. Furthermore, it was also demonstrated that κ-opioid receptor ligands, that are known to suppress neurochemical and neurobehavioral responses to cocaine, inhibited cocaine-induced potentiation of HIV-1 replication in microglial cells. This effect was mediated by down-modulation of CCR5, a coreceptor of HIV-1, involving the extracellular signal-regulated kinase 1/2 [40]. Intriguingly, similar to findings reported in PHA-activated PBMCs, cocaine-mediated upregulation of HIV-1 replication in microglia also involved the binding of cocaine to its cognate σ-1R and the cytokine TGF-β [41].

2.2 HIV Replication in Astrocytes

It has become evident in recent years that astrocytes previously thought of as refractory to HIV infection are also susceptible to HIV-1 infection in the presence of relevant cytokines [42–47]. Astrocytes are integral components of the CNS since they

maintain a homeostatic environment and actively participate in bidirectional communication with neurons and microglia [42, 48, 49]. Following initial infection with HIV-1, astrocytes exhibit a transient surge of viral replication that subsequently diminishes to low levels and often persists [42–44]. It has been estimated that up to 20 % of astrocytes can be infected with the virus in HIV-infected patients making these cells a potential reservoir for the latent virus [43]. The effect of cocaine on astrocytes in the context of HIV-1 infection has been reported by Nair et al. [39]. Previous studies have demonstrated that in normal human astrocytes pretreatment with cocaine prior to HIV-1 infection significantly upregulated viral replication as assessed by a significant increase in LTR-R/U5 gene expression [39], representing early stages of reverse transcription of HIV-1. Furthermore, exposure of astrocytes to cocaine exhibited increased virus replication at day 15 postinfection with a concomitant upregulation of viral proteins. Specifically, induction of these proteins, that are critical in the neuropathogenesis of HIV-1 infection, comprised the intracellular signaling molecules, translation elongation factor, and molecular chaperones [39]. These findings suggested that the response of astrocytes to cocaine and/or HIV-1 can lead to increased viral load and subsequent toxicity in the CNS.

3 Effect of Cocaine on Glial Functions in the CNS

3.1 Microglia

Microglia play a critical role in host defense. However, if the response of these cells is unabated, it can lead to the deleterious effects as had been observed in HIV-1-associated neuroinflammation and neuropathogenesis. Cocaine-mediated induction of the chemokine MCP-1/CCL2 has been demonstrated in rodent microglia. This effect of cocaine is transduced via the translocation of the σ-1R to the lipid raft microdomains of the plasma membrane, with subsequent sequential activation of Src, mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3′ kinase (PI3K)/Akt, and nuclear translocation of the nuclear factor kappa B (NF-κB). This ultimately culminates into binding of NF-κB to the promoter of MCP-1 leading to transcription of the chemokine [50]. Functional implication of cocaine-induced MCP-1 expression was validated using in vitro assays of monocyte transmigration across the human brain microvascular endothelial cells (HBMECs). In these assays conditioned media from cocaine-exposed microglia increased monocyte transmigration, an effect that was blocked by antagonists to CCR2 or σ-1R. These findings were also corroborated in vivo wherein cocaine injections in mice resulted in increased monocyte transmigration in the CNS, and this effect was attenuated in mice pretreated with the σ-1R antagonist. Interestingly, cocaine-mediated transmigratory effects were not observed in CCR2 knockout mice as expected. These findings led to the conclusion that cocaine-mediated induction of MCP-1 accelerates monocyte extravasation across the endothelium which could have a possible role in increased neuroinflammation associated with cocaine abuse in HIV-infected individuals [50].

In addition to upregulating inflammatory chemokines, exposure of microglial cells (BV2 or rat primary microglia) to a higher dose of exogenous cocaine has been shown to result in apoptosis with a concomitant generation of intracellular reactive oxygen species. Intriguingly, endoplasmic reticulum (ER) signaling mediators such as PERK, Elf2α, and CHOP were involved in decreased cell viability induced by cocaine as evidence by the fact that blockage of CHOP expression significantly ameliorated cocaine-mediated cell death. These findings thus underscore the importance of ER stress in modulating cocaine-induced microglial cell toxicity [51]. Understanding the link between ER stress, oxidative stress, and apoptosis could lead to the development of therapeutic strategies targeting cocaine-mediated microglial death/dysfunction.

3.2 Astroglia

In addition to enhancing virus replication in astroglial cells in vitro, cocaine administration in mice also resulted in increased proliferation and expression of glial fibrillar acidic protein (GFAP) in the dentate gyrus [52], suggesting thereby that cocaine abuse correlated with activation of astrocytes. Since cellular activation is a hallmark of HAND, this could have implications for development of disease progression in cocaine abusers that are HIV infected. In addition to inducing cell activation, cocaine has also been shown to potentiate toxicity of the viral envelope gp120 in rat primary astrocytes via upregulation of oxidative stress [53]. Molecular mechanisms involved in this process included activation of the JNK, p38, ERK/ MAPK, and NF-kB pathways [53].

4 Effect of Cocaine on Neurotoxicity

It is well recognized that although neurons are susceptible to the affects of HIV, the virus does not infect these cells per se. Various elegant studies have demonstrated that viral protein products such as the transactivator protein (Tat) and the envelope (gp120), which are released from the infected cells, can exert neurotoxicity in both in vitro and in vivo model systems [54–60]. Interestingly, ample evidence now suggests that cocaine can amplify the neurotoxic responses of viral proteins leading to enhancement of neurotoxicity in the CNS [56, 61–63]. The mechanism by which cocaine potentiates HIV protein toxicity involves augmentation of oxidative stress [64, 65] and activation of caspase-3 pathways. The downstream cell signaling pathways involved in this process included activation of JNK, p38, ERK/ MAPK, and NF-kB pathways [66]. In vivo evidence for cocaine-mediated potentiation of gp120 neurotoxicity was validated by Bagetta et al. [67]. These authors demonstrated that injection of rats with cocaine in combination with intracerebral ventricle injection

of recombinant HIV gp120 resulted in induction of expression of inducible nitric oxide synthase (iNOS) expression with concomitant increase in neuronal apoptosis in the neocortex. Furthermore, this effect was ameliorated in animals pretreated with an iNOS inhibitor, thereby underscoring the role of iNOS in gp120 and cocaine-mediated apoptosis of neurons. Consistent with the findings on gp120, cocaine also potentiated Tat-mediated neurotoxicity in hippocampal neurons, an effect that was ameliorated by a D1 dopamine receptor antagonist [68]. These studies thus imply that cocaine exerts its neurotoxicity in the setting of more than one HIV protein, thus manifesting an increased ability to exert neurotoxicity within the CNS.

5 Effect of Cocaine on the Blood-Brain Barrier (BBB)

The BBB plays an essential role in the development of HAND since it serves as the conduit by which free virus and/or infected immune cells enter the brain from the circulatory system [69–71]. It has been shown that cocaine can enhance HIV-1 neuroinvasion in HAND via either its direct effect on human brain microvascular endothelial cells (HBMECs) or through its paracrine effects on the BBB via release of the pro-inflammatory cytokines [1, 72–74]. One mechanism of cocaine-mediated increase in leukocyte migration across the endothelium is via upregulation of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin [72, 75]. Upregulation of yet another adhesion molecule—activated leukocyte adhesion molecule (ALCAM)—has also been demonstrated in HBMECs exposed to cocaine. In these cells following cocaine treatment, σ-1R translocated to the plasma membrane with subsequent phosphorylation of platelet-derived growth factor (PDGF)-β receptor. Involvement of mitogen-activated protein kinases, Akt, and NF-κB pathways subsequently resulted in induction of ALCAM expression. Expressed ALCAM, in turn, was essential for monocyte adhesion and migration, and these effects were ameliorated in cells or animals pretreated with an ALCAM neutralizing antibody.

In addition to upregulation of adhesion molecules, cocaine also increased the permeability of brain endothelial cells by regulating the expression of the vascular permeant PDGF-B [76]. Recent findings have also identified PDGF-B chain as a new member of the Notch target gene in HBMECs, thereby lending importance to the role of Notch signaling as one of the key players in the maintenance of BBB integrity. It has been shown that cocaine-mediated activation of Notch1 signaling leading to targeted upregulated expression of PDGF-B involved activation of the downstream effector CSL, providing the first evidence of involvement of Notch1 activation in cocaine-mediated regulation of PDGF-B expression [77]. Deeper understanding of the regulation by cocaine of endothelial barrier permeability could provide insights into the development of potential therapeutic targets for neuroinflammation associated with HIV infection and/or cocaine abuse.

6 Concluding Remarks

In summary, cocaine can be classified as a multifactorial agent that mediates its effects on several pathways in HIV-1 infected cells. Not only does the drug promote virus replication in PBMCs, macrophages, microglia, and astrocytes, but it can also modulate glial function and activation. Cocaine also causes interactive neurotoxicity with viral proteins, Tat and gp120, thereby exacerbating neuronal apoptosis. Additionally, cocaine exerts potent effects on microvascular permeability leading to increased influx of virus-infected inflammatory cells in the brain parenchyma. In summary, cocaine abuse in HIV-1-infected individuals exerts deleterious effects on the CNS resulting in exacerbated disease pathogenesis via multiple pathways.

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Methamphetamine Neurotoxicity and Neuroinflammatory Processes

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Abstract Methamphetamine (Meth) is a widely abused psychostimulant known to cause neurotoxicity. Traditionally, the toxic effects of Meth were thought to be restricted to dopaminergic and serotonergic axon terminals, but more recently the targets of Meth have been found to include dopaminergic and GABAergic neurons, brain endothelial cells, and the liver. In addition to the neuronal and nonneuronal targets of Meth, mechanisms responsible for damage including oxidative stress, excitotoxicity, and mitochondrial dysfunction will be discussed. The focus of this chapter will be to integrate the known targets of Meth and mechanisms of Meth-induced damage with more recently identified neuroinflammatory markers found after exposure to Meth. Various inflammatory mediators and their temporal expression after Meth exposure will be reviewed as well as supporting evidence for the role of neuroinflammation in Meth-induced damage.

Keywords Methamphetamine • Neuroinflammation • Neurotoxicity • Cyclooxygenase (COX) • Cytokines • Microglia • Striatum • Motor proteins

1 Methamphetamine Neurotoxicity and Neuroinflammatory Processes

Methamphetamine (Meth) is a widely abused psychostimulant traditionally thought to selectively damage dopamine (DA) and serotonin (5-HT) neuron terminals. The nature of the damage to the DA and 5-HT system, the established mechanisms associated with this damage, and the more recently identified targets of Meth will be discussed in

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this chapter. These observations will be evaluated and integrated within the context of a role for neuroinflammation and how it underlies the neurotoxic profile of Meth.

2 Meth-Induced Toxicity

It is well accepted that Meth use causes damage to terminals of DA and 5-HT neurons in rodents, nonhuman primates, and humans. Positron emission tomography (PET) and proton magnetic resonance spectroscopy (MRS) studies of abstinent Meth users illustrate that Meth produces long-term neuronal damage, marked by decreases in the DA transporter (DAT) [1–3] and the 5-HT transporter (SERT) [4] that persist for up to 3 years. PET and MRS observations in abstinent Meth users are substantiated by studies of human postmortem brain tissue and illustrate decreases in DA, tyrosine hydroxylase (TH), and DAT in the caudate-putamen and nucleus accumbens [5–7] and SERT in the frontal cortex [8].

The long-term neuronal damage caused by Meth in humans is supported by rodent models. Meth-induced long-term neurotoxicity in mice and rats is also reflected by decreases in DAT, SERT, the vesicular monoamine transporter 2 (VMAT2), TH and tryptophan hydroxylase (TPH) immunoreactivity, and DA and 5-HT tissue content in brain areas densely innervated by DA and 5-HT terminals, such as the striatum, hippocampus, and prefrontal cortex [9–12]. Furthermore, complete recovery of all presynaptic dopaminergic parameters, including DA tissue content, DA uptake, and evoked DA release from the striatum, does not occur until 12 months after the neurotoxic regimen of Meth [13]. Collectively, both rodent and human studies indicate long-lasting monoaminergic damage caused by Meth.

More recent studies have suggested Meth-induced damage extends beyond the damage to DA and 5-HT terminals. A broader scope of injury is indicated by decreases in the neuronal marker *N*-acetylaspartate [14, 15], a loss of gray matter, decreased volume of the hippocampus, and hypertrophy of white matter in the human brain [16]. In addition, rodent studies have shown that Meth produces damage to cell bodies and does not specifically target dopaminergic or serotonergic terminals [17–23]. Although decreased TH staining has been observed in the substantia nigra after Meth administration [24–27], it remains unclear whether Meth causes death of DA neurons or simply downregulates protein expression of TH in the substantia nigra [24, 26]. In addition, Meth increases caspase-mediated proteolysis and TUNEL staining in areas of the striatum and hippocampus [17–19]. A study by Zhu and Angulo [20] co-localized TUNEL staining with dopamine- and cAMP-regulated phosphoprotein (DARPP-32), parvalbumin, and choline acetyltransferase in the striatum after Meth administration. These data indicated that 21 % of DARPP-32-positive projection neurons, 45 % of parvalbumin-positive γ-aminobutyric acid (GABA) interneurons, and 29 % of cholinergic interneurons undergo apoptosis after a single administration of 30 mg/kg Meth to mice [20]. In addition, glutamatergic neurons in the somatosensory cortex are also decreased after treatment of rats with a binge regimen of Meth (10 mg/kg, ip, q 2 h 4×) [21].

Therefore, the toxic effects of Meth are not specific to DA and 5-HT neurons but include damage to GABAergic, cholinergic, and glutamatergic neurons.

Other recent studies have illustrated that the toxic effects of Meth are not limited to neurons. Meth produces damage to brain microvascular endothelial cells in culture by decreasing expression of the tight junction proteins occludin, claudin-5, and zonula occludens (ZO) [28, 29]. Moreover, the in vivo exposure of Meth to mice and rats also produces a decrease in expression of tight junction proteins including occludin, claudin-5, and ZO [30, 31]. These observations are paralleled by the extravasation of Evan's blue-bound albumin, endogenous IgG, or 10,000 kDa FITC-dextran from the cerebral vasculature into the brain parenchyma [30–35] and illustrate that Meth causes disruption of the blood–brain barrier (BBB).

The liver is a more recently identified target of Meth that can have an impact on the brain. Administration of a neurotoxic dose of Meth produces increases in the liver enzymes, aspartate aminotransferase (AST), and alanine aminotransferase (ALT), which are released into the circulation upon damage to the liver [36]. The consequence of such damage decreases the metabolism of ammonia with a subsequent elevation of plasma and brain ammonia concentrations that promotes glutamate-mediated excitotoxicity [36, 37]. Overall, the above studies illustrate that the toxic effects of Meth extend well beyond neurons to include other cells such as endothelial cells of brain capillaries and hepatocytes.

3 Established Mechanisms of Meth Neurotoxicity

Many of the long-term neurotoxic effects of Meth are the consequences of its acute actions. These include neurotransmitter release, hyperthermia, oxidative stress, and mitochondrial dysfunction, all of which have been demonstrated to contribute independently or convergently to monoaminergic terminal damage.

Hyperthermia plays a prominent role in the toxic effects of Meth [38–40]. This is evidenced by the fact that Meth-induced monoaminergic terminal damage is not observed in the absence of Meth-induced hyperthermia [39, 41]. Furthermore, pharmacological interventions that attenuate hyperthermia also attenuate Meth-induced monoaminergic damage. Antagonism of NMDA receptors using MK-801 or D1 or D2 dopaminergic receptors using SCH23390 and haloperidol, respectively, prevents Meth-induced hyperthermia and toxicity; however, when hyperthermia is maintained in the presence of these pharmacological agents, toxicity remains [39, 42]. Hyperthermia is also highly correlated with the extent of Meth-induced BBB disruption [33, 34]. In contrast, several studies suggest that hyperthermia is not the sole mechanism, since pharmacological interventions that do not alter hyperthermia are capable of attenuating Meth toxicity [31, 43–46].

The role of oxidative stress in Meth-induced monoaminergic terminal damage and BBB disruption is also well established. Meth increases ROS [47, 48], whereas the attenuation of oxidative stress prevents Meth toxicity. Meth also produces an increase in oxidized glutathione that is indicative of oxidative stress resulting from

the interaction of hydroxyl radicals with the endogenous antioxidant glutathione [49]. Conversely, antioxidants such as *N*-acetyl-L-cysteine, ascorbic acid, and vitamin E attenuate Meth-induced striatal DA and 5-HT depletions [50–52]. In addition, mice that overexpress Cu/ZnSOD or MnSOD are refractory to the striatal damage produced by Meth [53–56], whereas inhibition of SOD enhances Meth-induced DA and 5-HT depletions in the rat striatum [51]. The effects of oxidative stress can also impact cells other than neurons. The antioxidant Trolox attenuates the decreases in occludin and transendothelial electrical resistance (TEER) of primary human brain microvascular cells as well as Meth-induced increases in extravasation of fluorescein into mouse brain tissue that are suggestive of a compromise in the BBB [28]. Collectively, oxidative stress has been convincingly shown to mediate damage to monoamine terminals and the BBB.

Some of the oxidative damage to cells produced by Meth can be attributed to neurotransmitter activity such as the direct actions of oxidative products of DA metabolism and the activation of glutamate receptors. Inhibition of these processes can attenuate Meth-induced oxidative stress and toxicity. For example, inhibition of DA synthesis with α-methyl-ρ-tyrosine treatment attenuates Meth-induced oxidation of DA and DA depletions [57–59]. Dopamine-induced oxidative stress to DA terminals by Meth can result from a dysfunction in VMAT2 and the inability to sequester DA into vesicles, with a resultant increase in cytosolic DA and DA-derived free radicals [60, 61]. In addition, the activation of glutamate receptors, the subsequent increase in reactive nitrogen species (RNS), and the ensuing toxicity to monoamine terminals can be prevented by nitric oxide synthase (NOS) inhibitors [61–63] or by nNOS knockout [64, 65]. Furthermore, inhibition of glutamate release from the corticostriatal pathway or blockade of glutamate receptors prevents Meth-induced damage to striatal DA terminals [66–70]. Thus, there is substantial evidence for the role of DA and glutamate in Meth-induced monoaminergic terminal damage.

Altered mitochondrial function and bioenergetics also contribute to Meth neurotoxicity. Decreases in levels of adenosine triphosphate (ATP) are observed acutely in the brain after high-dose Meth administration [71]. The decreases in ATP are potentiated by inhibition of glucose uptake and utilization, both of which are associated with an enhancement of long-term decreases in tissue content of DA in the striatum [71]. A role for energy impairment is further substantiated by findings showing that Meth toxicity is potentiated by metabolic inhibitors. For example, central administration of Meth, which alone does not produce toxicity, synergizes with the mitochondrial complex II inhibitor, malonate, to deplete striatal DA [72]. Conversely, Meth toxicity is attenuated by supplementation with the energy substrates, ubiquinone and nicotinamide, when administered after exposure to Meth suggesting an ongoing and protracted but reversible mitochondrial dysfunction that contributes to the long-term depletions in striatal DA content [73]. Subsequent studies have provided a more direct association between mitochondrial function and Meth toxicity. The first investigation of Meth-induced alterations in the function of specific ETC complexes was by Burrows et al. [74], illustrating that Meth decreases levels of cytochrome oxidase, a marker of ETC complex IV activity. In addition, studies demonstrate changes in other mitochondrial electron transport chain complexes. Specifically, a decrease in complex II–III but not I–III activity was observed

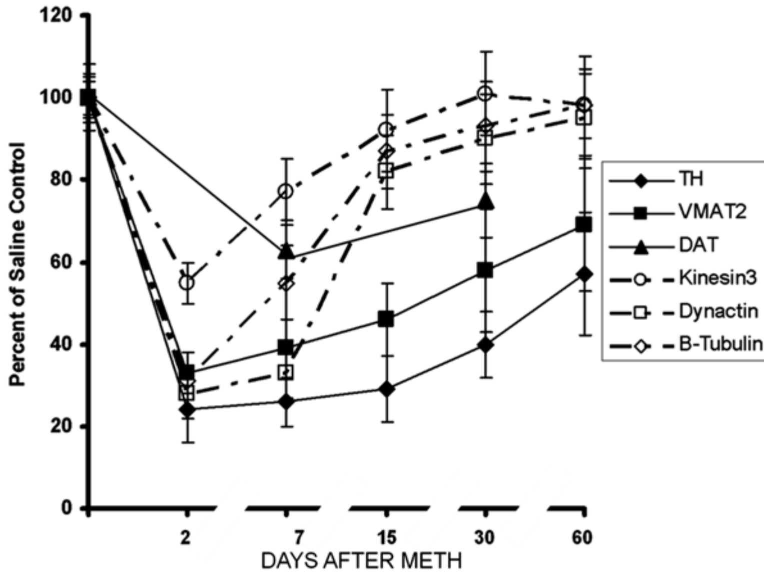

Fig. 1 Alterations in striatal proteins after Meth exposure. Rats were treated with Meth (10 mg/kg, ip, q 2 h 4×) and killed by live decapitation at the indicated time points. Motor proteins (*dashed lines*) and proteins associated with dopaminergic terminals (*solid lines*) were measured via Western blot in whole striatal homogenates. The motor proteins kinesin3, dynactin, and **β**-tubulin are significantly decreased compared to saline-treated controls at 2 and 7 days, and not significantly different from controls at 15, 30, or 60 days. The dopaminergic terminal-associated proteins, TH, VMAT2, and DAT, are significantly decreased at 2, 7, 15, 30, and 60 days after Meth treatment, compared to saline-treated controls (adapted from [77])

in the striatum after Meth [75]. This effect was attributed to glutamate since the NMDA antagonist MK-801 blocked the Meth-induced decreases in complex II–III activity. A role for RNS, specifically $ONOO^-$, was also demonstrated. In vitro application of $ONOO^-$ decreased complex II activity, and the $ONOO^-$ scavenger blocked Meth-induced decreases in complex II–III activity measured ex vivo, implicating a role for glutamate-mediated $ONOO^-$ production in Meth-induced decreases in mitochondrial activity through excitotoxic processes [75].

The above mechanisms have been focused on the degenerative changes to nerve terminals and/or axons. Indeed, Fink-Heimer silver grain staining studies [76] are suggestive of damage to axons with a resultant loss of phenotypic markers of nerve terminals such as the DAT, SERT, and VMAT2. Our recent findings indicate that there is also a loss of motor proteins that could disrupt the transport of DAT and VMAT2 as well as TH to nerve terminals resulting in a decreased dopaminergic phenotype. Figure 1 shows that there are decreases in the motor proteins kinesin and dynactin as well as the axonal protein β-tubulin that recover before the gradual return of DAT, VMAT2, and TH to control values [77]. These results suggest that mechanisms involving motor and axonal transport proteins are not only involved in the toxicity but may be important in the apparent recovery of dopaminergic terminals from the toxic effects of METH.

4 Neuroinflammation and the Neurotoxicity of Meth

The underpinnings that mediate the neurotoxicity to Meth have been evaluated and considered independently, but it is apparent that most of them share common mechanisms. For example, inhibition of NMDA receptors with MK-801 prevents Meth-induced hyperthermia, increases in protein nitrosylation, decreases in mitochondrial complex II–III activity, and damage to monoaminergic terminals in the striatum [39, 66, 75, 78]. In addition, the damage caused by local administration of ammonia and Meth is attenuated by the AMPA antagonist GYKI 52466 [36]. These data suggest an interaction and convergence of glutamate, hyperthermia, mitochondrial dysfunction, oxidative stress, and dopaminergic damage, all of which are likely consequences of neuroinflammation.

Neuroinflammation often accompanies the actions of Meth and is a consequence of the oxidative stress evidenced by increased ROS and RNS, as well as increased extracellular glutamate and/or ammonia that are observed shortly after exposure to Meth. The neuroinflammation is marked by activated microglia and astrocytes [15, 79, 80], whereas antioxidants and free radical scavengers such as edaravone and sulforaphane can prevent Meth-induced astrocytic and microglial activation, respectively [81, 82]. Meth-induced neuroinflammation may also be mediated by glutamate. In fact, exposure to glutamate, kainate, or NMDA activates microglia and produces pro-inflammatory cytokines [83–85]. Conversely, antagonism of NMDA receptors with MK-801 and dextromethorphan attenuates Meth-induced microglial activation [86]. Lastly, Meth-induced neuroinflammation may be a result of liver damage [87] and the resultant increases in brain ammonia concentrations [36]. Increases in microglial and astrocytic activation and pro-inflammatory cytokines are evident in rodent models of acute liver failure [88]. Similarly, chronic, moderate hyperammonemia created by feeding rats an ammonium-containing diet activates microglia and increases IL-1β, iNOS, and prostaglandin E2 (PGE2) [89]. Ammonia can also potentiate the effects of inflammatory cytokines on mitochondrial permeability transition [90] and swelling of cultured astrocytes [91].

As with the neuroinflammatory response that occurs in other neurodegenerative diseases, the neuroinflammation produced by Meth is similar to that of the innate immune response in the periphery. Loftis et al. [92] found that abstinent Meth users have long-lasting immune dysfunction as measured by increased mRNA expression of pro-inflammatory cytokines and chemokines in blood. In addition, PET scans of human Meth users indicate prominent microglial activation in the brain [15, 93]. These observations in humans are substantiated by rodent experiments that also observe glial activation [80, 94–96] and increases in inflammatory mediators in various brain regions after Meth administration [92, 97–101]. See Table 1 for a summary of the neuroinflammatory mediators altered after Meth exposure.

Microglial cells are the resident brain macrophages and along with astrocytes are traditionally thought to be the initial responders to an immunological challenge in the brain [105]. Along these lines, microglial and astrocytic activations are observed after exposure to neurotoxic doses of Meth in rodents. Activation of microglia is first observed around 24 h, peaks around 48 h, and returns to basal levels around 7–8 days after Meth [80, 95, 96, 102]. On the other hand, activation of astrocytes after Meth is

Table 1 List of neuroinflammatory mediators increased by Meth exposure

Time point after meth exposure	Common neuroinflammatory mediators increased by meth exposure	Meth treatment type	Reference
30 min	TNFα mRNA in hippocampus and frontal cortex	Single dose	[100]
	IL-6 mRNA in frontal cortex and striatum	Single dose	[102]
1 h	IL-1β mRNA in hypothalamus	Single dose	[97]
	IL-6 mRNA in hippocampus	Single dose	[102]
	IL-6 mRNA in striatum	Single dose	[103]
	TNFRI protein	Single dose	[102]
	GFAP	Single dose	[102]
12 h	IL-1β protein in striatum	Single dose	[103]
	TNFα mRNA in striatum	Single dose	[101, 103]
	TNFR1 and TNFR2 mRNA	Single dose	[101]
	TNFR1 protein	Single dose	[102]
1 day	Microglia	Binge dosing	[100]
	GFAP	Single dose	[100]
		Binge dosing	[79]
	COX-2	Binge dosing	[86, 99]
2 days	Microglia	Binge dosing	[80, 95, 96]
	GFAP	Binge dosing	[79]
3 days	GFAP	Binge dosing	[21, 79, 101]
	COX-2	Binge dosing	[104]
7 days	GFAP	Binge dosing	[79]
	Microglia	Single dose	[102]
	TNFα protein in hippocampus	Single dose	[102]
21 days	GFAP	Bing dosing	[79]

The inflammatory mediators are listed according to the time point at which they have been observed, and the Meth treatment paradigm and references are indicated

more protracted than Meth-induced microglial activation. For example, a neurotoxic binge regimen of Meth (10 mg/kg, q 2 h, ip, 4×) administered to mice or rats produces an increase in glial fibrillary acidic protein (GFAP) that is observed as early as 24 h, peaks around 48 h in the striatum and 72 h in the cortex, and lasts up to 21 days after Meth exposure [79]. The time course of astrocytic activation is slightly different after exposure to a single high dose of Meth. A single injection of high-dose Meth (30 mg/kg) increases GFAP immunoreactivity from 1 to 24 h after exposure but is not observed 3 or 7 days later [102]. Overall, it is clear that neurotoxic doses of Meth induce activation of both microglia and astrocytes to produce a neuroinflammatory state regardless of the temporal responses of microglia and astrocytes to Meth exposure.

Neurons may also play a role in the inflammatory process in the brain [106, 107]. Neurons and glia can be stimulated by many factors, including ROS and RNS that are known to be increased after Meth. ROS and RNS increase NFKB or AP1 translocation to the nucleus [108] and subsequent release of neuroinflammatory mediators including cytokines and chemokines, as well as increases in cyclooxygenase (COX), prostaglandins, and cell surface receptors for inflammatory molecules, such as cytokine receptors and toll-like receptors (TLRs) [106, 109, 110]. Interleukin-1β (IL-1β) mRNA transcripts are increased in the hypothalamus at 1 h after a single

intraperitoneal injection of 10 or 15 mg/kg Meth [97]. Although Meth-induced IL-1β mRNA expression in the striatum and hippocampus is not observed within 1 h after Meth [100], IL-1β protein expression in the striatum is observed 12 h after Meth exposure [103]. Collectively, these findings indicate that Meth induces IL-1β mRNA and protein expression in a dose-, time-, and brain region-dependent manner.

Meth-induced increases in mRNA expression of other pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) are also brain region dependent, increase within minutes after Meth exposure, and last for hours. Increased expression of TNFα mRNA in the hippocampus and frontal cortex occurs as early as 30 min after Meth exposure [100]; however, increased TNFα mRNA expression in the striatum is not observed until 12 h after Meth exposure [101, 103]. While most studies have observed immediate changes in cytokine gene transcripts, Goncalves et al. [102] observed an increase in TNFα protein in the hippocampus 7 days, but not at 1 h or 3 days, after exposure to a single high dose of Meth. Other evidence supporting the time- and region-dependent activation of pro-inflammatory mediators has been reported. IL-6 mRNA expression is increased in the hippocampus, striatum, and frontal cortex. Increases in gene transcripts of IL-6 in the frontal cortex and striatum are observed within 30 min, while that in the hippocampus is observed at 1 h after Meth exposure [100]. The Meth-induced increases in IL-6 mRNA expression in the striatum last at least 12 h after exposure to Meth [101, 103]. O'Callaghan et al. [103] also identified an increase in the IL-6 class cytokines oncostatin M (OSM) and leukemia inhibitory factor (LIF) and the chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemotactic protein-1 (MCP-1) in the striatum 12 h after a single 20 mg/kg dose of Meth.

In addition to increases in cytokines, the expression of cytokine receptors is increased after Meth. Protein expression and mRNA of the TNFα receptors, TNFR1 and TNFR2, are observed as early as 1 h and as late as 24 h after Meth exposure [101, 102]. Therefore, it appears that an increase in activity of the pro-inflammatory cytokines, chemokines, and their receptors after exposure to Meth occurs in a time-dependent manner within the same brain regions that exhibit monoaminergic terminal damage to Meth.

The cellular source of the cytokines and chemokines and the location of their receptors that are affected by Meth remain unclear. Meth can directly activate microglia and astrocytes in culture [111–113]; however, the stimulation of cytokine release from these cells by Meth is variable. Meth directly activates astrocytes in culture to produce IL-6 and IL-8 [111] and induces an increase in IL-1β, TNFα, and IL-6 mRNA expression in HAPI microglial cells [112], but does not affect IL-1β or TNFα mRNA expression in BV2 microglial cells [113]. While it was originally thought that cytokines and chemokines were released only from glial cells in the brain, it is now known that neurons can also participate in the inflammatory response [114–117] and may be the source of Meth-induced cytokines and chemokines. In fact, when dopaminergic cells in culture are treated with Meth, gene transcripts for cytokines are increased within these cells [118]. These data and findings indicating that the induction of cytokine and chemokine mRNA transcripts typically occurs much earlier than glial activation suggest that Meth acts initially to produce a cytokine response from neurons rather than glial cells, the latter being activated subsequently by cytokines or chemokines to produce additional inflammatory mediators that perpetuate the immune response.

Cyclooxygenase (COX) is another inflammatory mediator induced by Meth. COX is the enzyme that converts arachidonic acid (AA) into prostaglandin H2 (PGH2), which subsequently is converted to other prostaglandins and thromboxanes and can participate in the neuroinflammatory responses produced by Meth. The protein expression of COX-2, but not COX-1, is induced by Meth in the striatum within 24 h [99] and can be observed as long as 72 h later [104]. No studies have identified the trigger or the cellular source of Meth-induced COX-2 expression; however, Meth induces COX-2 protein expression likely in neurons, glia, and even BBB endothelial cells [31, 99, 104] through the activation of NFKB by proinflammatory cytokines and ROS [119, 120].

No studies have observed an increase in the products of COX activity, namely, the prostaglandins and thromboxanes, even though it is known that Meth produces an increase in COX-2 expression. Thomas and Kuhn [99] observed no change in PGE2 expression when COX-2 was increased in the striatum after Meth. Changes in thromboxanes have yet to be evaluated. Despite the finding that prostaglandins do not appear to be increased by Meth via the prostaglandin synthase activity of COX, the peroxidase activity of COX may be increased and result in oxidant formation and perhaps DA quinones that have been implicated in the toxic effects of Meth [121, 122].

In addition to prostaglandins, thromboxane, and cytokine receptors, toll-like receptors (TLRs), on both glia and neurons, are other cell surface receptors that likely mediate the inflammatory response [123]. TLRs recognize pathogen-associated molecular patterns common to microorganisms [124] but are also activated by endogenous ligands such as reactive oxygen species, heat shock proteins, and extracellular matrix components [125, 126]. Endogenous ligands of the TLRs, such as ROS and heat shock proteins, exist in the brain, and TLRs are associated with some neurodegenerative processes, such as Parkinson's disease, Alzheimer's disease, and epilepsy [127–129]. While no evidence exists for the increased expression of TLRs by Meth, Meth increases ROS [47, 48] and HSP70 [130, 131], both of which are known to activate TLR4 [125] and produce downstream activation of the transcription factors NFκB and AP1 [132]. This signaling cascade is similar to IL-1 receptor activation, which causes and potentiates neuroinflammation.

Lastly, the complement protein cascade system also has been implicated in several neurodegenerative disease states [133], but its relationship to Meth toxicity has yet to be evaluated. Since the complement system is intimately involved in microglial activation through its effects on complement receptors on microglia, it is reasonable to postulate that it plays an important role in the neurodegenerative causes or consequences of Meth toxicity.

5 Neuroinflammation: A Cause or Consequence of Meth-Induced Toxicity

Meth-induced monoaminergic damage has been associated with microglial activation, and lack of microglial activation is typically associated with lack of monoaminergic damage. Neurotoxic doses of Meth produce microglial activation, and

microglial activation precedes damage to DA and 5-HT terminals [80, 95, 96]. In addition, pharmacological inhibition of NMDA receptors with MK-801 prevents Meth-induced microglial activation and damage to DA and 5-HT terminals, suggesting that microglia may play a role in Meth-induced terminal damage. On the other hand, a dose of Meth (2 mg/kg) that does not produce long-term decreases in DA and 5-HT tissue content produces a slight but significant increase in microglial activation [96]. This finding indicates that microglial activation can occur at low doses of Meth that do not cause DA and 5-HT terminal damage and suggests that a dose-dependent threshold of microglial activation is required for damage to occur.

Activation of astrocytes may also be involved in Meth-induced damage. As mentioned previously, doses of Meth that produce dopaminergic and serotonergic damage also produce an increase in the astrocytic marker GFAP [39, 79]. Furthermore, prevention of Meth-induced increases in GFAP by MK-801 also prevents decreases in TH and DA tissue content [79], suggesting a role for astrocytes in mediating the toxicity to Meth. However, the Meth-induced decreases in DA and 5-HT tissue content and TH occur prior to increases in GFAP [79] and suggest that astrocytes can be activated in response to or as a consequence of Meth-induced terminal damage and could play a role in limiting monoaminergic terminal damage. For example, it is thought that astrocytes play a protective role in multiple sclerosis by releasing anti-inflammatory cytokines and limiting T-cell activation [134]. In addition, ciliary neurotrophic factor (CNTF)-mediated activation of astrocytes 2 days prior to kainate treatment significantly attenuates epileptic activity and hippocampal neuronal death [135]. More relevant to Meth-induced monoaminergic damage, astrocytes release molecules, such as 14,15-epoxyeicosatrienoic acid, a metabolite of arachidonic acid, which protects DA neurons in culture from oxidative damage induced by hydrogen peroxide [136]. Together these data suggest a protective rather than a destructive role for astrocytes in Meth-induced monoaminergic terminal damage.

The role of inflammatory cytokines in Meth-induced damage is also undefined. The findings that pro-inflammatory cytokines are increased within minutes after Meth exposure suggest a causative role for cytokines in mediating the long-term damage produced by Meth. Consistent with these findings is that monoaminergic terminal damage is prevented in IL-6 knockout mice [44]. On the other hand, TNFα seems to reduce Meth-induced dopaminergic toxicity as intrastriatal injections of TNFα attenuated Meth-induced increases in extracellular DA, potentiated DA uptake into vesicles, and prevented Meth-induced decreases in DA tissue content and TH [137]. Moreover, TNFα knockout mice exhibit enhanced Meth-induced DA depletions and hyperthermia [137]. Therefore, it remains unclear whether the seemingly protective role of TNFα is due to the direct pharmacological effects of TNFα or due to the attenuation of Meth-induced hyperthermia. The role of IL-1 is also unclear. Treatment with the interleukin-1 receptor antagonist, IL-1ra, attenuates Meth-induced lethality and peak body temperature, but does not prevent Meth-induced DA depletions [39]. Thus, it remains unclear whether cytokines prevent or mediate Meth-induced damage and point to the fact that there may be distinct roles for each of the cytokines.

The mediators of Meth-induced damage may be downstream from the action of the inflammatory cytokines. The COX inhibitor indomethacin prevents Meth-induced decreases in neuronal-specific class III beta-tubulin (Tuj-1) in the hippocampus [102]. Furthermore, the nonspecific COX inhibitor, ketoprofen, and an antagonist of the substance P receptor, NK1, attenuate Meth-induced increases in microglial cells and decreases in DAT [138–140]. However, the attenuation of Meth-induced DA depletions in COX-2 knockout mice is associated with attenuation of hyperthermia and may not be due solely to the deletion of COX-2 [99]. Furthermore, although the broad-spectrum anti-inflammatory minocycline inhibits microglial activation and attenuates Meth-induced increases in markers of neuroinflammation identified by gliosis and increased mRNA transcripts of inflammatory cytokines, it does not attenuate Meth-induced decreases in DA tissue content [101]. Thus, while anti-inflammatory drugs have successfully mitigated some markers of Meth-induced damage, no studies have shown that an anti-inflammatory drug can successfully prevent Meth-induced DA or 5-HT depletions independent of hyperthermia.

It remains to be determined how the relatively generalized effects of glial activation and neuroinflammation can explain the specific effects of Meth on DA and 5-HT terminals. Clearly, neuroinflammation per se does not cause specific damage to DA and 5-HT terminals, but we have hypothesized that in the presence of the more selective pharmacodynamic actions of Meth (e.g., at DAT and SERT), neuroinflammatory mediators can synergize with Meth to selectively render DA and 5-HT terminals vulnerable [141]. Regardless, evidence exists for the role of Meth-induced neuroinflammation in damage to neurons other than the monoaminergic terminals. Depletion of glutamate-positive neurons in the somatosensory cortex is associated with an increase in GFAP [21]. In addition, death of GABAergic and cholinergic neurons in the striatum is mediated by substance P, an inflammatory mediator, and its actions at the NK1 receptor, as evidenced by the prevention of cell death in the striatum after NK1 receptor antagonism [142] or ablation of NK1 receptor-containing neurons [143]. Consequently, Meth-induced neuroinflammation may play a role beyond that of toxicity to monoaminergic terminals.

Neuroinflammation could have independent or indirect effects that can potentiate Meth-induced damage even though it may not play a direct role in Meth toxicity. Local administration of lipopolysaccharide (LPS) into the striatum prior to Meth exposure results in an enhancement of Meth-induced decreases in TH and DA tissue content and increases in DA turnover and neuroinflammation as measured by gliosis and increases in IFNγ, IL-1β, TNFα, and COX-2 expression while having no effects on the serotonergic or noradrenergic systems in the striatum [144]. In addition, prior exposure to chronic unpredictable stress or chronic corticosterone administration enhances Meth-induced neuroinflammation, monoaminergic damage [103, 122], and BBB function [31]. Moreover, the COX inhibitor ketoprofen or the prostaglandin EP1 receptor antagonist SC-51089 prevents the long-term monoaminergic and/or BBB damage caused by serial exposure to chronic unpredictable stress and Meth [31, 122]. Collectively, preexistent neuroinflammation appears to potentiate Meth-induced damage to both monoaminergic terminals and the BBB.

6 Conclusion

Neuroinflammation plays a key role in the neurotoxic effects of Meth and is most likely both a cause and a consequence of Meth-induced damage. This dual involvement for neuroinflammation is evidenced by its role in initiating a perpetual cycle of oxidative and excitotoxic mechanisms that culminate in additional inflammatory effects and eventual toxicity to neurons and perhaps glia. As there are multiple inflammatory mediators and pathways, so are there multiple endpoints reflective of damage produced by Meth. It remains to be determined whether specific inflammatory mediators are responsible for the specific neurotoxicities associated with Meth. Further investigation is warranted into how neuroinflammation directly contributes to the toxicity as well as being a point of convergence of previously identified mechanisms of Meth toxicity. Regardless, there is strong evidence that neuroinflammation can enhance Meth-induced damage and strongly suggests that neuroinflammation is a factor in the comorbidity associated with stimulant abuse and contributes to Meth-induced brain injury and cognitive impairments.

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Drugs of Abuse and NeuroAIDS: Opiates

Thomas J. Rogers

Abstract Opiate abuse is a major global health problem, due in part to the fact that the HIV infection often occurs with intravenous drug abuse. There is strong clinical and preclinical evidence that opiate abuse promotes the neurodegeneration that can occur in association with HIV infection. Morphine or heroin can exert direct neurotoxic effects on neuronal cells and alter neuronal function. In addition, opiate administration after the virus infection has been established can exacerbate the neurotoxic properties of some of the HIV products. This can include the induction of pro-inflammatory mediators including both cytokines and chemokines and a loss of blood–brain barrier integrity. It is also clear that the activation of opioid receptors by agonists like morphine can initiate cross-talk interactions with other receptors, most notably the chemokine receptors CCR5 and CXCR4. Opiates clearly exert both pro- and anti-inflammatory activity, and our understanding of how these opposing influences are balanced in both the brain and periphery is rapidly advancing.

Keywords Opiate • Morphine • Heroin • Neurodegeneration • Blood–brain barrier • HIV-1 • Co-receptor • Heterologous desensitization • Glutamate • Chemokine

1 Introduction

Opiate drug abuse is a major contributing factor to the global AIDS epidemic. It is likely that over a third of the HIV infections in the USA can be linked to intravenous drug abuse, and global estimates suggest that almost 20 % of intravenous drug abusers are infected with HIV [1–4]. Chronic opioid abuse is a growing problem, due in

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part to the increase in the misuse of prescription opioid drugs in the USA [3]. Opioid abuse is associated with a decline in resistance to a number of opportunistic infections, and both direct and indirect processes are responsible for these immunosuppressive effects (reviewed in [5–7]). Work reported by a number of investigators, based on both clinical and laboratory research, has documented the capacity of heroin (or morphine) to inhibit adaptive and innate immune responses [5, 6, 8–10]. Moreover, experimental animal research shows that opioid administration leads to an increase in susceptibility to a number of infectious agents, including *Candida albicans*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, herpes virus, murine leukemia virus, and *Toxoplasma gondii* [11–19]. However, the effect of chronic opioid administration on resistance to HIV infection is less clear. Experimental animal studies carried out with SIV have provided conflicting results, and this is almost certainly due, in part, to inconsistencies in the properties of the viral strain and the dose of morphine administered [20–23].

It is important to recognize that the immunosuppressive activity of the opioids is not universal, since it is clear that these drugs can have strong pro-inflammatory properties in certain circumstances. For example, current evidence suggests that opiates promote the neuropathology which can be associated with HIV infection by increasing the toxicity of some of the HIV proteins, particularly in the brain [24]. The neurotoxicity of the opiates is likely due to an elevation in the expression of pro-inflammatory cytokines in the brain [24, 25]. There is also evidence that opioid administration results in the degradation of the integrity of the blood–brain barrier (BBB), which may promote the exposure of the brain to additional pro-inflammatory cytokines which may more easily pass across the BBB [24, 26]. These studies are consistent with clinical evidence which suggests that opiate abuse leads to higher rates of encephalopathy in HIV-infected patients, compared to infected nondrug abusers [27, 28].

2 Opiates and Opioid Receptors

Opium, derived from the seedpods of *Papaver somniferum*, has been utilized for medicinal purposes since prehistoric times. Heroin, or diacetylmorphine, is chemically synthesized from opium and is one of the common opiates employed by intravenous drug abusers. Heroin is metabolized to morphine, 6-monoacetylmorphine, morphine-6-β-D-glucuronide, and morphine-3-β-D-glucuronide. While morphine is utilized widely as an analgesic and is the major bioactive heroin metabolite, several of the metabolites also possess opiate activity [29]. Endogenous opioid peptides, including endomorphins 1 and 2, leu- and met-enkephalin, and dynorphin, are produced in both the brain and in the periphery, and levels of opioid peptides appear to increase in response to inflammatory stimuli [30]. The regulation of endogenous peptide expression in the periphery by leukocytes is not well defined, and this information is important for our understanding of the regulation of the immune system by opioids.

There are three opioid receptors, and these are designated μ-, κ-, and δ-opioid receptors (MOR, KOR, and DOR) [5, 31]. Each of these receptors is expressed

within the central and peripheral nervous system, although the relative expression may vary depending on the specific tissue site. The opioid receptors are also expressed by leukocytes, and molecular analysis of the opioid receptors expressed by leukocytes shows they are identical to those expressed in the CNS [32–34]. Experimental work on the modulation of the immune response is often conducted with one or more synthetic opioids, and these can have the advantage of being more receptor selective and/or possess a higher affinity for the respective opioid receptor type. The experimental use of these agonists can offer a major advantage in conducting experiments to understand the role of specific opioid receptors in the immune response, since opioid drugs of abuse are not highly receptor selective. For example, morphine has predominant binding activity for MOR but also activates both KOR and DOR. This means that the effects induced by morphine may be mediated by combinations of opioid receptor types. This can represent a critical issue since MOR and KOR can mediate opposing activities for cells of the immune system [35, 36].

3 Direct Mechanisms of Neurotoxicity of Opioids

The μ-opioids, particularly with chronic administration, can manifest detectable neurotoxic activity in the absence of other toxic stimuli. The overt toxicity of morphine appears to be modest [37], but it should be noted that there is evidence that this μ-opioid, and others, can directly induce neurotoxic effects. Mu opioids including both morphine and fentanyl exert direct toxic effects on Purkinje cells in vitro, and fentanyl administration to rats in vivo induces damage to the limbic system and exacerbates cerebral ischemia in the forebrain [38–40]. Finally, μ-opioids exert a proapoptotic effect when combined at relatively low doses with various other apoptotic agents [41–45]. There is evidence of astrogliosis in heroin abusers [46], and the dopaminergic function of tyrosine hydroxylase terminals in the nucleus accumbens is inhibited [47]. Chronic morphine or heroin administration to rodents results in reduced striatal levels of synaptic dopamine and dopamine transporter [48–50]. These effects in the brain have been associated with the accumulation of perivascular infiltrates of macrophages and lymphocytes, suggesting that at least a part of the toxicity in these studies was the result of a low level of inflammatory activity in regions of brain tissue. Of course, other mechanisms are almost certainly involved in the manifestation of the gliosis just described, including the recent observation of hyperphosphorylated tau in the hippocampal neurons of heroin abusers [51, 52].

Opioids may promote toxic effects in the brain by inducing a pro-inflammatory response. This is somewhat counterintuitive, since opioid administration has been well documented to exert immunosuppressive activity (reviewed in [5]). However, an evaluation of published work from a number of investigators shows that opioid receptor activation can exert pleomorphic effects on the immune system, particularly with respect to the inflammatory response. Studies to determine the effects of morphine, and other μ-opioids, on the production of pro- and anti-inflammatory cytokines have produced conflicting results, and this is almost certainly due to the

highly variable experimental systems employed for these studies (reviewed in [5, 36]). For example, morphine administration results in a reduction in the expression of interferon γ (IFNγ) and interleukin-2 (IL), cytokines that are critical for both acute inflammatory responses and adaptive immunity [53, 54]. Roy and her colleagues [55–57] working with both human blood leukocytes and murine splenocytes have shown that morphine polarizes toward a Th2 response, which would be expected to be less inflammatory. Sacerdote and her colleagues [58] have studied the effects of subcutaneous morphine administration on peritoneal macrophage function and reported a reduction in both baseline and lipopolysaccharide (LPS)-induced levels of IL-1β, tumor necrosis factor-α (TNFα), and IL-12. Recent work has also shown that morphine inhibits the expression of TNFα and IL-6 produced by human monocytes in response to bacterial peptidoglycan, but these effects required high concentrations of the opioid (10–100 μM) [59].

In contrast to these results, there are several reports which show that morphine, or other μ-opioids, induces the production of pro-inflammatory cytokines. Peng et al. [60] have reported an increase in the expression of both IL-12 and TNFα from murine peritoneal macrophages following morphine administration. These results are consistent with results with relatively low doses of morphine which show an increase in the expression of the pro-inflammatory cytokines IL-6 and TNFα, an effect which was due to the activation of the highly pro-inflammatory transcription factor NF-κB [61]. The latter results are particularly interesting in that the same investigation suggested that high morphine doses are inhibitory, suggesting that pharmacological doses of this drug may promote a more pro-inflammatory immune activity.

Morphine and other μ-opioids have been reported to upregulate NF-κB activity in neuronal cells. Treatment of rat cerebral cortex neurons with the MOR-selective agonist [D-ala^2, *N*-Me-Phe^4, Gly-ol^5]enkephalin (DAMGO) induces NF-κB activation [62], and morphine treatment of the NT2-N neuronal cell line induced NF-κB promoter activity [63]. The activation of NF-κB has significant implications since it is critical for the expression of a large number of pro-inflammatory cytokines, including IL-1β, IL-6, and TNFα, and the chemokines CXCL8, CCL2, and CCL5 [64–70]. Both morphine [61] and the endogenous μ-opioids endomorphin 1 and endomorphin 2 [71] have been shown to upregulate NF-κB activity in monocyte/macrophage cell populations. More recently, our laboratory has reported the upregulation of NF-κB functional activity following administration of nanomolar concentrations of DAMGO to primary human peripheral blood leukocytes and MOR-transfected HEK293 cells [72]. Moreover, we found that the induction of NF-κB activity was essential for the opioid induction of the pro-inflammatory chemokine CCL2. Finally, the latter studies showed that the MOR-initiated signaling pathway for the induction of NF-κB is dependent on the activation of PKCζ, and treatment with a PKCζ-specific pseudosubstrate inhibitor blocks both the MOR-induced activation of NF-κB and the induction of CCL2 expression. Our studies have shown that the activation of MOR initiates a signaling pathway which results in the potent activation of PKCζ, and this atypical protein kinase C is involved in regulating multiple leukocyte functional activities [72, 73]. Previous studies have shown that PKCζ directly phosphorylates IKKβ, activating IKKβ, leading to the degradation of IκB [74, 75].

Morphine also induces the expression of TGFβ [76] in human peripheral blood leukocytes, and while this cytokine exhibits pleiotropic activities for the immune system, it is predominantly immunosuppressive. Given the immunosuppressive nature of this cytokine, it is possible that at least some of the reported negative effects of morphine on cytokine expression may be mediated by the production of TGFβ. We have found that TGFβ expression is induced following DAMGO administration to either human peripheral blood leukocytes or purified blood monocytes [77]. Our studies have shown that DAMGO also induces expression of both CCL5 and CXCR4 by human peripheral blood T cells and monocytes [78, 79], and we have recently shown that the upregulation of both CCL5 and CXCR4 is dependent on the initial production of TGFβ by these cell populations [77]. Moreover, these studies showed that both T cells and monocytes respond to TGFβ treatment by upregulating CCL5 and CXCR4 expression. These results are interesting in view of the anti-inflammatory effects of TGFβ, since this cytokine is necessary for the upregulation of a pro-inflammatory chemokine (CCL5) and a potentially pro-inflammatory chemokine receptor (CXCR4).

In addition to the potential pro-inflammatory effects of morphine, this opioid has been shown to promote neurodegeneration by weakening the integrity of the blood–brain barrier (BBB). Mahajan et al. [24], working with an in vitro BBB model, have shown that morphine inhibits the expression of the tight-junction zona occludin (ZO) proteins, ZO-1 and occludin, and increases the expression of junctional adhesion molecule (JAM)-1, leading to an increase in BBB permeability. These studies also showed that morphine induces an increase in the transmigration of peripheral blood leukocytes, suggesting the potential for increased traffic of inflammatory leukocytes into the brain with morphine administration. More recent studies have shown that morphine induces the expression of platelet-derived growth factor (PDGF) from brain microvascular endothelial cells in an in vitro model of the BBB [80]. This cytokine is a potent mitogen, exhibits chemoattractant activity, is highly pro-fibrotic, and has been reported to impair BBB integrity during ischemic stroke [81, 82]. The mechanism of BBB impairment mediated by PDGF is not clear, but it is known that PDGF is preferentially produced within the immune system by alternatively activated (M2) macrophages [83].

4 Neurodegeneration Mediated by Opiates in Association with HIV

A review of the effects of HIV infection, or the impact of HIV products, on the process of neurodegeneration is beyond the scope of this review. These issues will be discussed at length in other chapters of this book. However, the intersection between the neurodegenerative activity of opiates and HIV products will be discussed.

There is growing evidence that the combination of HIV infection and opiate drug abuse creates a heightened level of neurodegeneration compared with HIV or opiate use alone. Of course, mu opiates are well documented to alter the functional activity of neurons, microglia, astrocytes, neuronal precursors, and oligodendrocytes [84–99]. This is not altogether surprising, since each of these cell populations expresses MOR,

albeit with diverse levels of expression in the various regions of the brain. However, the combination of HIV infection (and the release of HIV products into the brain milieu), with mu opiates, appears to target primarily the astrocytes and microglia and induce much greater pro-inflammatory and neurotoxic activity (reviewed in [100]).

4.1 Glial and Neuronal Cell Populations

Microglia play a critical role in HIV neuropathogenesis, and extensive activation of these cells (and infiltrating perivascular macrophages) is a common feature of the neurodegeneration associated with HIV infection [101, 102]. It is well known that the presence of activated macrophages and/or microglia correlates with the severity of the HIV-associated neurocognitive disorders (HAND); in fact, this correlation is stronger than the number of HIV-infected cells or viral load [103–106]. Both macrophages and microglia are subject to regulation mediated through opioid receptors, and these cells can exhibit substantial changes in functional activity with opiate administration. Experimental animal work has shown that systemic treatment with morphine induces an increase in the infiltration at sites of intrastriatal Tat injection [89]. These results are in agreement with published findings which show an association between increased numbers of microglia in the gray matter of the thalamus and hippocampus and encephalitis in opiate abusers [107]. Moreover, the accumulation of microglia expressing major histocompatibility complex type II (MHC II) and CD68 is increased in opiate abusers, when compared with non-abusers [108].

Astrocytes perform an essential set of functions in the development and maintenance of the brain and are important for the integrity of the BBB. Astrocytes are not susceptible to productive HIV infection (unlike macrophages and microglia), but these cells are important targets for the neurotoxic products of HIV. Astrocytes can exacerbate the neurodegenerative effects of HIV products by releasing mediators with potential toxicity such as nitric oxide, neurotransmitters, and pro-inflammatory cytokines, and collectively these serve to promote the HIV-mediated neuropathology. Of course, astrocytes also express opioid receptors, and morphine can augment, or accelerate, the neurotoxic activity of certain HIV products. For example, morphine administration together with HIV Tat can result in augmented cytokine and chemokine expression and, potentially, astrocyte death [90, 99]. Astrocytes are a major source of several of the chemokines within the brain, and opiate modulation of the expression of these mediators is an important aspect of the intersection between opioid receptors and the inflammatory response. The effects of opiates in combination with either HIV infection, or HIV products, will be discussed below.

The neurodegeneration observed in association with HIV infection leads to synaptodendritic injury which resembles the damage observed in other neurological diseases including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [109–111]. The normal synaptodendritic network is characterized by highly complex and branching dendrites. However, in HIV encephalitis, the dendrites exhibit pruning, with dendritic beading, atrophy, and vacuolization [112].

Nevertheless, while this damage is typically sublethal, it is likely to contribute to the neurobehavioral deficits which are characteristic of HAND [113, 114]. At the same time, neuronal loss is a characteristic of HIV-associated neuropathology, and this is likely due to bystander effects mediated by reactive oxygen species and other neurotoxic products released from astrocytes and microglia [115–118]. Nevertheless, recent reports suggest that opiate abuse exacerbates the HIV-induced synaptodendritic damage and promotes the development of more severe neurobehavioral abnormalities [100, 119]. It should be pointed out that several reports have described the ability of MOR agonists to lessen the complexity of dendrites and diminish the density of dendritic spines [38, 120, 121].

Neurons appear to be directly susceptible to the HIV products Tat, gp120, and vpr, and the release of these products into the brain interstitium can result in neuronal damage [122–126]. Since neurons are not a source of productive HIV infection, the source of these products is primarily perivascular macrophages and microglial cell populations. This serves to highlight the fact that HIV-associated neurodegeneration is the product of glial transmission of neurotoxic products to the neuron [100]. Because the microglia express opioid receptors, these cells respond to treatment with exogenous opioid agonists and exhibit altered glial cell function. Recent reports suggest that opiate exposure reduces the level of HIV products which are required to trigger more pronounced neuropathology [87, 127]. Moreover, these studies show that morphine administration exacerbates the neurotoxicity of HIV Tat and gp120.

It is clear that μ-opioid agonists can also promote a neurodegenerative outcome through indirect effects mediated through changes in the levels of various neurotransmitters. For example, HIV Tat activates astrocytes leading to potent cytokine and inflammatory mediator release leading to restrictive glutamate uptake [90, 128]. HIV gp120 induces a similar astrocyte inflammatory response, and the combination of either of these HIV products and morphine enhances these responses, exacerbates the glutamate release, and reduces the glutamate excitotoxic threshold [129, 130]. In addition, several drugs of abuse, including the opiates, induce an increase in the levels of dopamine in the CNS. Morphine induces a twofold increase in levels of dopamine in the nucleus accumbens and caudate nucleus in rats [131]. Recent reports suggest that an elevated dopamine level, or administration of exogenous dopamine, alters monocyte/macrophage and T cell function [132–134]. Moreover, recent work suggests that dopamine administration promotes the replication of HIV in primary human macrophages [133]. In addition, the administration of either L-DOPA (the precursor of dopamine) or selegiline (inhibitor of dopamine catabolism) to SIV-infected macaques increases the SIV load in the brain [135, 136].

4.2 Inflammation and Immune Activation

As mentioned above, the capacity of MOR agonists to modulate the expression of pro-inflammatory cytokines has been the subject of a great deal of research (reviewed in [5]). The influence of opiates on chemokine expression is particularly important

because these chemotactic cytokines are likely to be critical in the development of HIV-associated neurodegeneration. On the one hand, these factors are important for the traffic of infected monocytes across the BBB, and these cytokines may also promote the migration of infected T cells to lymph nodes to promote contact with noninfected target cells. The chemokines CCL2, CCL3, CCL4, CCL5, and CX3CL1 are chemokines that have been identified as contributors to the traffic of monocytes across the BBB, and CX3CL1 is particularly important for the migration of CD16+ monocytes that are highly susceptible to HIV infection [118, 137]. This relatively minor monocyte subpopulation that expresses both CD14 and CD16 has been reported to expand during HIV infection [138–143], and increased percentages of these cells correlate with HAD [144]. Monocytes and macrophages expressing high levels of CD16+ have been reported to be preferentially infected with HIV in brain tissue at autopsy [137]. In addition, the expression of the HIV co-receptor CCR5 is elevated on the CD16+ monocyte subset [145]. Recent analysis of monocyte subsets suggests that CD14+ CD16 cells exhibit a greater pro-inflammatory capacity, and the CD14+ 16+ cells possess strong responsiveness to viral pattern recognition epitopes and perform "patrolling" activity [146].

It is widely accepted that CCL2 is critically involved in directing the migration of infected monocytes across the BBB [147, 148]. With the accumulating inflammation in the underlying brain tissue, there is activation of the vascular endothelial cells in the BBB and secretion of IL-6 [149]. It is likely that the IL-6 produced at the BBB accelerates the transit of monocytes across the BBB. Both the virus infection and the accumulation of viral products induce elevated CCL2 expression from both the perivascular macrophages and astrocytes [150]. With greater accumulation of infected monocytes and macrophages, there is an expanding source of additional CCL2, resulting in biological amplification of the neuroinflammatory response.

As mentioned above, we have examined the effect of opiate administration on the expression of CCL2 by human peripheral blood leukocytes and purified monocytes [72, 77, 78]. In additional analysis, we also observed a significant induction of the chemokines CCL5 and CXCL10 following mu opiate treatment in vitro, and we suggest that the expression of these chemokines could promote the trafficking of noninfected target monocytes or T cells to the site of infected cell populations [78]. These results are consistent with more recent studies with mice which show that the combination of morphine and HIV Tat treatment of astrocytes upregulates the expression of the chemokines CCL2, CCL3, and CCL5 [88, 90]. These results suggest that the accumulation of Tat in the brain drives a pro-inflammatory chemokine response, and this response is accelerated by the administration of morphine. Additional studies showed that the morphine exacerbation of Tat-induced CCL2 expression is diminished in CCL5-knockout mice, suggesting that the regulation of CCL2 expression is mediated through a CCL5-dependent cooperative expression process [96]. It should be pointed out that these results are in contrast to a report showing downregulation of the expression of both CCL2 and CCL4 following morphine treatment of normal human astrocytes [151]. Additional work will be required to explain the divergent results in these studies.

Inflammation is a fundamental component of the neurodegenerative processes that are responsible for HIV-associated neurocognitive disorders. It is becoming apparent that elements of systemic inflammation are an important part of HIV pathogenesis, both in the CNS and the periphery [152–155]. Recent studies show that systemic inflammation promotes the development of HAND, and the augmentation of the pathology in the CNS can be independent of HIV replication [118, 147, 156]. For example, a recent analysis of a cohort of 922 HIV-infected subjects (the Study of Fat Redistribution and Metabolic Change in HIV infection [FRAM] study cohort) has shown that the pro-inflammatory biomarkers fibrinogen and C reactive protein (CRP) are significant and independent predictors of mortality [155]. Indeed, this study showed that these measures of inflammation retained predictive significance independent of circulating CD4 counts. A second study with the Strategies for Management of Antiretroviral Therapy trial showed a significant association for the inflammatory biomarkers IL-6, D-dimer, and CRP with mortality [157]. The persistent evidence of immune activation in these subjects, in the current highly active antiretroviral therapy (HAART) era, has been proposed as a significant contributor to disease progression [158]. The fact that chronic systemic inflammation is strongly associated with morbidity and mortality suggests that anti-inflammatory therapeutics may be beneficial as an adjunct to the standard ARV therapy currently in use.

The immune activation state that occurs with HIV infection and neurodegeneration is due in large part to the microbial translocation that is now believed to be common in these patients. Results from studies reported by Brenchley et al. [159] suggest that during HIV infection, a breakdown in the follicle-associated epithelium in the gut occurs, and this leads to translocation of gut flora through the gut wall, resulting in entry of microbial products into the bloodstream. This process is associated with depletion of leukocytes from the Peyer's patches and a loss of lymphocytes from the lamina propria and mucosa-associated tissue (MALT). In healthy adults, approximately 80 % of the total lymphocytes of the body are contained within the MALT, and depletion of cells from these lymphoid structures can result in a substantial reduction of T cells following infection [160]. Recent analysis has shown that circulating LPS, LPS-binding protein, and sCD14 levels correlate significantly with progression of the disease, and monocytes obtained from these individuals exhibit a refractory response to LPS stimulation in vitro, suggesting that these cells had been stimulated in vivo with LPS [159, 161, 162]. It is now apparent that the toll-like receptor family is an important contributor to the persistent immune activation. This is not surprising given the entry of microbial components into the bloodstream following the gastrointestinal damage, but in addition, viral toll ligands also participate in the activation of TLR7 [163, 164]. Work with mice has demonstrated that sustained activation of TLR7 induces a state of chronic immune activation which resembles immune activation associated with HIV infection [165].

Studies reported by Hillburger et al. [166] have shown that mice treated with morphine using slow-release pellets develop bacterial sepsis as a likely result of microbial translocation. This study is particularly significant given the critical role for microbial translocation in the process of immune activation observed with HIV infection. However, this study did not examine the combined effect of morphine and

HIV infection on the process of microbial translocation, and additional work on this issue would be valuable for our understanding of the influence of opiate use on immune status in HIV-infected patients.

The immune system is programmed to control the development of an inflammatory response, in part, through the production of immunosuppressive mediators such as IL-10 and TGFβ. Indeed, both TGFβ1 and IL-10 are upregulated in the CNS of patients who suffer with neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease, and TGFβ is upregulated in the CNS of patients with AIDS [167–170]. Analysis of brain tissue shows that TGFβ is readily detectable in macrophages, astrocytes, and microglial cells in the frontal cortex in patients with AIDS [170, 171]. The production of TGFβ in the CNS is considered anti-inflammatory and protective since there is evidence that it attenuates the level of astrocytosis which is characteristic of brain tissue in patients with HIV-associated dementia. In vitro analysis with astrocyte cultures shows that TGFβ inhibits cell proliferation and reduces glutamine synthetase [170]. Administration of TGFβ to microglial cell cultures results in downregulation of proliferation in response to either GM-CSF or M-CSF, and TGFβ inhibits the microglial expression of a number of pro-inflammatory cytokines and chemokines including IL-1, TNFα, CCL5, and CXCL8 [170, 171]. Moreover, TGFβ appears to inhibit expression of both complement factor 3 and inducible nitric oxide synthase, two potentially neurotoxic factors [172–175], and there are several reports which show that TGFβ inhibits microglial free radical production [176, 177]. Finally, TGFβ has been shown to inhibit HIV gp120-induced neuronal death, as well as calcium overloading, providing a degree of neuroprotection in HIV-infected brain tissue [178].

Based on reports from several investigators, the activation of MOR by opioid agonists appears to target TGFβ expression in leukocytes. For example, morphine treatment of human peripheral blood leukocytes downregulates the LPS- or PHA-induced expression of TNFα, and this effect is attenuated with the addition of anti-TGFβ antibodies [179]. In addition, morphine administration to human peripheral blood leukocytes upregulates TGFβ expression in response to either PHA or LPS [76]. More recently we observed that both human peripheral blood mononuclear cells and isolated peripheral blood monocytes upregulate TGFβ expression following activation of MOR [77]. It should be pointed out that TGFβ can exert pro-inflammatory activity in certain circumstances. For example, we have recently reported that mu opiates induce the expression of the pro-inflammatory chemokine CCL5, and the induction of expression is dependent on the initial expression of TGFβ [77]. In addition, TGFβ induces chemoattractant activity for monocytes and upregulates the expression of LFA-1 and the fibronectin receptor on monocytes [180–182]. These effects would be expected to promote adhesion of monocytes to endothelial cells and potentially promote traffic of monocytes across the BBB. Finally, there is evidence that TGFβ can induce monocyte expression of several pro-inflammatory cytokines [180, 183, 184]. While it is clear that the dominant role of TGFβ is to dampen the inflammatory response, it should be appreciated that the network of cytokines both in the periphery and in the brain can be quite complex, and TGFβ may exert a combination of effects as a part of a neurodegenerative disease process.

4.3 Interactions Between Opioid and Chemokine Receptors

It is well established that the chemokine receptors CCR5 and CXCR4 are the major HIV-1 co-receptors, and HIV strains can be distinguished based on the use of these co-receptors for target cell attachment and infection. The gp120 region of the HIV gp160 envelope protein possesses the capacity for binding to CD4, and one or more of the co-receptors, and this dictates cellular tropism for the virus. Virtually all HIV isolates from brain tissue use CCR5 rather than CXCR4 for viral attachment and are predominantly monocyte/macrophage tropic (R5 strains). Both of these chemokine receptors, and their chemokine ligands, are constitutively expressed in the brain.

Curiously, it appears that the expression of neuronal CXCR4 is upregulated, while the expression of CCR5 is reduced, in patients with HAND [185]. Both CXCR4 and CXCL12 are critical contributors to the development of the brain and play important roles in the maturation and maintenance of neuronal function in both the developing and adult brain [186, 187]. In the mature adult brain, CXCL12 has been shown to downregulate the expression of proapoptotic pathways and enhance neuronal survival [188, 189]. The role of CXCR4 in maintaining neuronal homeostasis is also mediated by modulation of the subunit composition of the NMDA receptor. Activation of CXCR4 leads to a reduction in the NR2B subunit of the NMDA receptor, and this substantially reduces excitotoxicity [190]. While X4 gp120 binds and activates CXCR4, the resulting signaling pathway is not identical to that which is induced by CXCL12 [191, 192]. Most notably, gp120 induces signaling elements which promote apoptosis and lead to a greater level of neuronal damage and cell death [193, 194].

Chronic morphine administration has recently been shown to inhibit the expression of the MAP kinases ERK1/2 and potentially attenuate the antiapoptotic activity of these kinases [195]. Moreover, morphine induces apoptosis in several regions of the brain, including both the frontal cortex and hippocampus [196]. Morphine has also been reported to significantly diminish dendritic spine complexity by reducing dendrite length and spine density [121, 197, 198]. Recent studies suggest that morphine mediates these effects on dendrite structure by attenuating the activity of the neurogenic differentiation 1 transcription factor (NeuroD), which is required for maintenance of dendritic spine stability [199]. As mentioned above, dendritic injury is a common feature of HAND, and evidence that morphine promotes dendritic simplification has significant implications.

The opioid receptors have the capacity to interact with the chemokine receptors that are expressed both within the CNS and in the periphery. We have reported studies which show that the activation of MOR leads to a significant upregulation of CCR5 and CXCR4 expression by human peripheral blood monocytes and T cell lymphoblasts [79]. This increase in the expression of CCR5 and CXCR4 was associated with an increase in susceptibility to infection with R5 and X4 strains of HIV-1, respectively [79]. These results are consistent with earlier work which has demonstrated that upregulation of co-receptor expression results in a corresponding increase in HIV replication [200, 201]. Treatment with morphine has also been reported to upregulate the expression of the chemokine receptors CCR2b, CCR3, and CCR5 by normal human astrocytes [151].

In addition to the capacity of the opiates to regulate the *expression* of chemokine receptors, there is considerable evidence that chemokine receptor *function* can be regulated through the action of opioid receptors. One of the mechanisms for the regulation of G protein-coupled receptor (GPCR) function is heterologous desensitization, a process in which the activation of one GPCR by its ligand results in the cross-inactivation of a second (unrelated) GPCR in the absence of the ligand for the second receptor (reviewed in [202]). Our laboratory and others have demonstrated that MOR can mediate cross-desensitization of several chemokine receptors, including CCR1, CCR2, CCR5, CXCR1, and CXCR2 [203, 204]. The biochemical basis for these interactions involves the sequential activation of multiple kinases, which leads to the activation of one or more members of the second messenger-dependent kinase family [202]. In the signaling pathway between MOR and CCR5, we have found that MOR activates PKCζ and this kinase phosphorylates and inactivates CCR5 within a period of less than 10 min [73]. The desensitization of CCR5 induced by MOR inhibits CCR5 function as measured by loss of chemotactic activity or a calcium mobilization response. In addition, HIV co-receptor function for cross-desensitized CCR5 is also lost when analyzed with R5 (but not X4) strains of HIV-1 [204].

Examination of the cross-talk between a number of GPCRs has led to the conclusion that there is a hierarchy which defines the interactions between these receptors [202]. In general, some GPCRs are strong cross-desensitizers but tend to be less sensitive as targets for the desensitization. On the other hand, certain GPCRs exhibit the opposite characteristics. For example, the formyl peptide receptor (FPR) is a relatively strong desensitizer, but this GPCR is difficult to cross-desensitize. Our laboratory has examined the interaction between MOR and CXCR4, and we have found that MOR is unable to cross-desensitize this receptor through this second messenger-dependent kinase pathway [204]. However, the Meucci laboratory [194] has described a cross-talk process in neuronal cells in which the activation of MOR results in inactivation of CXCR4, based on the loss of CXCR4 signaling activity. Their studies showed that the cross-desensitization induced through MOR resulted in the loss of the neuroprotective activity of CXCR4 in NMDA neurotoxicity studies. These results suggest that the cross-talk induced through activation of MOR would be very likely to contribute to the neurodegeneration associated with HIV infection.

We have reported results which show that the cross-desensitization between MOR and some susceptible chemokine receptors is bidirectional [205]. These studies show that MOR is cross-desensitized by CCR2, CCR5, CCR7, CX3CR1, and CXCR4, but not by CXCR1 or CXCR2. Moreover, the activation of CCR1, CCR5, or CXCR4 results in the loss of MOR-mediated analgesic activity in vivo [205–207]. These results suggest that in situations where the levels of pro-inflammatory chemokines are elevated in the brain, the threshold for sensation of pain is reduced. Clearly the neuroinflammation that is associated with HIV infection involves significantly increased levels of many pro-inflammatory chemokines. It is well known that heightened pain sensitivity (hyperalgesia) is associated with systemic inflammatory "flu-like" symptoms that include joint and muscle pain, fever, and somnolence [208, 209].

Finally, the results from our laboratory indicate that the MOR-induced cross-desensitization of CCR5 is apparent within 10–15 min and persists for at least 4–6 h. As mentioned above, this results in a loss of CCR5 co-receptor activity and a substantial reduction in susceptibility to R5 HIV infection [204]. The cross-desensitization of CCR5 can be prolonged if the MOR activation is sustained, but with acute opioid administration, the loss of co-receptor function is lost for the first several hours. This is followed by an *increase* in co-receptor function and increased R5 HIV susceptibility at 24–48 h [79], and this persists for several days. This suggests that the impact of opiates on R5 HIV susceptibility is likely to be complex, with confounding influences occurring at the level of co-receptor function.

5 Conclusion

The administration of opiates in the drug abuse population, in the context of HIV infection, promotes most of the neurodegenerative processes that take place as a part of the underlying viral infection. The brain would appear to be particularly susceptible to these effects because of the abundant number of cells which express MOR at relatively high levels. While opiates like morphine can exacerbate (or attenuate, depending on the conditions) systemic inflammatory processes, the data for the effects of these opiates would appear to be much less certain for the brain. The cells of the immune system in the periphery express much lower levels of the opioid receptors, and the impact of opiate administration is much more variable outside of the CNS. However, there are still many issues which need to be resolved in terms of the influence of opiate abuse on the development and progression of HIV-associated neurodegeneration. Of course, analysis of the progression of the disease in this organ is difficult because of the absence of tissue for longitudinal studies. Moreover, the drug-abusing population is very diverse, and controlled studies are extremely difficult because of the absence of subjects who do not abuse additional drugs. In fact, perhaps the most important questions that remain to be addressed will involve analysis of the effects of drug combinations, since this situation is much more relevant to the actual condition of patients. The most common drug combinations which should be studied are the combinations of opiates with tobacco or alcohol. The effects of these drug combinations on neurodegeneration in the context of HIV infection are almost entirely unknown.

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Cannabinoids

Paige S. Katz, Scott Edwards, and Patricia E. Molina

Abstract Cannabinoids play a significant role in several physiological and pathophysiological processes including cognitive and immune function. Reports have identified cannabinoids as a potential pharmacological therapy for treatment of neuroinflammation following injury or neuroinflammatory diseases. Cannabinoids, whether phytocannabinoids, endocannabinoids, or synthetic analogs, signal primarily through two cannabinoid receptors, CB1 and CB2. These lipophilic compounds easily cross the blood–brain barrier and have low levels of toxicity. This chapter reviews the current understanding of the endocannabinoid system and the potential therapeutic applications for treatment of traumatic brain injury, HIV encephalitis, Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, and chronic pain.

Keywords Alzheimer's • Amyotrophic lateral sclerosis • Cannabinoids • Endocannabinoids • Cannabinoid receptors • HIV encephalitis • Multiple sclerosis • Traumatic brain injury

Abbreviations

Δ9-THC	Δ9-THC-tetrahydrocannabinol
2-AG	2-Arachidonoylglycerol
Aβ	β-Amyloid
AD	Alzheimer's disease

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AEA	Arachidonoyl ethanolamide or anandamide
AIDS	Acquired immunodeficiency syndrome
ALS	Amyotrophic lateral sclerosis
BBB	Blood–brain barrier
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CBD	Cannabidiol
CBN	Cannabinol
CNS	Central nervous system
ERK	Extracellular signal-regulated kinase
FAAH	Fatty acid amide hydrolase
FAK	Focal adhesion kinase
FALS	Familial ALS
GABA	Gamma-aminobutyric acid
HIV	Human immunodeficiency virus
HIVE	HIV encephalitis
IL	Interleukin
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAGL	Monoacylglycerol lipase
MS	Multiple sclerosis
NAPE	*N*-Arachidonoyl-phosphatidylethanolamine
NAT	*N*-Acetyltransferase
NFTs	Neurofibrillary tangles
NK	Natural killer
PI3-K	Phosphatidylinositide 3-kinase
PKB	Protein kinase B
PLC	Phospholipase C
PLD	Phospholipase D
PPAR	Proliferator-activated receptor
SALS	Sporadic ALS
SIV	Simian immunodeficiency virus
TBI	Traumatic brain injury
TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1

1 Introduction

The *Cannabis sativa* plant (i.e., marijuana) contains over 60 different chemical constituents called cannabinoids. The cannabinoids include cannabinol, cannabidiol, Δ8-THC-tetrahydrocannabinol, as well as the major psychoactive constituent Δ9-THC-tetrahydrocannabinol (Δ9-THC). These compounds are known to induce euphoria, impaired perception and memory, and mild sedation. An endogenous

cannabinoid system consisting of arachidonoyl ethanolamide (anandamide; AEA) and 2-arachidonoylglycerol (2-AG), derivatives of arachidonic acid, has also been described. These endocannabinoids, like those that are plant derived, signal through two principal cannabinoid G-protein-coupled transmembrane receptors; the CB1 receptor expressed throughout the brain, and the CB2 receptor distributed predominantly in cells and tissues of the immune system. In addition to the plant-derived and endocannabinoids, synthetic cannabinoids have been under intense investigation to determine their pharmacological actions and further understand the physiological role of the cannabinoid system. Several reports on their psychoactive effects have more recently been followed by reports of their analgesic, antiemetic, and orexigenic effects. Moreover, several lines of evidence have also demonstrated their role in immunomodulation, suggesting their potential for therapeutic use in chronic inflammatory diseases. Thus, advanced understanding of cannabinoid pharmacology, major cannabinoid receptor subtypes (CB1 and CB2), and their expression levels and localization (CB2 predominantly on B lymphocytes, macrophages, and natural killer cells), in addition to identification of endogenous cannabinoids (AEA and 2-AG), suggests that in addition to the neurobehavioral effects, cannabinoids may have a potential role in modulating immune response and specifically neuroimmune response to injury, disease, and aging.

2 Cannabinoids

2.1 Phytocannabinoids

Cannabis sativa is a flowering plant, commonly called marijuana, that contains over 400 different chemicals of which 60 are collectively called cannabinoids [1, 2]. The primary plant-derived cannabinoids include Δ9-THC-tetrahydrocannabinol (Δ9-THC), cannabinol (CBN), and cannabidiol (CBD). Cannabinoids are highly lipophilic compounds that share structural and chemical similarities and are found mainly in the resin secreted from the flowering portions of the plant. Reports on the medicinal properties of cannabis date to over 5,000 years ago, as evidenced by descriptions in Chinese texts from the third millennium BC where it was recommended for malaria, constipation, rheumatic pains, and female disorders [3]. In addition, references to cannabis use as a therapeutic agent to alter mood, cognitive function, and memory have been found in Assyrian records, Egyptian hieroglyphics, and Greek and Roman medical texts [3, 4]. CBN was the first phytocannabinoid to be isolated, and its structure was elucidated in the early 1930s followed in 1964 by the elucidation of Δ9-THC's structure and later its synthesis [5, 6]. Clinical pharmacology studies increased once the structure of Δ9-THC was elucidated and analytical techniques were available to measure its concentrations [7–9]. Moreover, it led to the synthesis and testing of active cannabimimetic analogs, of which the dimethylheptyl derivative

of 11-hydroxy-Δ8-THC (HU-210) has been shown to have a much higher affinity for cannabinoid receptors than Δ9-THC [10]. Pharmacological preclinical and clinical studies have determined that Δ9-THC is the major psychoactive constituent, CBN has lower psychotropic activity than Δ9-THC, and CBD has no psychotropic activity [7, 11, 12].

Cannabis is the most widely cultivated, trafficked, and abused illicit drug [13]. Recreational cannabis consumption can be through smoking or inhalation or through lacing of food products, with smoking being the most common route of cannabis recreational use. The systemic level of cannabinoids achieved varies according to the route of consumption, frequency of intake, and overall amount or potency of the drug consumed. Cannabis is often rolled into joints resulting in wide variation of the concentrations achieved partly due to the range of Δ9-THC concentrations found in smoking preparations (0.3–13 %), due to the source and quality of cannabis, as well as due to loss in the smoke or pyrolysis and to exhalation from the pulmonary dead space [2, 14, 15]. Oral cannabis ingestion in the form of baked goods like cakes or cookies is also associated with loss through digestion.

Pharmacokinetics of Δ9-THC varies based on the route of administration, with smoking (inhalation) and intravenous (i.v.) having the fastest onset when compared to oral administration [16, 17]. Comparative studies of Δ9-THC—intravenous, smoked, and oral administration—indicate that peak plasma concentrations are achieved faster (3 min) following intravenous or smoked delivery than following oral ingestion (60–90 min) [16]. Following cannabis smoking or inhalation, aerosolized Δ9-THC is rapidly absorbed within seconds resulting in rapid and efficient delivery to the brain due to its lipid solubility. Cognitive, mood, and behavioral effects are apparent within minutes and can last for up to 2–3 h. Controlled studies have demonstrated that Δ9-THC administration to humans produces a spectrum of effects, including increased pulse rate, decreased blood pressure, muscle weakening, increased appetite, euphoria followed by drowsiness, depersonalization, altered time sense, decreased memory recollection, decreased hearing discrimination, and sharper distorted visual signals [2]. Nevertheless, due to the encouraging findings from several preclinical studies, synthetic analogs of Δ9-THC have been developed and subjected to clinical trials for several disease conditions [18]. Nabilone and dronabinol are used clinically for treatment of several conditions including attenuation of cancer chemotherapy-induced nausea and vomiting and appetite stimulation in human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) patients [19, 20]. More recently, Sativex, a combination of Δ9-THC and cannabidiol, available as an oral spray and proposed to produce less psychotropic effects than Δ9-THC, has been approved to treat pain and spasticity in multiple sclerosis (MS) patients [21]. Additional routes of cannabinoid administration that have been tested under different clinical conditions include intravenous, rectal, and sublingual [22]. Cannabinoids are hydrophobic and are retained in adipose tissue when consumed or administered chronically. Release into the circulation can continue after cessation of consumption, accounting for a relatively long half-life (1–3 days) in the body. Δ9 undergoes phase I hepatic detoxification mainly by hepatic cytochrome P450 isoenzymes [17].

2.2 Endocannabinoids

The endocannabinoid system consists of lipid-derived mediators, cannabinoid receptors, uptake receptors, and enzymes involved in their synthesis and degradation. Endocannabinoids were discovered approximately 60 years after the discovery of Δ9-THC and almost 10 years after identification of CB receptors. Endocannabinoids are amides, esters, and ethers of long-chain polyunsaturated fatty acids derived from arachidonic acid with short half-lives that likely function as neuromodulators at or near their site of synthesis. The first endocannabinoid isolated and described in 1992 was *N*-arachidonoyl ethanolamine (anandamide; AEA) [23]. The second endocannabinoid, 2-arachidonoylglycerol (2-AG) [24, 25], was identified soon after, and a third endocannabinoid, 2-arachidonyl glyceryl ether, was reported almost a decade later [26]. More is known about AEA and 2-AG [27, 28], and studies have shown 2-AG to be the more abundant and bioactive endocannabinoid in the brain [29, 30]. These endocannabinoids are the primary endogenous agonists of the CB1 and CB2 receptors [31, 32], and their behavioral and physiological effects are similar to those of Δ9-THC [33, 34].

Unlike preformed and packaged neurotransmitters, endocannabinoids are synthesized in postsynaptic neurons "on demand" from distinct phospholipid precursors in response to specific stimuli. Biosynthesis of endocannabinoids from arachidonic acid is stimulated by increased intracellular calcium concentration resulting from either neuronal depolarization or activation of G_q-coupled metabotropic receptors [35]. While the endocannabinoids have similar chemical structures, their biosynthesis occurs through distinct pathways. Biosynthesis of AEA involves two enzymatic reactions; the first reaction is mediated by *N*-acetyltransferase (NAT) generating an intermediate *N*-arachidonoyl phosphatidylethanolamine (NAPE), followed by cleavage of membrane-bound NAPE by phospholipase D (PLD) to form AEA [25, 36] (Fig. 1). 2-AG synthesis is produced by the cleavage of inositol-1,2-diacylglycerol, by phospholipase C (PLC) to form 1,2-diacylglycerol (DAG) or 2-arachidonoyl-lysophospholipid, followed by hydrolysis by DAG lipase or lysophospholipase, respectively (Fig. 1). Once released, endocannabinoids undergo quick deactivation through cellular uptake through a selective membrane transporter followed by enzymatic degradation mediated by fatty acid amide hydrolase (FAAH) for AEA and by both FAAH and a serine hydrolase, monoacylglycerol lipase (MAGL) [27, 37–40] (Fig. 1).

2.3 Cannabinoid Receptor Signaling

Cloning of the first cannabinoid receptor was completed in 1990 from rat cerebral cortex. Originally named the "neuronal" cannabinoid receptor, it was later renamed CB1 [41]. A few years later, a second receptor, CB2, was cloned and deemed the "peripheral" cannabinoid receptor based on its initial identification in the spleen [42]. While the amino acid sequence of the CB1 receptor is highly conserved across

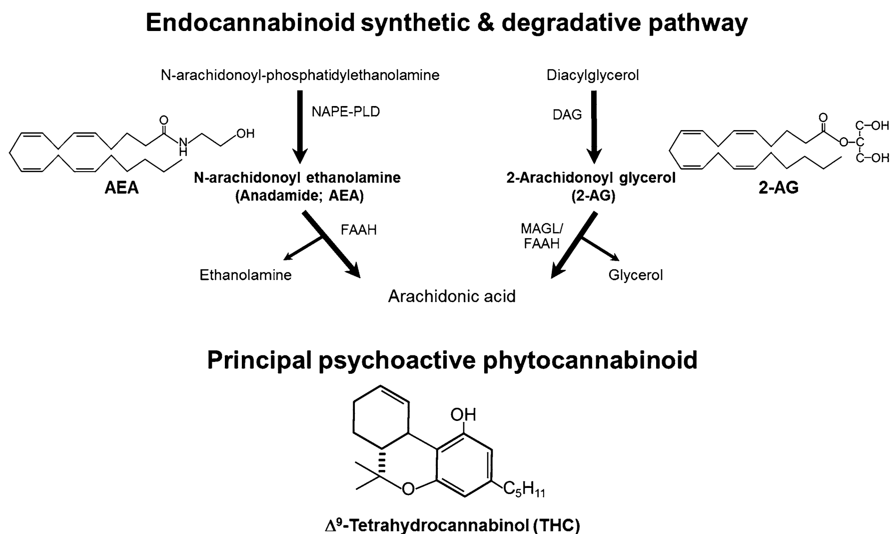
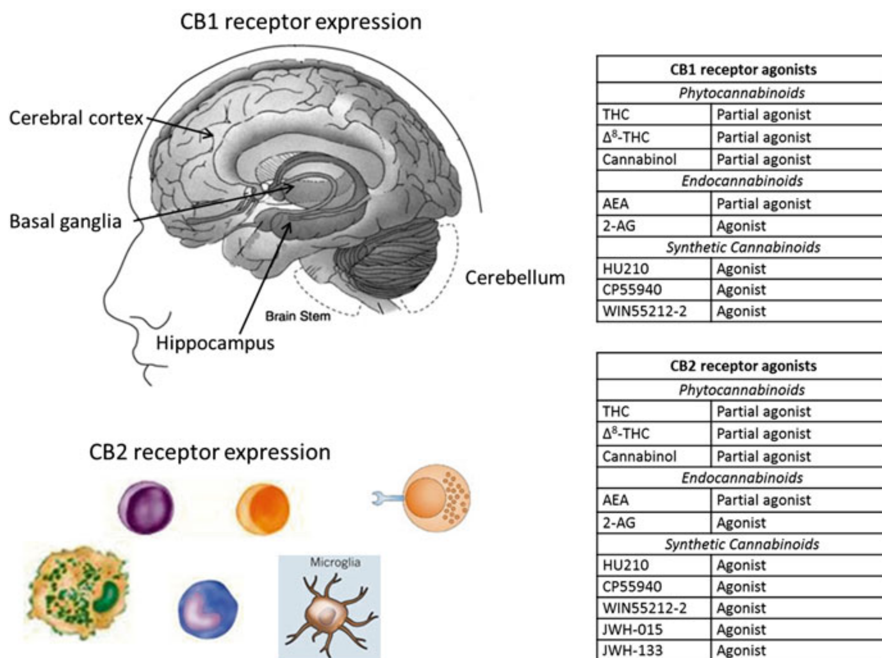


Fig. 1 The endocannabinoids *N*-arachidonoyl ethanolamine (anadamide; AEA) and 2-arachidonoylglycerol (2-AG) are synthesized from phospholipid precursors, *N*-arachidonoyl phosphatidylethanolamine (NAPE) and diacylglycerols (DAGs). Anandamide is formed from the N-arachidoylation of phosphatidylethanolamine by *N*-acyltransferases followed by transformation of NAPE into anandamide by a *N-acyl-phosphatidylethanolamine*-selective phosphodiesterase (NAPE-PLD). 2-AG is produced by the hydrolysis of DAGs via sn-1-selective DAG lipases (DAGLs) alpha and beta. Following cellular reuptake, anandamide is metabolized via fatty acid amide hydrolase (FAAH) and 2-AG via monoacylglycerol lipase (MAGL). CB1 and CB2 receptors are the most studied molecular targets for anandamide and 2-AG. Anandamide has the highest affinity, whereas 2-AG has the highest efficacy. In the brain CB1 receptors are often expressed in presynaptic terminals so that endocannabinoids participate in retrograde signaling, inhibiting neurotransmitter release

mammalian species, the CB2 receptor's amino acid sequence in not conserved. Human CB1 and CB2 receptors share only 48 % amino acid homology [43]. Cannabinoid receptors are $G_{i/o}$-protein-coupled receptors that result in dose-dependent adenylate cyclase inhibition upon ligand binding [44–46]. Cannabinoids can induce activation of members of all three multifunctional MAPK families including extracellular signal-regulated kinase (ERK) [47–49], c-Jun N-terminal kinase (JNK) [50], and p38 [51]. CB1 and CB2 receptor-dependent signal transduction attenuates adenylate cyclase and cAMP production [52], activates mitogen-activated protein kinases (MAPK) and focal adhesion kinase (FAK), and stimulates phosphatidylinositide 3-kinase/protein kinase B (PI3-K/PKB) [53]. In addition, activation of CB1 receptors inhibits different types of calcium (Ca^{2+}) channels, activates certain potassium (K^+) channels, and suppresses neurotransmitter release [54]. This is of particular relevance to endocannabinoid signaling, resulting from binding to presynaptic CB1 cannabinoid receptors leading to inhibition of voltage-sensitive Ca^{2+} channels and activation of K^+ channels [55]. Thus, endocannabinoids act through retrograde signaling to blunt membrane depolarization and exocytosis of neurotransmitters such as glutamate, dopamine, and gamma-aminobutyric acid (GABA).



CB1 receptor agonists	
Phytocannabinoids	
THC	Partial agonist
Δ^8-THC	Partial agonist
Cannabinol	Partial agonist
Endocannabinoids	
AEA	Partial agonist
2-AG	Agonist
Synthetic Cannabinoids	
HU210	Agonist
CP55940	Agonist
WIN55212-2	Agonist

CB2 receptor agonists	
Phytocannabinoids	
THC	Partial agonist
Δ^8-THC	Partial agonist
Cannabinol	Partial agonist
Endocannabinoids	
AEA	Partial agonist
2-AG	Agonist
Synthetic Cannabinoids	
HU210	Agonist
CP55940	Agonist
WIN55212-2	Agonist
JWH-015	Agonist
JWH-133	Agonist

Fig. 2 Distribution of cannabinoid receptor expression relevant to neuroimmunomodulatory effects. The CB1 receptor, the mediator of cannabis-induced neurobehavioral effects, is preferentially expressed in the brain localized to the basal ganglia, hippocampus, cerebellum, cerebral cortex, and brain stem. Localization of CB2 is predominantly in the immune system cells including B lymphocytes, macrophages, monocytes, and natural killer (NK) cells, in addition to CB2 receptors that are expressed in peripheral tissues like the thymus and tonsils, and primarily mediates anti-inflammatory and immune-modulated-dependent actions. Principal ligands for CB1 and CB2 receptors are summarized. Walter L, Stella N. Cannabinoids and neuroinflammation. Br J Pharmacol. 2004; 141: 775–785

The CB1 receptor is preferentially expressed in the brain and has been identified as the mediator of cannabis-induced neurobehavioral effects [56–58]. Radiolabeling studies have demonstrated specific binding sites in the brain, with expression localized to the basal ganglia, hippocampus, cerebellum, cerebral cortex, and brain stem [56, 57, 59] (Fig. 2). CB1 receptors are expressed on neuronal dendritic spines and axon terminals, on mature oligodendrocytes and their progenitors, and on astrocytes [60–62]. Localization of CB2 is predominantly in the immune system cells including B lymphocytes, macrophages, monocytes, and natural killer (NK) cells, in addition to peripheral tissues like the thymus and tonsils, and primarily mediates anti-inflammatory and immune-modulated-dependent actions [42] (Fig. 2). More recent studies have identified expression of the CB2 receptors in specific regions of the brain, spinal cord, and dorsal root ganglia and cells including oligodendrocytes and their progenitors, cerebellar neurons, and microglia. Overall central nervous system (CNS) expression levels of CB2 are lower than that of CB1 receptors [52, 61, 63]. Additional evidence suggests the existence of an additional cannabinoid receptor, but to date the lack of strong pharmacological and functional data has failed to confirm its identity.

2.4 Physiological Effects of Cannabinoids

Cannabinoid signaling through both the CB1 and CB2 receptors plays a significant role in several physiological and pathophysiological processes including cognitive and immune function [64]. The psychotropic effects of cannabis, commonly known as a feeling of "high," are mediated primarily via the CB1 receptor [65]. Cannabinoids have been shown to inhibit nociception, suppress motor activity, impair cognitive processes and short-term memory, and reduce body temperature [58, 66]. Contrasting reports have shown cannabinoids to induce aggressive behavior, hyperalgesia, increased motor activity, and elevation of body temperature [67–69]. These contrasting reports on cannabinoids in the CNS highlighting both inhibitory and stimulatory actions could be due to dose-dependent effects, with low CNS concentrations inducing stimulatory and higher concentrations inducing the conventional inhibitory effects [70, 71].

Cannabinoids, including Δ9-THC, have been shown to have significant immunomodulatory effects on cytokine production and lymphocyte phenotype, function and survival, as well as cell-mediated immunity [72–76]. In addition, cannabinoids have been reported to render protection from injury resulting from release of toxic mediators by infected cells [77–80]. For example, pulmonary alveolar macrophages of moderate cannabis smokers show suppressed antimicrobial activity, cytokine production, and cytokine responsiveness [81, 82]. Similar immunosuppressant effects on lymphocyte and alveolar macrophage function have been reported in nonhuman primates [83]. Early studies using mice and rats or rat and human immune cell cultures treated with Δ9-THC demonstrated suppression of cytotoxic T cell killing and phagocytosis and killing by macrophages [84–86]. Furthermore, the potential of cannabinoids to regulate the activation and balance of human Th1/Th2 cells by a CB2 receptor-dependent pathway has been supported by findings from several studies [87, 88]. Thus, CB2 receptor agonists have been proposed as effective therapeutic agents in chronic inflammatory diseases, such as inflammatory bowel disease, multiple sclerosis, HIV-1 infections, stroke, and Alzheimer's disease (AD), to name a few [64]. Not all clinical studies have demonstrated efficacy, which could be partly due to receptor specificity in the current compounds [89].

2.5 Cannabinoid Modulation of Neuroinflammation: Importance of Microglia

Neuroinflammation is a complex physiological process which involves removal of the injurious stimuli and culminates in repair of damaged tissue. Neuroinflammatory responses primarily involve neurons and glia (microglia, astrocytes, and oligodendrocytes). Glial cells play an active role supporting neurons and can also respond to stress and insults by inducing inflammatory processes to protect the brain. Specifically, following an injury and or insult, microglia, the resident macrophages in the CNS, actively migrate and proliferate, phagocytose and process antigens, and

upon activation synthesize and secrete proinflammatory cytokines including interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α). Anti-inflammatory processes are activated in an effort to maintain homeostasis and counteract any excessive inflammation. When disrupted, this balance in glial activation results in severe chronic neuroinflammation, which if left uncontrolled promotes and propagates neurodegeneration. Evidence strongly suggests that activation of microglia and astrocytes and the accompanying increased expression of proinflammatory cytokines and chemokines often are associated with disease-, trauma-, and toxicant-induced damage to the CNS. While there are multiple risk factors for the development of neurodegenerative diseases, including genetic vulnerability, age, and the presence of chronic disease states (hypertension, diabetes, metabolic syndrome, obesity), several lines of evidence suggest that neuroinflammation is an underlying common mechanism during the initial stages of disease as well as throughout disease progression in several conditions including traumatic brain injury [90], chronic pain [91, 92], HIV encephalitis [93], multiple sclerosis (MS) [94] and neurodegenerative diseases such as AD [95], and amyotrophic lateral sclerosis (ALS) [96]. Increasingly, reports in the literature strongly support an anti-inflammatory role of the cannabinoid system, with potential of modulation of progression of these inflammatory diseases [62, 97–100].

The importance of microglia as a central player in progression from neuroinflammation to neurodegeneration has received considerable attention [101]. Microglia primed by prior insults or genetically predisposed to vigorously respond to subsequent inflammatory insults lead to neuronal damage and dysfunction underlying several disease states. Cannabinoid receptors are highly expressed in microglial cells [102], and several lines of evidence indicate that a significant number of their neuroprotective effects are mediated through CB receptors on microglial cells. Several mechanisms are involved in cannabinoid modulation of microglial function including decreased cytokine production, chemotaxis, oxidative stress, and excitotoxicity [103, 104]. Hence, disease states characterized by chronic neuroinflammation progressing to neurodegeneration have been studied in preclinical and clinical settings to determine the potential role of cannabinoids in exerting protection or amelioration of disease progression. Salient findings supporting a role for cannabinoid modulation of disease are summarized in the following section.

2.6 Cannabinoid Immunomodulation in Disease Processes

2.6.1 Traumatic Brain Injury

Brain damage following traumatic brain injury (TBI) results from mechanical disruption of the brain tissue followed by an acute inflammatory response, breakdown of the blood–brain barrier (BBB), edema formation and swelling, infiltration of peripheral blood cells, and microglial activation of resident immunocompetent cells. This process leads to intrathecal release of numerous immune mediators such

as cytokines and chemokines. The neuroinflammatory cascade characterized by activation of astrocytes and microglia and an increased production of immune mediators, together with the excitotoxic and oxidative responses, are the principal underlying mechanisms of cell injury [105]. Although the early inflammatory response plays an important role in tissue repair and recovery [106], its sustained duration contributes both to the acute pathologic processes following TBI including cerebral edema and the longer-term neuronal damage and cognitive impairment [107]. Thus, timely modulation of neuroinflammation without interfering with the reparative contribution of activated glia is likely to improve outcomes. Reports in the literature indicate that CB agonists decrease glutamatergic toxicity, oxidative stress, inflammation, and brain edema [108]. Furthermore, the synthetic, nonpsychotropic cannabinoid HU-211 (dexanabinol) has been reported to effectively improve motor function recovery, reduce BBB breakdown, and attenuate cerebral edema in a model of closed head injury in rodents [109]. These preclinical findings have been confirmed in phase II clinical trials in severe closed head injury [110] but have not been confirmed in phase III clinical trials [111]. CB neuroprotective effects have also been reported in other injury models including optic nerve crush and global and focal ischemia [112]. Attenuation of proinflammatory responses has been identified as a potential mechanism responsible for improved outcomes [113]. The administration of the endocannabinoid 2-AG has also been shown to reduce brain edema and hippocampal cell death and to improve clinical recovery from closed head injury [114]. However, improvements are largely lost after the first 24 h following injury, and better outcomes were observed when the availability of endocannabinoids was enhanced by coadministration of glycerol esters, which are thought to inhibit their uptake and hydrolysis. Thus, endocannabinoid's short half-life limits their utility as a therapeutic intervention, but approaches that delay their degradation may be more effective in providing neuroprotection [115].

The BBB has emerged as an important site of cannabinoid immunomodulation. In vitro studies have demonstrated that synthetic cannabinoids downregulate vascular cell adhesion molecule-1 (VCAM-1) in brain endothelial cell cultures [116]. CBD, in particular, has received much attention recently due to its distinct actions and potential for anti-inflammatory effects. The ability of CBD to function as an inverse agonist at both CB1 and CB2 receptors and in addition to inhibit FAAH, the major enzyme for endocannabinoid breakdown, makes it an attractive therapeutic agent. CBD exerts immunosuppressive actions on macrophages and microglial cells and attenuates oxidative and nitrosative stress and has been shown to attenuate transendothelial migration of monocytes and barrier disruption [117].

2.6.2 Alzheimer's Disease

Alzheimer's disease (AD), the most common neurodegenerative disease, involves neuronal cell death, progressive cognitive decline, and senile dementia. The series of events and the underlying mechanisms remain poorly understood. The neuropathology of AD includes formation of extracellular *β-amyloid (A*β) plaques on neurons and intracellular neurofibrillary tangles (NFTs). The combined formation of

plaques and NFTs is accompanied by select neuronal and synaptic loss, neuronal death, and gliosis in the cortex and limbic system [118–120]. Neuroinflammation is present early in the disease and progresses to cognitive impairment with accelerated formation of amyloid plaques and NFTs. AD is characterized by extracellular accumulation of the Aβ as amyloid deposits preferentially in the hippocampus. Aß deposits promote inflammation, caspase activation, and oxidative stress [121] and are often surrounded by activated microglia and astrocytes increasing local inflammation [122]. Preclinical studies show that subchronic administration of cannabinoids attenuates proinflammatory cytokine gene expression in β-amyloid-injected mice and modulates microglial cell function, suggesting protective effects from AD pathology. Similarly, central cannabinoid agonist (WIN55212-2) administration has been shown to effectively prevent Aβ-induced microglial activation, cognitive impairment, and apoptosis [123]. More recent studies have provided evidence that cannabinoids inhibit the rise in microglial intracellular calcium resulting from high ATP concentrations such as those resulting from cell death and moreover and decrease the lipopolysaccharide (LPS)-mediated nitric oxide release [124]. In vitro studies also provide support for the role of microglia in the immunoprotective effects produced by cannabinoid agonists, involving inhibition of Aβ-induced activation and reduced neurotoxicity [125]. This is further supported by studies demonstrating CB2-mediated removal of Aß deposits in human brain sections through microglial-mediated mechanisms. Moreover, those studies showed upregulation of CB2 receptor expression in microglial cells located in the vicinity of plaques. Similar cannabinoid receptor upregulation has been reported in brains of patients with HIV encephalitis as discussed below. The proposed mechanism for cannabinoid-mediated amelioration of AD-like symptoms in preclinical models involves suppressed localized inflammation and enhanced microglial-mediated Aß phagocytosis [126]. An additional target for cannabinoid-mediated neuroimmunomodulation recently identified is the peroxisome proliferator-activated receptors (PPAR). PPAR agonists have been shown to improve cell survival, attenuate inflammatory responses, reduce amyloid plaque burden, and reverse behavioral impairment associated with AD [127–129]. Results from a rodent model of AD indicate that the neuroprotective effects attributed to synthetic cannabinoid agonists involve both CB1 and CB2 receptors and activation of PPAR-γ [130].

2.6.3 HIV Encephalitis

The approval of medicinal cannabis and the synthetic cannabinoid dronabinol for the treatment of anorexia in HIV/AIDS patients has raised the possibility of cannabinoid modulation of HIV disease progression. The rigorous examination of the impact of chronic cannabinoid administration on the course and progression of HIV infection is virtually impossible. Few clinical studies have attempted to obtain insight into this comorbid condition, and observations have been limited to the effects of short-term THC administration (21 days) [131]. While short-term use of cannabinoids, either oral or smoked, does not substantially elevate viral load in HIV-infected individuals receiving stable antiretroviral regimens, no significant

advantageous effects have been reported in the clinical literature. However, more recent investigations have provided significant evidence of cannabinoid modulation of multiple mechanisms and processes underlying HIV disease progression, including viral replication [132, 133]. In addition, studies have shown an increase in CB1 and CB2 receptor expression in brains from HIV encephalitis (HIVE) patients [134]. Similar findings have been reported in brains of simian immunodeficiency virus (SIV)-infected macaques. Specifically, expression of CB2 receptors has been identified mainly in perivascular macrophages, microglial nodules, and T lymphocytes of SIV-infected macaques [135] and on monocytes/macrophages in perivascular cuffs of postmortem HIV-1 encephalitic cases [133].

Controlled studies conducted in chronically THC-treated SIV-infected macaques have shown attenuated tissue inflammation and viral load and significant reduction of early morbidity and mortality [132, 136]. The close correlation between tissue inflammation and viral replication strongly suggests that attenuated inflammation is a relevant mechanism for cannabinoid modulation of SIV/HIV disease progression [137]. The potential role for cannabinoids in modulation of HIVE is complimented by reports from in vitro studies showing cannabinoid agonists inhibit HIV-1 Gp120-induced calcium influx and attenuate disruption of human brain microvascular endothelial cell tight junction proteins while also inhibiting the transmigration of human monocytes across the BBB and restoring barrier integrity following a challenge with HIV-1 Gp120 [138]. Additional reports have provided supporting evidence for CB2-mediated attenuation of macrophage migration to the HIV-1 protein Tat [139, 140]. Moreover, studies have shown that CB agonists and FAAH inhibitors, leading to higher levels of endocannabinoids, prevent the downregulation of tight junction proteins zonula occludens-1 and claudin-5 as well as inhibit HIV Gp120-mediated damage of brain endothelium [138]. Thus, suppression of inflammation and protection of BBB integrity have been proposed as relevant mechanisms for cannabinoid-mediated modulation of HIV disease progression.

In addition to suppression of inflammation and protection of BBB integrity, alternative mechanisms may contribute to cannabinoid modulation of HIV disease progression. Reports in the literature suggest that cannabinoids may regulate chemokine receptor signaling, specifically the activity of CXCR4, one of the co-receptors for viral entry. This has led to the prediction that CB2 receptor signal transduction can interact with that of CXCR4, leading to alterations including receptor desensitization, allosteric modulation, and dimerization. Support for this possibility comes from studies demonstrating that CB2 agonists decrease CXCR4-activation-mediated G-protein activity and MAPK phosphorylation, which is associated with suppression of viral replication [141].

2.6.4 Multiple Sclerosis

Multiple sclerosis (MS), a chronic inflammatory autoimmune disease, is characterized by degeneration of the myelin sheath (demyelination) covering axons leading to sensory disturbances, muscle weakness, uncontrollable muscle spasms, ataxia,

cognitive deficits, bladder dysfunction, fatigue, and problems with speech (dysarthria), swallowing (dysphagia), and sight [142]. Autoreactive T cells have been implicated in the initial process of myelin degradation and inflammation, leading to activated glial and astrocytes which produce proinflammatory cytokines and antibodies. Preclinical studies using rodent models of MS have shown that cannabinoid treatment ameliorates or reduces inflammation, improves motor function, delays clinical signs, and improves survival [143–146]. This clinical improvement and enhanced survival was shown to be associated with marked reduction of neuroinflammation in Δ9-THC-treated animals. Additional studies in a similar preclinical model have shown a significant reduction in neurodegeneration and the associated increased glutamate in the cerebral spinal fluid [147].

Encouraging findings from clinical studies have provided support for cannabinoid-mediated amelioration of disease. A clinical trial using cannabis extracts as a treatment for MS proved to be effective in reducing spasticity in a small cohort of patients [148]. Subsequent studies using blinded, randomized, placebo controls have not provided consistent results. One study using 16 MS patients with severe spasticity demonstrated no differences in spasticity after 4 weeks of treatment between placebo- and Δ9-THC-treated patients [149], while another study demonstrated improvements in pain, bladder dysfunction, and spasticity in some patients receiving Δ9-THC [150]. Among the factors that could contribute to the inconsistency of the results are doses of cannabinoids used (Δ9-THC vs. cannabidiol), route of administration (oral vs. smoked), and heterogeneity of the disease state at which the trial was conducted. More recently in the MUSEC (multiple sclerosis and extract of cannabis) trial, oral cannabinoid treatment for 12 weeks confirmed the positive finding in the CAMS (cannabinoids in multiple sclerosis) trial with a significant reduction in an 11-point spasticity scale in cannabinoid-treated patients [151–153]. While these are encouraging findings, long-term studies are warranted to establish whether cannabinoids will impact long-term disease management.

2.6.5 Amyotrophic Lateral Sclerosis

ALS, the most common form of adult motor neuron disease, is a chronic neuromuscular disease with progressive degeneration of both upper and lower motor neurons, with accompanying muscle weakness, wasting, and spasticity leading to complete paralysis and ultimately death from respiratory failure [154]. The majority of all ALS cases are sporadic (SALS) with a small percentage, 5–10 %, occurring as inherited or familial (FALS) forms of the disease. Evidence suggests the proposed mechanisms driving motor neuron cell death in the brain and spinal cord involve increased oxidative stress due to free radical toxicity and/or excessive glutamate activity and chronic inflammation [155, 156]. Mutations in several genes have been implicated in FALS with about 20 % involving the cytosolic copper-zinc superoxide dismutase (SOD1) gene. Accumulating evidence supports a role for the endocannabinoid system in pathophysiology and disease progression in rodent models of ALS. Chronic CB receptor agonist administration as well as genetic deletion of FAAH, leading to

increased levels of AEA, results in significant delay of disease progression [157] and prolonged survival [158, 159]. Relative increase in the spinal cord CB2 expression and receptor binding and function has been reported in rodent models of ALS [159]. This observation has been confirmed in postmortem spinal cords of ALS patients [160]. Finally, ALS patients self-report that cannabis improved symptoms associated with the disease, including alleviating pain and muscle spasticity, increasing appetite, and reducing depression [161]. Taken together, these findings provide support for a role for cannabinoid and particularly CB2 receptor mechanisms as a potential target for pharmacological therapy in patients with ALS.

2.6.6 Chronic Pain

While acute pain plays an adaptive role in human physiology, persistent or unresolved pain serves little purpose and imparts a devastating burden on individual lives and society as a whole. The Institute of Medicine estimates that pain-related medical care and compromised economic productivity costs the United States around one trillion dollars a year [162]. Chronic pain conditions are expected to increase with the aging population and given medical advances in reducing mortality after various traumas. Cannabis has been used for centuries to alleviate various inflammatory disease states, including pain [163]. Recent findings have postulated a role for cannabinoids in homeostatic pain modulation within the immune system [164] and along the neuraxis [165, 166] where they may act in a synergistic fashion with endogenous opioid systems [167, 168]. The homeostatic quality of cannabinoid analgesia refers to a particular potency in the context of hyperalgesic and inflammatory conditions [169], although cannabinoids may also play a critical role in placebo effects [170]. Various cannabinoid sources and formulations have been proven useful in the management of different types of pain, including oral THC/cannabidiol (Sativex) for arthritis [171] and smoked marijuana for HIV-associated sensory neuropathy [172]. A systematic review of randomized, controlled trials examining the use of cannabinoids for the treatment of chronic noncancer pain revealed that cannabinoids are safe and effective in a variety of pain conditions [173].

Nevertheless, the psychoactive properties of cannabinoids appear to represent a substantial obstacle to their beneficial use in chronic pain states, and this concern has driven the search for peripherally selective cannabinoid ligands [174–176]. According to their almost complete restriction to spinal neurons and immune cells, the CB2 receptor appears to represent the most viable receptor target for analgesic intervention, and CB2R activation is indeed antinociceptive in preclinical models of both mechanical and thermal hyperalgesia in the absence of cognitive disruption [177–182]. In addition, selective inhibition of either FAAH [115, 183] or MAGL [184] also appears to produce robust antinociception without untoward psychomimetic effects. Unfortunately, Schlosburg and colleagues found that chronic MAGL inhibition led to analgesic tolerance and even physical dependence [185]. However, the same study revealed that analgesic efficacy was preserved following chronic FAAH inhibition, pointing to FAAH as a more suitable target for analgesia without

an elevated risk of dependence. Interestingly, a recent report suggests that the ability of THC to reduce the unpleasantness of pain is regulated by the amygdala [186], suggesting the importance of cannabinoid signaling within central brain regions in mediating the emotional dimension of pain, which may play a substantial role in chronic pain conditions [187, 188].

3 Perspectives

Cannabinoid effects, whether resulting from phytocannabinoids, endocannabinoids, or synthetic analogs, are now known to encompass significant organ system physiological modulation that extends beyond the initially described psychoactive responses. The identification of an endogenous cannabinoid system and the improved understanding of the mechanisms regulating the availability of these ligands have provided an additional approach for capitalizing on their therapeutic value. Recognition of systemic cannabinoid receptor expression and their tissue-specific distribution has unveiled the possibility of therapeutically targeting their function to modulate disease progression, particularly in conditions associated with chronic inflammation. Continued progress in understanding of cannabinoid pharmacology and pharmacokinetics is likely to lead to additional medical conditions that can benefit from targeted cannabinoid system modulation.

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Alcohol and Neurodegeneration

Yuri Persidsky, Larisa Gofman, and Raghava Potula

Abstract Strong association between alcohol abuse and an increase in both systemic and brain levels is evident from both humans and animal models. This chapter comprehensively reviews the interplay between progression of neuroinflammation and neurological disorders initiated by alcohol abuse. Recent observations of excitotoxicity associated with excessive neurotransmitter release, oxidative stress leading to free radical damage, and cell death through an enhanced inflammatory response provide important clues to the mechanisms that could mediate alcohol's toxic effects on brain cells. Chronic alcoholics have the temporal hallmark of neurocognitive deficits, neuronal injury, and neurodegeneration. Studies suggest that the initiation and progression of alcohol-mediated neurodegeneration is driven in part by release of pro-inflammatory factors from activated microglia, oxidative stress, impairment of blood–brain barrier (BBB), and glutamate-associated neurotoxicity. Recent observation of strong associations between cannabinoid systems within the central nervous system in regulating neuroinflammation via the cannabinoid receptor 2 highlights the importance of this pathway in alcohol-driven neuroinflammation. Regulatory mechanisms that regulate alcohol-induced neuroinflammation, oxidative neuronal injury, and altered BBB are examined, as well as modalities to ameliorate these processes are discussed.

Keywords Alcohol • Neurodegeneration • Neuroinflammation • BBB • Neuron • Glia

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Alcohol abuse continues to be a major morbidity factor, causing approximately 1.3 million deaths globally (3.2 % of all deaths) each year and accounts for approximately 4 % of the disease burden of all diseases [1]. While the effects of alcohol exposure on liver function and end-organ injury are well accepted, the significant association between neurodegeneration and alcohol exposure is less established. New intriguing data recently were acquired to suggest a relationship between chronic inflammatory responses as underlying causes for alcohol-associated neurodegeneration as well as elements of alcohol addiction [2]. Since alcohol dependence and abuse are important health problems, therapeutic strategies to overcome this addiction are urgently required to reduce the burden of such conditions on society.

Newer neuroimaging techniques have shown significant alteration of brain structure, including atrophy of subcortical and cortical areas, thalamus, corpus callosum, and cerebellum [3]. Substantial progress has been made in brain imaging in chronic alcoholics, indicating global reduction in gray matter and white matter and an increase in cerebral spinal fluid volume and diffusional abnormalities among alcoholics and heavy to moderate drinkers [4]. Chronic alcohol consumption has been shown to be related to shrinkage of different parts of the brain and impairment of the decision-making process. When compared to normal controls, two predictors (gray matter changes and decision-making measure) were significantly altered in alcoholics. Recent studies address the issue whether amelioration takes place during 2 weeks of abstinence from alcohol [5]. This study indicated gray and white matter recovery after few days of abstinence, but it varied between different brain regions. These findings offer a unique insight into potential therapeutic interventions, promoting structural changes in the CNS of alcoholics. The changes were attributed to a recovery of myelin in the corpus callosum [6].

According to Zahr et al. [3], brain tissue loss consists of two components, transient and permanent. It has been suggested that in tissue shrinkage secondary to neuronal loss, there is no complete brain tissue recovery. Magnetic resonance spectroscopy revealed that despite prolonged abstinence, individuals that chronically consume alcohol demonstrate persistent diminution of *N*-acetylaspartate (a neuronal marker) in the frontal lobe, thalamus, and cerebellum [7]. Other studies demonstrated improvement in the level of *N*-acetylaspartate and choline (a metabolite associated with re-myelination) during abstinence [8].

Corresponding neuropathology studies indicated a loss and destruction of white matter in the same brain regions. Structural changes in the brain and the associated functional consequences that occur with chronic alcohol exposure can be grouped into "uncomplicated alcohol-related brain damage." [3] Neuropsychological manifestations of chronic uncomplicated alcohol exposure are characterized by the heterogeneity of severity and type of deficits. It has been shown that the pericerebral space with respect to intracranial cavity changed from 8.3 % of total intracranial volume in healthy controls to 11.3 % in patients with chronic alcohol exposure [9]. Previous stereologic studies indicated that this reduction occurred mainly due to decreased white matter volume. The morphologic substrate of white matter loss is currently unknown; however, this phenomenon is probably associated with a loss of myelin and axonal integrity. Common alcohol-associated CNS lesions encompass

white matter loss (leukoencephalopathy), enlarged ventricles, cerebellar degeneration, and neuronal demise in the superior frontal association cortex, anterior cingulate area, hippocampus, entorhinal cortex, and hypothalamus, which contribute to cognitive and motor deficits [10, 11].

In patients with uncomplicated alcoholism, neuropathology studies reveal up to 25 % loss of pyramidal neurons in the superior frontal cortex [3]. Much is unknown about neuronal loss in the primary motor cortex in uncomplicated alcoholism; however, silver impregnation techniques showed that neurons in the superior frontal and motor cortex featured dendritic arbor shrinkage, indicating a compromise of interneuronal communications. No changes were detected in the number of neurons in the basal ganglia, hippocampus, or serotonergic raphe nuclei in uncomplicated alcoholism.

In recent years, genomic and proteomic analysis of samples of human frontal cortex identified several groups of alcohol-associated genes encoding myelination, synaptic structure, mitochondria, signal transduction and intracellular metabolism, protein trafficking, and transcriptional regulation [12–14]. The data acquired in these studies point to the involvement of multiple pathways in the effects of alcohol on the CNS. Changes in expression of proteolipid protein and myelin basic protein (participating in stabilization of the myelin sheath) could provide additional insights into white matter changes in chronic alcoholics. It has been suggested that chronic liver injury occurring in chronic alcoholism and its associated hepatitis results in production of toxic substances (such as ceramides, ammonia) and enhanced insulin resistance, promoting neurodegeneration [15].

A number of molecular mechanisms have been proposed for ethanol-associated brain injury. These encompass alcohol-specific effects, including toxic metabolites (production of acetaldehyde and fatty acid ethyl esters), defects in mitochondrial function, generation of reactive oxygen species, decrease in brain-derived neurotrophic factors, and effects on excessive glutamate on synaptic transmission (resulting in excitotoxicity) [3]. Increased gut permeability for bacterial byproducts, such as LPS (so-called bacterial translocation), and liver dysfunction can be additional factors leading to brain injury in chronic alcoholism [16]. The latter is of significant interest, providing links between alcohol exposure and development of systemic inflammatory responses.

A significant body of evidence indicates that there is a close association between alcohol abuse and an increase in both systemic and brain levels of inflammation. The levels of cytokines in systemic circulation were increased in alcoholic subjects [17–19]. It has been shown that chronic alcohol consumption in humans is associated with increases in serum pro-inflammatory cytokines [20, 21]; monocytes isolated from the blood of alcoholics produce greater amounts of tumor necrosis factor α (TNFα) spontaneously and in response to endotoxin [22]. In experimental animals (rats), several months of alcohol administration increased the number of inflammatory factors [interleukin (IL-1β), inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX-2)] [23]. Recent publications from the group of Dr. Crews suggested significant upregulation of pro-inflammatory genes in the brains of alcohol-exposed animals with secondary inflammatory stimulus (such as LPS administration) [19].

They found upregulation of the β-chemokine, CCL2, a key innate immune factor, in multiple regions of postmortem alcoholic brains as compared to age-matched controls. These changes are accompanied by profound microglial activation in different brain regions [16, 17]. An additional indication of inflammatory response was the upregulated expression of cannabinoid receptor 2 in the brain endothelium, changes seen in human brain tissues affected by encephalitis [16]. Chronic alcohol administration to mice caused a sustained increase in brain CCL2 and an increase in microglial activation paralleling human studies.

Studies indicating that genes involved in the immune stress response exhibited differential expression in the frontal cortex of human alcoholics and normal controls. It has been shown that expression of the same genes is also related to genetic predisposition of alcohol consumption in mouse strains, pointing to a role for pro-inflammatory mediators in the regulation of alcohol intake [24]. Gene expression data sets led to the selection of six genes related to inflammation, and behavioral testing of gene-deficient mouse strains showed that animals defective in such genes drink less alcohol. Conversely, injection of LPS prior to alcohol testing resulted in a sustained increase in alcohol consumption. It has been shown that one of the key inflammatory pathways, toll-like receptor (TLR) 4, is critical in enhanced neuroinflammation associated with alcohol exposure. Several labs found that alcohol can activate TLR4 receptors in the brain in different types of glial cells [25]. Furthermore, alcohol-exposed animals lacking these receptors showed evidence of protection from astrocyte and microglia activation, increased expression of pro-inflammatory factors, and neuronal injury. These animals did not show long-lasting memory impairment or anxiety-like behavior. These studies collectively point to the role of neuroinflammation, not only in neurodegeneration associated with alcohol exposure, but also to promotion of addictive behavior. Development of therapeutics diminishing inflammation, therefore, may protect the CNS from alcohol injury and mitigate addiction.

1 How Microglia Are Affected by Alcohol and Implications for Neurodegeneration

Maintaining the integrity of neurons and neuronal circuits in the CNS is imperative for proper signaling and communication. Neurodegeneration is characterized by the loss of structure or function of brain cells and comprises assembly of pathophysiological events that include cellular damage, disease development, and cellular death [26]. Glial cells, including astrocytes, microglia, and oligodendrocytes, are important to neuronal function as both supporting cells for proper neural circuit transmission and an alert mechanism during injury or infection. Over the years, glial cells have been implicated in modulating and influencing neuronal health. Microglia, the immunocompetent cells of the CNS, respond to any homeostatic modification and play a pivotal role in regulation of neuroinflammatory processes, including neurodegenerative disease [27]. Although they are historically

considered to be quiescent in the healthy brain and active only during brain injury or disease, recent literature has demonstrated microglia as a dynamic entity undergoing morphologic changes to maintain healthy brain function. As the primary effector cells of the brain, microglia are indispensable for maintenance and clearance of foreign material and debris [28]. Microglia activation is a highly regulated process and is important in all aspects including proliferation, cell cycle, migration, and apoptosis during the response to inflammation, ischemia, trauma, injury, and neurodegenerative diseases [28]. Importantly, microglia can cause both neuroprotection and neurodegeneration in the CNS. Most of the known functions of microglia, including neurotoxic and neuroprotective properties, are attributed to morphologically activated microglia [29].

Microglial cell dysfunction plays an important role in pathogenesis of many neurodegenerative and neuroinflammatory diseases [30]. Constant alterations in the CNS lead to dynamic changes in microglial activation, contributing to inflammation and consequently leading to neurodegenerative outcomes. Prolonged microglial cell activation due to aberrant signaling alters the function of microglia in neurocognitive disorders, including Alzheimer's disease and Parkinson's disease [31]. Alcohol abuse and alcoholism represent substantial problems that affect a large portion of the general public [32]. Alcohol is a common dietary constituent that modulates the immune system [17]. It has been reported that the number of risk factors associated with chronic alcohol abuse has significantly increased, especially in disorders of the CNS [33]. Recent studies have enumerated the deleterious effects of alcohol on the brain, including the immune cells found within the CNS [32, 34–37]. Alcohol attenuates phagocytosis [34, 38, 39], proliferation [35], expression of brain-derived neurotrophic factor (BDNF) in the hippocampus [40], and apoptotic action in microglia [41]. Alcohol consumption has an impact on the structure and function of the brain [3].

In the mature brain, resting microglia exhibit a ramified morphology and are responsible for immune surveillance [42]. The classical phenotype of activated microglia is very important in the clearance of pathogens, as well as the release of soluble factors that act as signaling molecules to combat injury and infection [27]. When microglia are activated due to brain injury or immunological stimuli, they undergo dramatic morphologic alterations, from a ramified cell with a small soma and long processes to an activated amoeboid cell with a large soma and shorter processes. Microglia activation can be differentiated based on morphology, marker expression, and cytokine secretion [43]. Microglia phenotype varies with the type of insult, the extent of damage, and the time of recovery post-injury. Addressing the effects of alcohol on microglia-mediated neurodegeneration is important to understand their role in neuroinflammation. In the healthy brain, microglial cells are highly sensitive to changes in their microenvironment and readily become activated in response to infection or injury. Activated microglia upregulate a variety of surface receptors, including the major histocompatibility complex (MHC-II), CD11b, Iba-1, and 18 kDa mitochondrial translocator protein [43]. Expression of these surface molecules is one component of microglial cell activation associated with release of factors that are important for signaling in the brain during injury, including the release of pro-inflammatory cytokines.

Pro-inflammatory cytokine release is a hallmark of activated microglia and contributes to chronic neuroinflammation [17]. Studies by many groups have shown a significant increase in the release of these factors in alcohol models, both in vitro and in vivo, and excessive quantities of these individual factors produced by activated microglia can be deleterious to neurons [17, 44, 45]. Alcohol-related studies on microglia conclude that these surface receptors are upregulated in alcohol-treated brains as compared to normal non-treated brains [43]. CCL2, a member of the β-chemokine family that signals through G protein-coupled receptor CCR2, is important for neuroinflammation pathways in microglia. Crews et al. has demonstrated that the CCL2 signal is increased in the human alcoholic brain; this can potentially play an important role in driving microglia activation and therefore indirectly lead to excessive production of pro-inflammatory cytokines such as IL-1β and TNFα [17]. Lastly, CCL2 can act as a "priming" stimulus for microglia (lowering their "threshold sensitivity"), enhancing their synthesis of pro-inflammatory cytokines in response to subsequent stimulation [43, 46]. From these data, we can speculate that alcohol can dramatically affect the release of CCL2 in microglia, potentially orchestrate the release of cytokines, and generate a prolonged pro-inflammatory immune response.

While several groups have demonstrated neuroinflammation in microglial cell reaction to blood–brain barrier (BBB) injury [47], Marshall and colleagues [43] suggested that activated microglia produced anti-inflammatory cytokines (such as IL-10 and TGFβ) and were not fully activated in the binge drinking model (7 days of alcohol exposure). They were unable to demonstrate increased permeability of the BBB in their in vivo model. These investigators proposed that partial activation of microglia following binge ethanol exposure suggests that microglia have homeostatic roles, rather than directly contributing to neurodegeneration, and are a consequence of alcohol-induced damage, rather than the source of the damage.

Specialized functions such as migration and phagocytosis are the main characterizing features of microglia, and the integrity of neurons in the CNS depends on their proper function. Alcohol alters microglial cell migration and phagocytosis [34, 48]; however, the exact mechanisms remain elusive. Likewise, classically activated microglia show increased phagocytic activity as seen in postmortem alcoholic brains stained for ED-1, classically used to detect phagocytic microglia [49]. The level of activation achieved and cytokines released influences whether microglia exacerbate injury or promote recovery. The effects of alcohol on microglia are poorly understood; however, there are two potential mechanisms by which alcohol may influence microglial neurodegeneration: toll-like receptors (TLRs) and purinergic receptors.

TLRs are a family of pattern-recognition receptors that enable the recognition of conserved structural motifs in a wide array of pathogens. TLRs recognize components released from stressed or damaged host cells including ATP, aggregated β-amyloid, and heat shock proteins [42]. Activation of TLRs triggers the downstream stimulation of nuclear factor-kB (NF-kB) encoding molecules associated with inflammation [50]. Multiple studies have shown that alcohol induces microglial cell activation in vitro by stimulating TLR4 enhancing phagocytosis and leading to neuronal

death, indicating that activation of the TLR4 response by alcohol can be an important mechanism of alcohol-induced neuroinflammation and neurodegeneration [51].

Purinergic receptors (P2R), also known as purinoceptors, play a unique role in integrating neuronal and glial cellular circuits, as virtually every type of glial cell possesses receptors for purines and pyrimidines [52]. These receptors are ubiquitously expressed and mediate a remarkable variety of physiological and pathophysiological reactions [52–56]. Several signaling pathways are coupled to P2R in the CNS, including the MAPK/ERK pathway, NGF expression, and calcium mobilization [40, 53, 57–61]. P2R have been implicated in alcohol abuse disorders and shown to affect signaling in the CNS [62, 63]. Recent literature has shown the involvement of P2R in alcohol's action in microglia [63]. It is now generally accepted that microglia contribute to the neurodegenerative process through the release of a variety of neurotoxic factors that exacerbate the degeneration of neurons. It remains to be determined, however, how alcohol triggers microglial activation and if P2R have a role in regulating microglial activity.

Microglia, the CNS representatives of macrophages, partake in neuroinflammation in response to various intrinsic or extrinsic stimuli. It has been recently suggested that microglial signal transduction is one of the main targets of alcohol action in the brain: alcohol exposure selectively modulates intracellular signal transduction in microglia rather than globally inhibiting signaling pathways in a nonspecific manner. Deregulation of the inflammatory activation signaling of microglia by alcohol may contribute to the derangement of CNS immune and inflammatory responses [36]. Inflammation is a common denominator among the diverse list of neurodegenerative diseases. Previously, inflammation was considered to be a passive response to neuronal damage; however, an increasing number of reports demonstrate that prolonged inflammation in the CNS contributes to neuronal death. The importance of microglia, as inflammatory mediators of neurodegeneration, and their mechanisms require further study.

An ongoing controversy exists regarding whether microglia are neuroprotective or neurotoxic when activated. In their resting state, microglia provide "checks and balances" and safeguard mechanisms in the CNS, ensuring that the brain functions properly. Likewise, if a pathogen has breached the CNS or an injury has occurred, microglia assume a more central role in releasing pro-inflammatory cytokines and chemokines to combat the damage. Alcohol has been shown to play a role in modulating the activation of microglia and affecting their normal function, which may be potentially harmful in neuronal death. The exact mechanisms by which alcohol influences microglial cell activation are currently unknown; however, recent studies have shown evidence to support alcohol's effect on microglia-mediated neurodegeneration. Understanding the balance between neuroprotection and neurodegeneration is important in understanding the diseases of the CNS. Microglia possess a myriad of functions within the CNS, and emergence of their role in health and disease has become of interest in studying neurodegenerative diseases. Rather than classifying microglia as exclusively beneficial or deleterious, it is more likely that microglia function in both roles.

2 Alcohol Effects on the BBB

While a substantial amount of data has been acquired regarding the role of glia in alcohol-driven neurodegeneration, only recently it has become obvious that BBB compromise could be part of this process. Alcohol exposure (25–50 mM) of human brain endothelial cells results in a rapid (20–30 min) decrease of BBB tightness (measured by transendothelial electrical resistance and permeability to tracers of different molecular weights), formation of small gaps in monolayers, and redistribution of tight junction (TJ) proteins [64]. These effects are secondary to oxidative stress due to alcohol metabolism via induction of catalytic activity and expression of alcohol-metabolizing enzymes (CYP2E1 and alcohol dehydrogenase), which parallel enhanced generation of reactive oxygen species (ROS) in BMVEC. These changes lead to Ca^{2+} release (via stimulation of inositol 1,4,5-triphosphate receptor), activation of myosin light chain (MLC) kinase, and phosphorylation of MLC and TJ proteins [64–66]. These effects are reversible after alcohol withdrawal or inhibition of specific intracellular pathways. In addition, BBB compromise enhances migration of mononuclear cells across endothelial monolayers in vitro.

Longer periods of exposure (24–48 h) to alcohol stimulated the activity/expression of matrix metalloproteinases (MMP-1, MMP-2, and MMP-9) and decreased the levels of tissue inhibitors of MMPs (TIMP-1, TIMP-2) via activation of protein tyrosine kinase, modifications of TJ proteins, and disruption of basement membrane integrity [67, 68]. All these effects could be reproduced by exposure to acetaldehyde or donors of oxidative stress, indicating the importance of such effects by products of alcohol metabolism in the effects of alcohol on the BBB. Our more recent work indicated a compromise of antioxidative protective mechanisms in BMVEC exposed to alcohol and suggested protective approaches for the BBB. We studied whether stabilization of antioxidant enzyme activity would prevent ROS generation that results in barrier disruption. We determined the effects of alcohol on the kinetic profile of superoxide dismutase (SOD), catalase activity, and ROS/nitric oxide (NO) generation in primary human brain endothelial cells. Alcohol simultaneously augmented ROS generation and the activity of the antioxidative enzymes, SOD and catalase. SOD activity was increased for a much longer period of time than was catalase activity [69]. A decline in SOD activity and protein levels preceded elevation of oxidant levels. SOD stabilization by the antioxidant and mitochondria-protecting agent, *N*-acetyl-L-carnitine (ALC), and the anti-inflammatory agent, rosiglitazone, suppressed ROS levels. Mitochondrial membrane protein damage and decrease in membrane potential after alcohol exposure indicated mitochondrial injury. These changes were prevented by ALC. Importantly, a rapid increase in permeability can be demonstrated in animal models (mice, rabbits) exposed to pathophysiologically relevant doses of alcohol (1–2 h). In addition, alcohol promotes the pro-inflammatory phenotype in the brain endothelium: upregulation of COX-2, increased generation of prostaglandin E2, and enhanced expression of cannabinoid 2 receptor (unpublished observations).

In addition to structural tightness, alcohol exposure (50 mM) decreased glucose uptake and correlated with the reduction of glucose transporter protein 1 (GLUT1) in

BMVEC [70]. In vivo, chronic alcohol intake inhibited the transport of glucose into the frontal and occipital regions of the brain. These changes paralleled a marked decrease in GLUT1 protein expression in the BBB. In parallel, alcohol intake impaired BBB TJ proteins in the brain microvessels and enhanced permeability (measured by sodium fluorescein and Evans blue accumulation in brain tissue), thus confirming the leakiness of the BBB. The antioxidant, ALC, attenuated these effects of alcohol on glucose uptake and BBB. Such changes occurring on a repeated basis after exposure to alcohol could be one of the underlying mechanisms of neurodegeneration that warrants further investigation as a potential target for therapeutic interventions.

3 Neuronal Injury and Astrocyte Dysfunction Caused by Alcohol Exposure

Alcohol abuse-related neuronal injury and dysfunction are associated with increases in oxidative stress in the brain that coincide with the induction of pro-inflammatory cytokines and oxidative enzymes. We found that the metabolism of alcohol in primary human neurons by alcohol dehydrogenase (ADH) and cytochrome P450 2E1 generated ROS. In addition, alcohol metabolites further augment ROS/NO levels via induction of NADPH/xanthine oxidase (NOX/XOX) and nitric oxide synthase (NOS) in human neurons [71]. A marked increase in lipid peroxidation and a decrease in a neuronal-specific marker paralleled ROS generation. Increase in iNOS protein correlated with an upregulation of 3-nitrotyrosine protein levels in the frontal cortex of alcohol-fed mice [72]. Colocalization of neurofilaments and iNOS protein confirmed that iNOS was mostly expressed in neurons. Of note, neither astrocytes nor microglia exhibited colocalization of iNOS/3-NT in this brain region, further confirming not only that iNOS induction is a major source of peroxynitrite but also that the enzyme is not responsive in astrocytes and microglia. These findings indicate that chronic alcohol ingestion preferentially modulates iNOS protein levels in neurons, but not in astrocytes or microglia, validating our recent findings that alcohol/acetaldehyde exposure increased the level of iNOS protein in cultured primary human neurons [71].

It is accepted that alcohol administered acutely in a pathophysiologically relevant dose can selectively and potently suppress the function of *N*-methyl-D-aspartate (NMDA) receptors [73]. Until now, the exact mechanism or site of action is unknown. Prolonged administration of alcohol leads to an adaptive increase in the sensitivity of NMDA receptors in vivo and in vitro. Such changes potentially can result in an enhanced vulnerability for glutamate-induced cytotoxic response (excitotoxicity) [74]. Animal studies suggest that chronic alcohol exposure and withdrawal are accompanied by a hyper-glutamatergic state, leading to neurotoxicity [75]. Preclinical models have shown that “anti-glutamatergic” compounds can reduce neuronal cell death. Increased sensitivity of neurons to excitotoxic insults is one of the mechanisms underlying alcohol-induced brain damage. NMDA stimulation results in increased calcium influx that is associated with uptake into

mitochondria and causes the production of ROS that interfere with the function of mitochondria and plasma membranes. Direct suppression of the mitochondrial respiratory chain also indirectly induces further NMDA receptor stimulation. If the suppressive effect of alcohol on NMDA receptors is removed during withdrawal, the possibility of neuronal damage is significantly augmented through this receptor system, more so when increased and/or prolonged withdrawal signs after repeated withdrawal [76]. Alcohol-induced brain damage is mediated by glutamate-mediated transmission. Recently, the sulfur-containing amino acid, homocysteine, has been suggested to be neurotoxic in alcoholism [77]. The catabolism of homocysteine to methionine, a key step in detoxifying homocysteine, requires folate as a cofactor. Chronic alcoholics often have a low intake of folate resulting in a sustained hyperhomocysteinemia. Homocysteine is a partial or complete agonist at the glutamate and glycine binding sites within the NMDA receptor complex, respectively. Enhanced levels of homocysteine may lead to a pathological increase in receptor activity and subsequent excitotoxicity. From a clinical perspective, increased levels of plasma homocysteine can be used as a marker to predict alcohol withdrawal symptoms, so that therapeutic intervention can be initiated [78]. To date, little is known regarding gender differences in alcohol-mediated neuroinflammation and neurodegeneration. Recently acquired data suggest that there is more pronounced glial reaction (reflective of inflammation) and neuronal injury in female versus male mice in a binge model of alcohol administration. Alfonso-Loeches et al. [79] showed that chronic alcohol treatment induces inflammatory mediators (iNOS and COX-2), cytokines (IL-1β, TNFα), gliosis (GFAP), caspase-3 activation, and greater neuronal loss in the cerebral cortex of female mice when compared to male animals.

Astrocytes are altered by alcohol exposure in vitro and in vivo reflecting putative direct and indirect effects. Astrocytes play a significant role in supporting the function of neurons and brain endothelium. It has been reported that glial fibrillary acidic protein, (GFAP), a marker for reactive astrocytes, and vimentin (detecting hyperactive astrocytes) are substantially increased in animals subjected to chronic alcohol administration [80]. These changes appeared to be related to neuronal cell death in the same areas. Mechanisms underlying the effect of alcohol on astrocytes remain the subject of debate; however, several groups reported complimentary data pointing to TLR4 activation as one possible pathway [81]. Blanco and colleagues demonstrated that astrocyte activation with IL-1β or alcohol (10 and 50 mM) resulted in the translocation of IL-1 receptor, IL-1R, and/or TLR4 into lipid raft–caveolae-enriched fractions and the recruitment of signaling molecules (phospho-IL-1R-associated kinase and phospho-extracellular-regulated kinase) into these microdomains. Using cellular imaging techniques, they demonstrated that IL-1R was internalized by caveolar endocytosis via enlarged caveosomes after IL-1β or alcohol treatment, which sorted their IL-1R cargo into the endoplasmic reticulum–Golgi compartment and into the nucleus.

Using primary human astrocytes, we showed that activation of cytosolic phospholipase A2 (cPLA2) and cyclooxygenase (COX-2) by alcohol in astrocytes enhanced the secretion of inflammatory agents via the interactive tyrosine phosphorylation of TLR4 and Src kinase [82]. Alcohol exposure (20 mM for 48 h)

increased the activity of cytochrome P450 2E1, ROS levels, and secretion of prostaglandin E2 (PGE2). PGE2 generation was dependent on induction of cPLA2 activity/protein as well as COX-2 protein level. Src phosphorylation was necessary for these effects of alcohol. The interactive tyrosine phosphorylation of TLR4–Src complex at the cell membrane triggered the activation of cPLA2 and COX-2 in the cytoplasm through a Src signaling intermediate. Inhibition of alcohol metabolism and blockage of Src activity or TLR4 prevented the activation of cPLA2 and COX-2 as well as diminished PGE2 production, suggesting that interactive phosphorylation of TLR4–Src regulated the pro-inflammatory response in astrocytes. Alcohol-driven changes were reduced in TLR4 knockout mice underscoring its involvement in CNS alcohol effects [79].

Another possibility of alcohol-induced astrocyte dysfunction is its effects on adenosine signaling. Lee et al. [83] demonstrated that mice lacking the ethanol-sensitive adenosine transporter, type 1 equilibrative nucleoside transporter (ENT1), consumed more alcohol compared with wild-type mice and had elevated striatal glutamate levels. ENT1 inhibition or knockdown reduces glutamate transporter expression in cultured astrocytes. Inhibition or deletion of ENT1 reduced the expression of type 2 excitatory amino acid transporter (EAAT2) and the astrocyte-specific water channel, aquaporin 4 (AQP4). EAAT2 and AQP4 colocalization was reduced in the striatum of ENT1 null mice. Ceftriaxone, an antibiotic increasing EAAT2 function, elevated not only EAAT2 but also AQP4 expression in the striatum. Furthermore, ceftriaxone reduced alcohol drinking, suggesting that ENT1-mediated downregulation of EAAT2 and AQP4 expression contributes to excessive alcohol consumption in a mouse model. These observations have significant implications as AQP4 regulates water content in the brain and could be another factor contributing to neurodegeneration.

Concentrations of extracellular glutamate were increased in animals exposed to alcohol for 4–8 days, suggesting deficits in glutamate transport [84]. Increased gene expression for EAAT1 was shown in the brains (frontal cortex) of alcoholics, while no results were presented for EAAT2 [85]. In contrast to discrepant experimental results, a number of clinical studies showed efficacy of anti-glutamatergic approaches for treating alcohol withdrawal symptoms [86] and dependence [87]. Furthermore, increased glutamate levels in animals with defective glutamate transporters enhanced their alcohol consumption [88]. Taken together, these data indicate multifaceted effects of alcohol on astrocyte function and suggest potential interventions.

4 Summary

It is clear that prolonged and excessive alcohol exposure affects all cell types in the brain via both direct and indirect effects. Importantly, new data suggest that inflammatory responses play a significant role in alcohol-associated neurodegeneration and alcohol addiction.

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Part V
Novel Approaches to Therapy

Cell-Based Drug Delivery for Improving Antiretroviral Therapeutic Outcomes

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Abstract During the past decade, our laboratories pioneered the use of monocyte-macrophages as Trojan horses for the uptake, trafficking, and delivery of nanoformulated antiretroviral therapy (nanoART) to improve drug delivery to tissue, cell, and subcellular sites of human immunodeficiency virus infections. Particles were manufactured with excipients and coated with sugars or peptides to facilitate particle uptake and sequestration in mononuclear phagocytes. Particles housed as cellular depots in recycling endosomes and free bioactive medicines dissociated from particles were released over periods of days to weeks. This enabled long-lived drug tissue reservoirs where peripheral monocytes and tissue macrophages activated by the nanoparticles are able to sequester ART for periods of weeks and longer. Indeed, following parenteral injection of the particles high concentrations of drug are found in the reticuloendothelial system with limited systemic toxicities enabling prolonged drug-dosing intervals. Target coating of the particles further facilitated pharmacokinetic and pharmacodynamics improvements over native or "naked uncoated" particle drug administrations, especially in reaching reservoirs of viral infection, such as the brain, gut, and lymphoid organs. The future of such therapies towards improving drug compliance, diminishing viral resistance, and facilitating residual virus reductions is timely and important and expanded upon within the current review.

Keywords Macrophage • Nanoparticle • Cell-based drug delivery • Antiretroviral Therapeutics • NanoART • HIV • Humanized mice models

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1 Introduction

Nanomedicine is an emerging discipline designed to provide improved biomedicines that can facilitate drug efficacy, reduce drug toxicity, engage antimicrobial and anticancer immunity, and target sites of infection, inflammation, degeneration, and other disease-related processes [1–3]. Overall, we posit that there is a strong likelihood that such technologies will yield improvements in delivery of therapeutics which can be realized within the current decade [4]. Notably, nanoformulations of drugs, proteins, enzymes, and antibodies have already been developed and demonstrate improved absorption and circulating half-lives with reduced toxicities and enhanced delivery to target sites of disease [2, 3]. Moreover, a large number of nanoparticle-based drug delivery systems are designed to evade the immune system in order to more efficiently deliver drug to the desired target, as in nanoformulations for cancer treatment. This precludes untoward immune reactions. When the targets themselves are the cells of the immune system, novel strategies can be developed for specific targeting. This concept formed the basis of the development of early liposomal formulations of amphotericin B for leishmaniasis and for cryptococcal and other fungal infections [5–7]. Liposomal amphotericin is preferentially taken up by phagocytic cells, the same cells infected by the Leishmania parasite or the fungus [8]. However, the main benefit of the liposomal formulation was a decrease in systemic toxicity, allowing for administration of higher doses and enhancing efficacy [5, 8]. In the past half-decade, targeting of nanoparticles to mononuclear phagocytes (MP; monocytes, macrophages, and dendritic cells), lymphocytes, and stem cells to be used as Trojan horses for delivery of anti-inflammatory and anti-infective medicines has been increasingly explored to facilitate drug delivery beyond parasitic infections [2, 9]. For example, our own laboratories have developed this concept for MP delivery of nanoformulations of antiretroviral drugs (nanoART) [10–13]. The promise of such a delivery system and the difficulties inherent in translating it for clinical use are discussed.

2 What Is NanoART?

As of 2011, there were more than 34 million people worldwide infected with HIV [14]. Notably, in the USA alone there are over one million infected patients with an estimated 50,000 new cases per year [15]. Treatment with combination antiretroviral therapy (cART) has proven effective in reducing the development of serious complications from HIV-1 infection but has not succeeded in eradicating virus from protected reservoir sites such as the lymphoid tissues, gut, and central nervous system [16–18]. While antiretroviral therapy leads to reduced morbidity and mortality for human immunodeficiency virus type one (HIV-1)-infected people [19–21], other major limitations are noteworthy, resting in systemic drug toxicities as well as the need for lifelong daily drug regimens. This type of dosing regimen can be difficult to follow and if not adhered to leads to increased viral resistance to therapy.

Suboptimal adherence causes increased risk of resistance and treatment failures [22–24]. Advanced disease, gastrointestinal absorbance, opportunistic infections, and drug abuse are all highly correlated with sporadic ART adherence commonly resulting in accelerated disease progression [25–28]. Because of concerns about promoting virologic resistance, providers are often reluctant to prescribe ART in settings associated with poor patient compliance. To counter the need for multiple dosing, single-dose multi-class cART such as Atripla™ (emtricitabine/tenofovir/efavirenz), Complera™ (emtricitabine/tenofovir/rilpivirine), and Stribild™ (Quad; elvitegravir/cobicistat/emtricitabine/tenofovir) [29–31] have been developed. However, these medicines have shown serious toxicities including lactic acidosis, hepatotoxicity, flare-up exacerbation of hepatitis after sudden discontinuance, peripheral neuropathy, and demonstrable renal impairments [32–36]. The need for long-acting and slow-release (once-a-month) formulations of standard and new ART regimens that can positively impact these concerns is clear and would be welcomed by HIV-1-infected patients [37–39]. The development of a such a drug delivery system could revolutionize ART treatment. To this end we have developed antiretroviral nanoparticles (nanoART) that are carried within circulating MP and delivered to virus-target tissues and hard to reach viral depots such as the central nervous system (Fig. 1) [10–13, 40, 41]. Cell-based nanoART can travel to sites of virus-associated inflammation, extend drug half-life by sequestering it away from hepatic metabolism, and release drug slowly with limited tissue toxicities [2, 9, 42]. However, in order for such a system to be realized for clinical use, there are limitations that need to be overcome. The drug-laden cells must migrate to the site of disease and deliver a sufficient amount ("payload") of drug as to provide effective therapy. Importantly, the cell's normal function and movement should not be compromised by the presence of the drug and excipients.

Many of the limitations for cell-based drug delivery and the targeting of specific cells for carriage can be overcome by the careful and systematic development of drug nanoformulations. The physicochemical characteristics of a nanoparticle drug carrier will influence its suitability for cell-based drug delivery. Nanocarriers for drugs are usually composed of a polymeric shell surrounding an inner core for drug carriage. The polymeric structure of a nanocarrier determines the drug loading capacity, imparts particle stability, and determines the interaction with environmental components and cell surfaces [9, 43]. Nanocarriers that have high drug-to-carrier ratios offer the potential for carrying many drug molecules per carrier and can thus maximize the efficiency of drug delivery. The charge status of the nanocarrier outer core affects the interaction of the nanoparticle with cell surfaces through interaction with cell surface receptors [9], with charged carriers being internalized by MPs to a greater extent than neutral particles [44–46]. The size and shape of the nanoparticle can also profoundly affect cell uptake. Particles in the range of 200–1,000 nm are readily taken up by MPs [47], and rod-shaped particles are internalized to a greater extent than spherical particles [48–50]. Once internalized and to be effective for drug delivery, the nanoparticle must be stored in non-degrading compartments for long-term carriage and subsequent drug release [9].

For treatment of HIV infection, harnessing the normal MP functions, including phagocytosis, cell–cell communication, and movement throughout the body and to

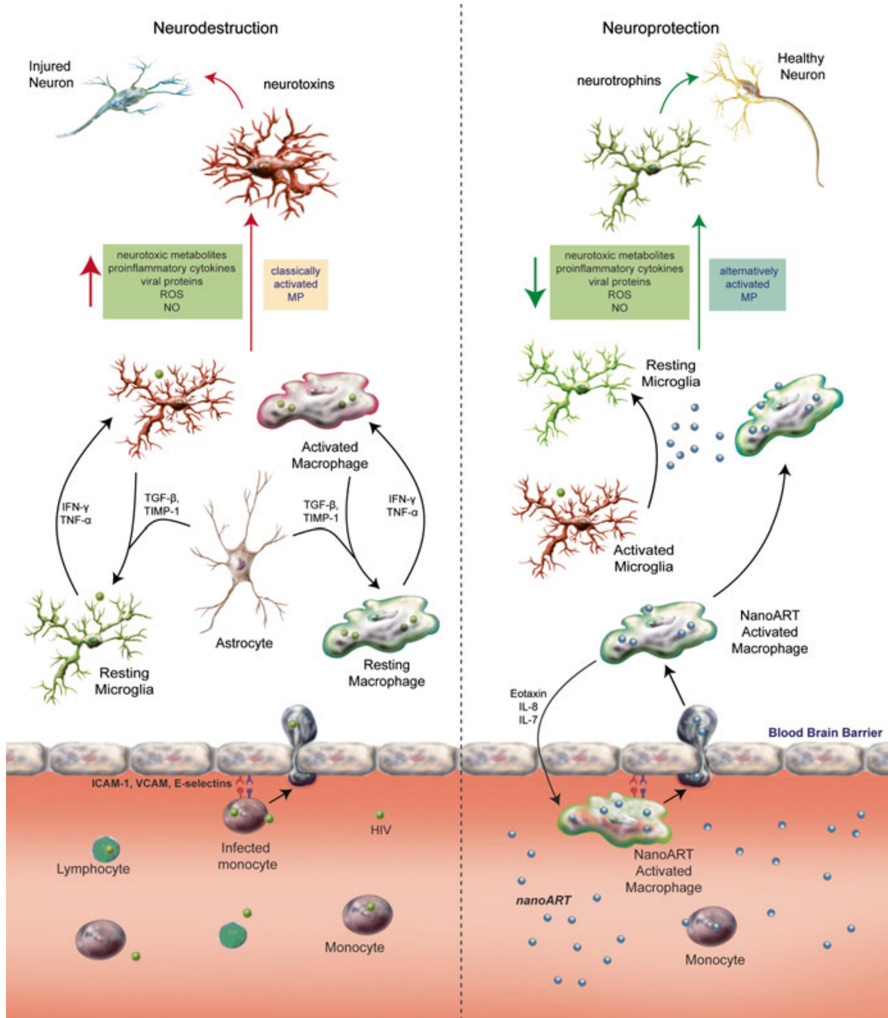

Fig. 1 Innate immune activity in HIV-1 neuropathogenesis and protection afforded by macrophages serving as Trojan horses for nanoART delivery. Activated, HIV-infected monocytes are attracted to the CNS by chemokines released from activated astrocytes and resident microglia. Spread of virus to neighboring glial cells induces neuroinflammation and neurodegeneration. NanoART-loaded macrophages are attracted to the CNS by chemokines. NanoART-loaded macrophages within the CNS release cytokines and chemokines that attract more monocyte-macrophages and enhance their migration across the blood brain barrier. NanoART and free ART are released from macrophages and suppress viral infection of microglia, resulting in a reduction in neuroinflammation and neurodegeneration

sites of injury, requires development of nanoparticles to be specifically engulfed by macrophages in select subcellular organelles. With these goals in mind, our laboratories have developed polymeric crystalline nanoparticles and polymeric micellar formulations of antiretrovirals for MP-based drug delivery [13, 51, 52]. Polymeric

crystalline formulations of antiretroviral drugs have been prepared by wet-milling and high-pressure homogenization. Synthesis of these formulations requires the free base form of the drug to have low aqueous solubility. The nanoformulations were prepared by fractionating crystalline drug in the presence of polymer surfactants, such as poloxamers 188 and 407 (P188 and P407, respectively), methyl-polyethylene glycol (mPEG), 1,2-distearoyl-phosphatidyl ethanolamine-methylpolyethyleneglycol2000 (DSPE-mPEG$_{2000}$), sodium dodecyl sulfate (SDS), and 1,2-dioleoyloxy-3-trimethylammoniumpropane (DOTAP) [40, 51]. Nanoparticles (200–500 nm in size) loaded with the protease inhibitors atazanavir, ritonavir, and indinavir or the non-nucleoside reverse transcriptase inhibitor efavirenz were produced with loading capacities of >80 % [12, 40, 51]. The shape of the nanoparticles is largely dependent on the drug itself and can affect internalization by macrophages; atazanavir particles, which are long slender rods, are internalized more rapidly and to a greater extent than the ellipsoid indinavir particles [13, 40]. The nanoformulations are stable in suspension for weeks to months; however, this is dependent upon the polymer coating. Drug was released from atazanavir particles coated with P407 more slowly than equivalent particles coated with P188 (unpublished observations).

Poly(lactic-co-glycolic acid) (PLGA) nanoformulations have been widely studied for drug delivery [53]. PLGA is attractive for drug delivery because of its relatively high loading capacity (up to 15 %), biodegradability and biocompatibility, ease with which formulations can be prepared, ability to incorporate both hydrophobic and hydrophilic drugs in conjunction with imaging agents, sustained drug release, and potential for targeting to specific cells [53]. PLGA-ART nanoparticles have been developed by a number of laboratories [51, 54–57]. By incorporating multiple drugs into a single PLGA particle, cART can be administered from a single nanoparticle dose [54, 55]. PLGA particles can also be used as carriers for drug and imaging contrast agents. PLGA nanoparticles containing antiretroviral drug and superparamagnetic iron oxide and coated with lipid and PEG-modified lipid (SMART particles) were developed in our laboratories as tools to monitor nanoparticle biodistribution in mice by magnetic resonance imaging (MRI) [52]. SMART particles were stable in PBS and released drug slowly over 10 days. Suitable relaxivity was maintained when particles were taken up by macrophages demonstrating their use for MRI.

Liposomes are also widely used for drug nanoformulations [58, 59]. High biocompatibility, low immunogenicity, and extended circulation times contribute to their use as drug delivery systems. However, their low loading capacity, relatively quick release profiles of hydrophobic drugs, and lesser physical stability than polymeric formulations limit their applications for specific drugs [60]. Nevertheless, liposomal formulations have provided extended pharmacokinetics for hydrophilic drugs such as the nucleoside reverse transcriptase inhibitor stavudine [61, 62] and can be modified with targeting moieties to provide enhanced receptor-mediated uptake by MPs.

Cell-based nanoparticle delivery can be improved by actively targeting nanoformulations to the carrier cells. Polymeric and liposomal nanoformulations are readily taken up by MPs by general phagocytic and endocytic mechanisms [2, 9]. By targeting surface receptors on the cells, nanoparticle uptake can be improved and may be directed to specific subcellular compartments for storage and protection

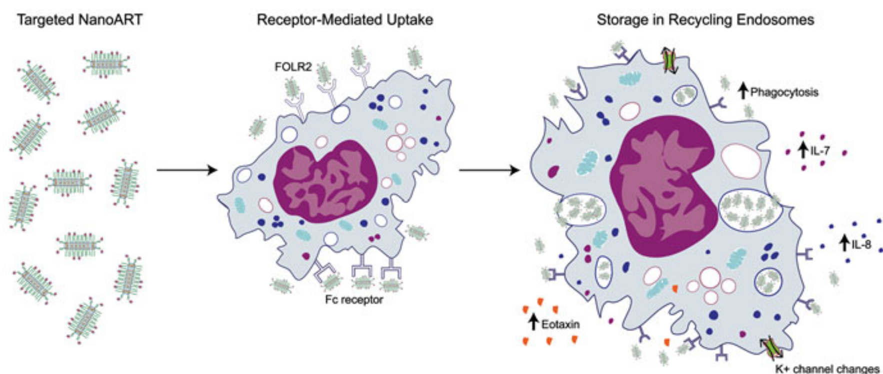

Fig. 2 Targeted nanoART production and macrophage interactions. Antiretroviral (ART) drug crystals are coated with a targeting ligand-conjugated polymer (such as folic acid-modified poloxamer 407; FA-nanoART) by high-pressure homogenization. Uptake of FA-nanoART can occur through binding to the Fc receptor and the folic acid receptor 2 (FOLR2). FA-nanoART is stored in recycling endosomes, where it is protected from degradation. NanoART carriage transforms the macrophage by enhancing phagocytosis, altering secretory functions, and stimulating the cell's migratory activity

from degradation (Fig. 2) [63–65]. By conjugating a targeting ligand onto the polymer or lipid, specific targeting of polymeric and liposomal nanoformulations can be achieved [59, 66]. In recent studies, nanoART targeted to folate receptors on macrophages were synthesized and demonstrated enhanced macrophage uptake, retention, and antiretroviral efficacy in cultured cells. Liposomal formulations of stavudine and zidovudine targeted with mannose and galactose provided increased macrophage uptake and enhanced liver, spleen, lung, and lymph node drug levels in rodents [61, 62, 67]. These targeting strategies aim at macrophages; however, it is conceivable that active targeting of T-lymphocytes may provide improved antiviral therapy and penetrance into viral reservoir sites, such as lymph nodes.

3 Testing NanoART Efficacy

A key for development of drug nanoparticles for cell-based delivery is screening in cell-based assays. Cell culture models have been used extensively for determining drug activity and toxicity and for assessing biologic effectiveness. In particular, for effective cell-based nanoformulated drug delivery, uptake of the drug by the carrier cell must be rapid and extensive, without affecting the normal functions of the cell or inducing toxicity. Once inside the cell the nanoformulated drug must be protected from degradation for effective delivery and for the cell to serve as a reservoir for long-term release of drug. Thus, prior to in vivo studies, the activity of nanoformulated antiretrovirals can be evaluated in cultured cells such as macrophages. Cell uptake, retention, release, toxicity, and antiviral efficacy of the nanoART in macrophage cell lines or primary cells isolated from animals or humans can be used as

predictive screening tools for nanoformulations prior to pharmacokinetic and _biodistribution studies in animals. A specific battery of tests should be designed in cell cultures that would prove predictive of in vivo behavior. Thus, for nanoART, activity in cultures of human monocyte-derived macrophages (MDM) has been shown to be generally predictive of pharmacokinetic performance in mice [40, 51, 68, 69]. Since macrophages are infected with the HIV virus in vivo, this screening system has also proven effective in monitoring efficacy in the same cells that are targeted for drug carriage [40, 51]. Macrophage uptake and retention of nanoART produced by high-pressure homogenization or wet-milling using various poloxamer and/or phospholipid surfactants was dependent to some extent on size, charge, shape, and coating; however, the greatest influence on these activities and on antiretroviral efficacy proved to be the drug itself [13, 40]. Preloading macrophages with nanoformulated atazanavir, ritonavir, or indinavir and then infecting the cells up to 15 days later with HIV-1_{ADA} provided 85, 80, and 40 % inhibition of progeny virion production and HIV-1 p24 staining, while treatment with an equivalent concentration of efavirenz nanoparticles resulted in nearly complete inhibition of viral infection [40, 51]. Addition of the targeting moiety folic acid to nanoformulations of atazanavir enhanced macrophage uptake and retention over twofold and enhanced antiviral efficacy in macrophages infected with HIV-1_{ADA} [68].

For effective cell-based delivery the function of the carrier cell should not be altered by carriage of the nanoformulated drug. Specifically for macrophages the normal functions of phagocytosis, migration, and release of immune-modulating cytokines and chemokines should be evaluated in cells loaded with nanoparticles. As a means of predicting global functional changes in carrier cells tools such as proteomics have proven useful [70, 71]. Pulsed stable isotope labeling of amino acids in cell culture (pSILAC) is once such tool that can identify dynamic protein changes in cells during drug treatment, intracellular trafficking, and storage that are indicative of cellular toxicities resultant from the drug itself, drug carriage, and drug release. In recent studies by Martinez-Skinner et al., pSILAC was used to determine dynamic global proteomic changes in macrophages loaded with nanoART [71]. Proteomic changes linked to immune cell migration and chemotaxis, cytokine and chemokine production, lipid metabolism, free radical scavenging, and cell differentiation were observed. These protein changes were substantiated by functional assays, including phagocytosis, migration, cytokine/chemokine secretion, and potassium channel activation, and indicated that nanoART uptake induced a unique activated macrophage phenotype that is primed for further nanoART uptake and storage and enhances cell migration [71].

4 Subcellular Distribution of NanoART

Intracellular trafficking of the nanoformulated drugs is a key factor in determining the stability of the drug once inside the cell carrier and drug release at the target site [65]. Cationic and anionic nanoparticles can have different fates once inside MP [9].

In general positively charged nanoparticles are less likely to be degraded than are negatively charged particles by decreasing acidification of lysosomal compartments [9]. Lysosomal degradation can also be reduced if the nanoparticles traffic to non-degrading endosomal compartments inside the MP. Ritonavir nanoART prepared with P188, DSPE-mPEG$_{2000}$, and DOTAP as surfactants was taken up by MDM via clathrin-mediated endocytosis and trafficked to Rab 11^{+} and Rab 14^{+} recycling endosomal compartments [68, 72] from which they were released intact at the cell surface. The released nanoparticles retained their ability to reduce HIV-1 infection of MDM. It is noteworthy that these particles were positively charged. Recent studies, however, have demonstrated similar trafficking of negatively charged atazanavir nanoART prepared with P407 and folate-modified P407 [68], suggesting a similar intracellular trafficking profile for crystalline nanoART regardless of charge state. Intracellular trafficking will also determine cell storage and release rate of the nanoART. A sustained and controlled release of drug at the target site is necessary in order to provide optimal therapeutic effectiveness [9]. Stimulated drug release at the target site is also possible by employing signals such as changes in intracellular calcium or mild hyperthermia [73, 74]. Of particular importance, however, active targeting of nanoART to specific cell compartments could not only enhance cell storage but could allow the drug to be directed to the cell compartments where HIV replicates [75].

5 Animal Models for NanoART Testing

New drug formulations are routinely tested in rodents prior to translating results to clinical trials. Determination of pharmacokinetics and biodistribution of nanoformulations for cell-based nanoART delivery is easily achieved using normal mouse strains. Uptake of parenterally injected nanoformulations by MPs and ART drug sequestration inside cells of the reticuloendothelial system can be monitored using rodent model systems [76]. Drug metabolism and enzyme induction and their contribution to ART pharmacokinetics, biodistribution, and toxicity can be determined in rodents and can be predictive of human events [77]. A particular challenge, however, in determining the pharmacodynamics and efficacy of nanoART is the specificity of HIV-1 infection for humans. The development of “humanized” mouse models that recapitulate HIV-1 pathogenesis has allowed the study of new ART therapies and mechanisms of HIV-1 pathogenesis [78, 79]. Severe combined immunodeficient (SCID) mice contain an autosomal recessive mutation in the *prkdc* (DNA-dependent protein kinase) gene that results in a deficiency in mature T and B lymphocytes [80]. Transfer of the SCID mutation onto the nonobese diabetic (NOD) background subsequently resulted in a mouse strain (NOD/SCID) with very few occurrences of spontaneous lymphocyte production (leakiness) [81]. Because of the lack of functional lymphocytes, transplantation of foreign tissues and cells is possible in these mice [82]. Mouse models have been developed using transplanted human thymus, fetal liver, and peripheral blood lymphocytes wherein human

immune cells are able to reconstitute the NOD/SCID mouse immune system [83, 84]. Immunodeficient mice transplanted with human immune cells are especially useful for assessing the pharmacokinetics and biodistribution of nanoART that are targeted to human macrophages and T-lymphocytes.

Results of studies in mice can be generally predictive of drug behavior in humans. However, there are differences in drug metabolism, immune responses, and metabolic rate that can impact the overall translation of experimental results to humans. Dose, bioavailability, frequency of administration, adverse drug reactions, and the responsiveness of reproductive organs to therapeutics can all differ from mice to humans. For nanoART testing nonhuman primates are a desired model for determining pharmacokinetics, injection route, systemic toxicity, distribution of drug to lymphoid tissues and female genital tissues, and importantly antiretroviral efficacy [85]. The expense and value of these animals, however, requires careful study design.

6 Pharmacokinetics, Pharmacodynamics, and Biodistribution

For a nanoformulated drug to be successful, it is essential that a desired pharmacokinetic (PK) and pharmacodynamic (PD) profile be achieved. The standard assessments designed for small-molecule PK may not be applicable to PK of nanomedicines. Because of the unique behavior of nanoparticles, including nanoparticle absorption, drug release kinetics, and nanoparticle clearance, using blood and plasma drug levels as a determination of PK may not provide a complete picture of nanoparticle distribution [86]. In particular for targeted and cell-based nanoparticle delivery, determination of levels of drug in plasma may not be an accurate indication of the PD of the nanoformulated drug [87]. Of significance, determination of drug concentrations at the target site or in target cells may be more predictive of therapeutic efficacy [88]. In addition when using MPs as carriers for drug nanoparticles, the activation state of the cells may influence their uptake of the nanoparticles and the delivery of drug to target sites. Thus, to develop an effective dosing regimen, determine optimal route of administration, and determine tissue and cell distribution, careful in vivo assessments of drug nanoformulations are required.

NanoART (atazanavir/ritonavir) that had passed in vitro screening for macrophage uptake, retention, toxicity, and antiretroviral efficacy was characterized for PK and biodistribution in mice and nonhuman primates [76]. In mice treated subcutaneously with a single dose of nanoART (atazanavir/ritonavir), plasma and tissue drug levels 14 days after injection were 10- and 40-fold higher than levels following administration of non-formulated drugs. Multiple doses provided up to 270-fold higher plasma and tissue levels after 6 weeks of weekly nanoART administration. The sustained plasma and tissue drug levels were attributed to localization of nanoART in non-lysosomal compartments in tissue macrophages, primarily Kupffer cells, providing a storage depot for extended drug release. Similar elevated and

extended plasma drug levels were observed following subcutaneous and intramuscular administration of nanoART as compared to native drugs to rhesus macaques. Active targeting of nanoART to macrophage folate receptors enhanced plasma and tissue drug levels by up to fivefold when administered by intramuscular injection to mice [68]. Of particular significance was the detection of drug levels in lymph nodes of up to 70 ng/g tissue in mice treated with folate-targeted nanoART. These results suggest that targeting nanoART to macrophage and even T-cell receptors can provide penetration of drug into previously inaccessible HIV viral reservoirs.

7 Antiretroviral Drugs for Nanoformulations

The advent of ART has greatly reduced the morbidity and mortality associated with HIV-1 infection. The serious subacute neurological pathology of HIV-associated encephalitis has been reduced to a milder often subclinical form [20, 21]. Initial and maintenance therapy has traditionally been a combination of reverse transcriptase inhibitors and protease inhibitors. Protease inhibitors are usually a combination of a therapeutic dose of lopinavir, atazanavir, or fosamprenavir and a much lower, boosting dose of ritonavir to inhibit metabolism of the therapeutic component by hepatic cytochrome P450 and extend plasma half-life ([37]). However, HIV infection-associated renal, cardiovascular, and peripheral neuropathic complications especially in light of coinfections such as hepatitis C and tuberculosis can affect continuation of certain drug regimens. Newer drugs such as integrase strand transfer inhibitors (raltegravir and elvitegravir), entry inhibitors (enfuvirtide), and CCR5 antagonists (maraviroc) provide improved virologic control with reduced associated toxicities [89–92]. Of importance, viral resistance may develop more slowly in patients receiving newer generations of these drugs [93–95]. Preexposure prophylaxis regimens have recently been approved by the FDA (tenofovir/emtricitabine) and show great effectiveness in reducing HIV transmission in adherent individuals [96, 97]. In spite of new therapeutics and therapeutic regimens, eradication of virus from protected sites, such as lymphoid tissue and brain, is still not possible and the incidence of minor neurologic deficits has not been reduced.

8 HIV Model Systems

As previously described a number of rodent systems have been developed for the study of HIV-1 infection and evaluation of new therapeutics. SCID mice injected with HIV-1-infected human MDM demonstrate key features of HIV-1 neuropathology [98, 99]. This mouse model has proven effective in determining effective ART therapies and in examining the interaction of the innate and adaptive immune systems in overall CNS response to HIV infection [11, 100, 101]. NOD/SCID mice repopulated with human peripheral blood lymphocytes and infected with HIV-1

have been used to evaluate pharmacokinetics, biodistribution, and antiviral efficacy of nanoART in a system of activated immune cells [69]. However, the usefulness of this model for long-term evaluation of nanoART is limited since the mice succumb to rejection of the human cell grafts within several weeks. Long-term human cell reconstitution mouse models have been developed [84, 102–105]. Newborn NOD/SCID mice are irradiated to deplete their normal bone marrow stem cells and then given human CD34+ polymorphonuclear stem cells from human fetal liver or human cord blood. Reconstitution of the mouse immune system with human immune cells occurs over a period of 12 weeks [102, 103]. In response to infection with HIV-1 these "humanized" mice demonstrate hallmark lymphocytic and humoral immune responses and support chronic HIV-1 infection. Because of the long-term survival of these mice and the stability of the immune reconstitution, chronic ART treatment regimens can be evaluated. In humanized mice chronically infected with HIV-1, weekly doses of nanoART (atazanavir/ritonavir) provided sustained plasma, liver, and spleen drug levels and suppressed viral infectivity to below the limit of detection during the course of nanoART treatment [106]. Upon cessation of treatment, however, plasma viral load rebounded and the presence of infected T-cells in lymph nodes was not suppressed. In this same model brain metabolite changes in response to chronic HIV infection correlated with microgliosis and were partially reversed with weekly nanoART treatment [107].

Immunodeficient mice reconstituted with human bone marrow $CD34^+$ stem cells, and liver and thymus cells (BLT mice) are useful for studying several tissue pathologies associated with HIV infection [84]. Many tissues in these mice are reconstituted with human cells, including the female reproductive tract. ART treatment regimens, including nanoART, to reduce vaginal transmission of HIV-1, including preexposure prophylaxis, are being explored using BLT mice [84, 108–110].

The most widely used animal model for study of the pathology of HIV infection has been the SIV-macaque model [111–113]. In particular the model lends itself to study of the neurological effects associated with chronic HIV infection, including development of encephalitis and neurological impairment. This model has been particularly valuable in determining the involvement of innate and adaptive immune functions in development of late-stage CNS disease. The actions of HIV genes in vivo can also be examined in a hybrid model where portions of the HIV gene have been inserted into SIV, creating hybrid simian-human immunodeficiency virus (SHIV) [114]. The SIV model has also proven valuable for exploring therapeutic options and identification of mechanisms of therapeutic insufficiency, viral latency, vaginal transmission, and preexposure prevention [115, 116].

9 Clinical Translation of NanoART

The promise of nanoART for treatment of HIV infection is real and of immediate need. However, to reach this goal a number of hurdles need be overcome. Translation of laboratory-based preparation of nanoART to large-scale

production for clinical trials will require the development of protocols following good manufacturing guidelines [117]. Difficulties in production scale-up will need to be overcome by carefully evaluating and establishing defined protocols for each step of synthesis. Production uniformity, purity, and activity, as well as formulation stability and shelf-life, will need to be determined and criterion for pass/fail defined. Protocols for product sterilization will need to be developed. Laboratory animal studies can define the most appropriate dose, route, and frequency of administration and can determine efficacy in animal models of HIV infection. For investigational new drug application approval, safety assessment studies in animals, both rodents and non-rodents, are required [118–120]. These studies may include some or all of the following categories: acute and repeated dose toxicity studies (90 days), reproductive toxicity, genotoxicity, local tolerance, and, for long-term use, carcinogenicity. Specific endpoints such as mortality, body weight gain/loss, clinical observation, complete blood counts, clinical metabolic panel, gross pathology, histopathology, injection site tolerance/irritation, and immunotoxicity (function and phenotypes) are usually included in preclinical toxicity assessments. These hurdles are not insurmountable, but do require time and sufficient resources.

Another important factor in determining the feasibility of translation of nanoART to clinical use is economics (reviewed in [121]). The populations in most critical need for new technologies for delivery of antiretroviral drugs are in developing countries where access is limited and often cost-prohibitive. Antiretroviral formulations can provide a means for improving pharmacokinetics of existing cheaper drugs, decreasing dose levels and frequency, reducing the occurrence of intolerable side effects, and reducing the development of resistant viral strains. ART nanoformulations as prophylactic preventatives could provide reduction in the current frequent dosing regimens and hence reduction in cost.

10 Potential for HIV Eradication

The development of nanoART as an effective therapeutic modality for HIV-1 infection offers many promises. The ability to maintain extended plasma drug levels from a single intramuscular injection would improve patient adherence and reduce the potential for development of resistant viral strains. The ability to specifically target cell and tissue reservoirs and to direct the nanoformulations to the same subcellular compartments where viral replication occurs offers for the first time the potential for viral eradication.

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Immunomodulatory Therapeutics

David J. Graber and William F. Hickey

Abstract Altered immune mechanisms occur in a number of neurological illnesses. These immune responses range from persistent or disproportionate reactivity of resident microglia cells within the CNS to the infiltration of potentially autoreactive peripheral leukocytes. Therapies directed at elements of peripheral immune system have been a successful strategy for treating the relapsing-remitting stage of multiple sclerosis. However, regulating the microglial activity within the CNS could be a promising approach for treating the progressive stage of multiple sclerosis and other neurodegenerative diseases for which effective therapies have remained elusive. Immunomodulating agents that have been evaluated in several neurological illnesses with a neurodegenerative and/or a neuroinflammatory component are discussed in this chapter.

Keywords Neuroinflammation • Anti-inflammatory • Immunosuppressive • Central nervous system • Microglia • Amyotrophic lateral sclerosis • Parkinson's disease • Alzheimer's disease • Multiple sclerosis

Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
COX	Cyclooxygenases
DAMPs	Damage-associated molecular patterns

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IFN-beta	Interferon-beta
MS	Multiple sclerosis
NSAIDs	Nonsteroidal anti-inflammatory drugs
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear-related factor E2-related factor 2
PD	Parkinson's disease
PPMS	Primary progressive multiple sclerosis
PTGS	Prostaglandin-endoperoxide synthases
SPMS	Secondary progressive multiple sclerosis
VCAM-1	Vascular cell adhesion molecule

1 Immune Mechanisms in Neurodegenerative Diseases Are Propitious Therapeutic Targets

Early use of the term "neuroinflammation" often described the infiltration of peripheral immune cells (e.g., lymphocytes) into the spinal cord in experimental autoimmune encephalomyelitis. The term "neuroinflammation" has evolved over time, however, to include reactive microglia and their production of inflammatory mediators within the CNS parenchyma—even in the absence of leukocytic infiltration. This seems to be a feature unique to the CNS because accumulation of leukocytes in a tissue is a defining characteristic of inflammation outside the nervous system.

Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS; "Lou Gehrig's disease"), and Alzheimer's disease (AD) are well-recognized neurodegenerative diseases lacking effective treatments to stop the neuron loss and gradual, irreversible neurological disability. In addition to the loss of select groups of neurons, common characteristics of these illnesses are an aged-related onset, formation of intracellular protein aggregates, signs of oxidative stress, and so-called neuroinflammation. Increased numbers of microglia, transformed glial cell morphology and immunological marker expression, and production of inflammatory mediators (i.e., cytokines, chemokines, and free radicals) are components of the neuroinflammation in such neurodegenerative diseases and in corresponding animal models. In the late 1980s and early 1990s, analysis of neural parenchyma near sites of neuron loss in postmortem CNS from patients with various neurodegenerative disease revealed microglia with highly activated phenotype which included having a rounded morphology and enhanced expression of immunological cell surface markers [1–4]. Evidence of elevated inflammatory mediators in these same tissue regions followed [5–8].

Whether the neuroinflammation associated with neurodegenerative disease is exclusively a reaction to neuron damage, or whether the subsequent production of inflammatory mediators is a key contributor to neuron death, is disputed. Aberrantly processed proteins, intracellular protein aggregates, and damage-associated molecular

patterns (DAMPs) released from injured neurons may be a driving force for neuroinflammation by interacting with pattern recognition receptors expressed on microglia or infiltrating macrophages. Experimental evidence shows that some of the inflammatory mediators produced during neuroinflammation can be neurotoxic [9–13]. A viscous cycle of neurotoxicity-mediated neuroinflammation and neuroinflammation-mediated neurotoxicity may be a basic pathogenic mechanism in neurodegenerative diseases. To confirm this, neuroprotection in human neurodegenerative diseases as a result from limiting parenchymal neuroinflammation is necessary.

Multiple sclerosis (MS) is an autoimmune demyelinating disease that is now considered to have neurodegenerative aspects based on axonal degeneration [14, 15]. The immunological mechanisms associated with MS are much more complex than that with other neurodegenerative diseases. Infiltration of leukocytes (e.g., T cells) into the affected myelin-rich white matter regions of the CNS is critical for the relapsing-remitting stage of MS. As discussed in more detail below, the frequency and severity of relapsing demyelinating episodes are reduced by immunomodulatory agents that have been developed during the last 30 years to regulate the immune system peripherally. Progressive MS is a more debilitating stage that frequently follows the relapsing-remitting stage ("secondary progressive MS," SPMS), but can sometimes dominate from the start ("primary progressive MS," PPMS). The progressive stage, which shares similarities to other classic neurodegenerative diseases, currently lacks effective treatments. There is a gradual worsening of irreversible neurological disability over months to years. In the chronic MS plaques, there is enhanced activity of reactive microglia, but with fewer signs of classic inflammation that is characteristic of acute, transient lesions found during the relapsing-remitting stage [16, 17]. As in other neurodegenerative diseases, chronic neuroinflammation controlled mainly by reactive microglia within the CNS may function as the destructive factor for the gradual decline evident in progressive stage of MS.

2 Therapeutic Immunomodulation

Immunomodulating drugs can be divided into three classes: (1) immunostimulants, (2) immunosuppressants, and (3) anti-inflammatory agents. Immunostimulants boost or instigate an immunological reaction. Both antigen and adjuvant in vaccines act as immunostimulants by mounting adaptive immunity to protect against specific pathogens. Imiquimod is an immunostimulant that binds to the pattern recognition receptor called toll-like receptor 7 on immune cells to enhance the synthesis of inflammatory mediators to aid in the immune response to clear warts or target cancerous skin cells. With respect to immunostimulatory treatments for neurodegenerative diseases, one strategy is to enhance the immune response for aberrant protein removal in the CNS. Bapineuzumab, gantenerumab, and solanezumab are humanized antibodies created to bind amyloid beta in an effort to instigate

phagocytosis-mediated clearing of plaques in AD. Anti-amyloid therapies have not been successful to date despite some evidence of lowering amyloid brain plaque [18] and tau protein in CSF [19]. A non-antibody-mediated approach to enhance clearing of aberrant proteins by phagocytosis has been demonstrated in a preclinical mouse model of AD upon treatment with a small molecule retinoid X receptor agonist called bexarotene [20]. Whether this effectiveness will translate in human disease is undetermined at this time. Opposite to enhancing the immune response as a therapeutic strategy for treating neurodegenerative diseases, attenuating the excessive or chronic neuroinflammation is also being pursued.

Immunosuppressive drugs tend to impede the adaptive immune response or limit the infiltration of leukocytes during inflammation. Such agents are commonly utilized to prevent tissue rejection following organ transplantation and to treat autoimmune diseases. The diverse mechanisms by which immunosuppressive drugs work include selectively killing certain types of leukocytes, shifting the balance of Th1 versus Th2 versus Th17 responses, inhibiting production of interleukin-2, or blocking tissue infiltration of immune cells at sites of inflammation. For CNS illnesses, immunosuppressive agents have been commonplace for treating relapsing-remitting MS. Infiltration of lymphocytes into white matter is well documented during this phase of MS and in the animal model experimental autoimmune encephalomyelitis. Immunosuppressive agents such as natalizumab, fingolimod, and teriflunomide are discussed in more depth below and summarized in Table 1.

Neurodegenerative diseases like AD, ALS, and PD do not appear to be mediated by adaptive immunity. They do, however, involve excessive innate immune activity most often represented by production of inflammatory mediators by microglia that can be toxic to neurons. This immune-mediated destruction of host cells has been referred to as auto-inflammatory and auto-toxicity, which is distinctive from autoimmunity. The progressive phase of MS, which is typically impervious to therapies that mitigate the relapse-remitting stage, may involve similar centralized neuroinflammation. Anti-inflammatory agents differ from immunosuppressants in that they target more of the synthesis of inflammatory mediators produced during innate immune responses or the function of selective inflammatory products. Mechanisms include inhibiting the synthesis of select mediators, blocking signaling pathways to prevent the synthesis of a group of mediators, and blocking the effects of soluble mediators individually. For drugs developed to limit the neuroinflammation in neurodegenerative disease, they will need to overcome the restrictive blood–brain barrier while retaining their anti-inflammatory activity within the unique environment of the CNS.

The majority of anti-inflammatory drugs are either glucocorticoid receptor agonists (steroid-based) or cyclooxygenase inhibitors (nonsteroidal anti-inflammatory drugs, NSAIDs). Additionally, drugs such as minocycline and thalidomide were originally developed for their non-immunological effects but were later reported to also have anti-inflammatory activity. These anti-inflammatory agents are discussed in more detail below and are summarized in Table 1.

Table 1 Immunomodulating drugs in neurodegenerative diseases

	Molecular target	Mechanism	RRMS	PPMS or SPMS	AD	PD	ALS
IFN-beta	Type I IFN receptor	Shift in adaptive immunity towards Th1; anti-inflammatory	Reduce relapse rate [24, 25]	NE [26, 27]	Und	Und	Und
Glatiramer acetate	Major histocompatibility complex	Shifts adaptive immunity towards Th2; interfere with antigen presentation	Reduce relapse rate [29]	NE [29]	Und	Und	NE [30]
Natalizumab	α4-Integrin	Block tissue infiltration of immune cells	Reduce relapse rate [31, 32]	Und	Und	Und	Und
Fingolimod	Sphingosine-1-phosphate receptor	Sequesters lymphocytes in lymph nodes	Reduce relapse rate [37, 38]	Und	Und	Und	Und
Teriflunomide	Dihydroorotate dehydrogenase	Toxic to active lymphocytes	Reduce relapse rate [40]	Und	Und	Und	Und
Dimethyl fumarate	Nuclear-related factor E2-related factor 2	Free radical protection; anti-inflammatory; reduce lymphocyte function	Reduce relapse rate [41, 42]	Und	Und	Und	Und
Glucocorticoid	Glucocorticoid receptor	Reduce lymphocyte function; anti-inflammatory	Improve recovery from severe relapse [43–45]	Inc [46–48]	NE [49]	Und	Und
NSAIDs	Prostaglandin-endoperoxide synthases (cyclooxygenases)	Reduce prostaglandins	Und	Und	NE [60]	Und	NE [61]
Minocycline	Unknown	Inc	Und	Und	Und	NE [68]	NE [67]
Thalidomide	Unknown	Inc	Und	Und	Und	Und	NE [72]

Outcomes of clinical trials for relapsing-remitting multiple sclerosis (RRMS), primary or secondary progressive MS (PPMS or SPMS), Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) are summarized. Interferon, IFN; nonsteroidal anti-inflammatory drug, NSAID; undetermined, Und; inconclusive, Inc; no efficacy, NE

3 Interferon-Beta1 (Betaseron®, Extavia®, Avonex®, and Rebif®)

Description. Interferon-beta (IFN-beta) is an endogenous protein and a 187 amino acid member of the IFN family of cytokines. IFNs are expressed in response to viral infections and function to interfere with viral replication. Double-stranded RNA that is produced during viral replication in host cells is the main signal for IFN expression. Recombinant IFN-alpha, IFN-beta, and IFN-gamma were developed as potential antiviral therapies. Since MS was hypothesized to be caused by an unknown viral infection, pilot trials with these recombinant IFNs were conducted. IFN-alpha did not improve relapse rates in MS [21] and IFN-gamma exacerbated relapses [22]. IFN-beta, a subtype normally produced by fibroblasts, showed promising results in relapsing-remitting MS [23]. This prompted the development of new ways to synthesize this polypeptide. Recombinant IFN-beta has been produced in bacterial expression systems (IFN-beta1b) or in mammalian cells (IFN-beta1a). The FDA approved subcutaneous injections of synthetic IFN-beta in 1993 for treatment of MS.

Efficacy. In relapsing-remitting MS, IFN-beta therapy increases the number of progression-free patients, lowers the number of newly formed CNS lesions based on MRI detection, and decreases the number of relapses (acute, transient worsening of disability status) [24, 25]. Yet, IFN-beta therapy does not appear to slow down the decline in disability in the progressive stage of MS [26, 27].

Mechanism of Action. IFN-beta activates heterodimeric IFN-alpha/beta receptor (type I IFN receptor). While IFN-alpha also activates this receptor, IFN-gamma activates type II IFN receptor and initiates a separate signaling pathway. IFN-beta engages the type I IFN receptor, initiates a signaling pathway involving receptor-associated kinases Jak1 and Tyk2, and signals the formation of a transcription factor complex IFN-stimulated gene factor 3. IFN target genes that have the IFN-stimulating response element are expressed. Many immunomodulatory and antiviral proteins are produced in response to activation of this pathway.

The precise therapeutic mechanism of IFN-beta treatment in relapsing-remitting MS remains unclear. It seems to be based on its immunomodulatory activity as opposed to its antiviral activity. This may include reduced T cell proliferation, reduced antigen presentation, altered expression of inflammatory mediators, and a shift in adaptive immunity towards Th1 (activation of CD8+ T cells).

Drawbacks. The method of administration for IFN-beta is by intramuscular or subcutaneous injection. This relies on self-administration and can cause adverse reactions (e.g., necrosis) at injection site. Some patients develop neutralizing antibodies against IFN-beta, resulting in loss of drug effectiveness. Finally, there is a subset of relapsing-remitting patients that do not respond to IFN-beta therapy.

4 Glatiramer Acetate (Copaxone® and Copolymer 1)

Description. Glatiramer acetate (Fig. 1) is a mixture of random polymers of four amino acids commonly found in myelin basic protein: glutamic acid, lysine, alanine, and tyrosine. Animals given this compound were shown to be resistant to the development of experimental autoimmune encephalomyelitis, and this protection could be adoptively transferred to normal syngeneic recipients [28].

Efficacy. Glatiramer acetate lowers the relapse rate in relapsing-remitting MS, but is ineffective in slowing the disability increase in the progressive stage of MS [29]. It was evaluated in ALS and showed no beneficial effect on the rate of neurological deterioration [30].

Mechanism of Action. The therapeutic mechanism of glatiramer acetate in relapsing-remitting MS is not fully understood. It may involve competitive inhibition with autoantigens for binding to major histocompatibility complex molecules to inhibit presentation to T cells. It is also suggested that glatiramer acetate shifts adaptive immunity towards Th2 and enhances expression of anti-inflammatory cytokines.

Drawbacks. It cannot be taken orally and is self-injected either intramuscularly or subcutaneously by patients. Adverse reactions (e.g., necrosis) can occur at the injection site. Some of the postinjection reactions include chest pain, flushing, dyspnea, palpitations, and/or anxiety.

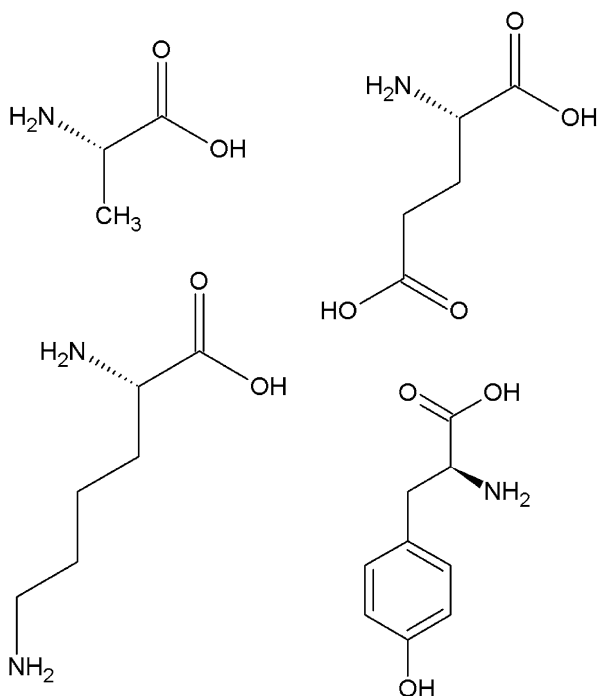

Fig. 1 Chemical structures of the amino acids in glatiramer acetate, which is a polymer consisting of random sequences of glutamic acid, lysine, alanine, and tyrosine. Structures generated using ACD/ChemSketch 12.01 (Advanced Chemistry Development, Inc.)

5 Natalizumab (Tysabri®)

Description. Natalizumab is a monoclonal antibody against the cell adhesion molecule α4-integrin. It is produced in murine myeloma cells and humanized by complementarity-determining region grafting—hypervariable region of the murine antibody gene is combined to a human immunoglobulin framework. It is an IgG4 subclass of antibody, which is preferred for therapeutic antibodies because it is a weak inducer of effector functions.

Efficacy. Natalizumab reduces the rate of relapses and limits the accumulation of new lesions [31, 32]. Its effect on the progressive stage of MS has not been fully elucidated.

Mechanism of Action. Natalizumab binds to α4-integrin expressed on immune cells and interferes with its binding to vascular cell adhesion molecule (VCAM)-1 expressed on vasculature endothelial cells at sites of inflammation. VCAM-1 expression is increased in active CNS plaques [33]. Natalizumab blocks infiltration of leukocytes into the central nervous system.

Drawbacks. A major limitation in disrupting leukocyte infiltration into the CNS with natalizumab treatment is the development of potentially fatal progressive multifocal leukoencephalopathy in patients infected with latent JC virus and subsequent development of immune reconstitution inflammatory syndrome after halting treatment [34]. This can now be managed by limiting natalizumab to patients negative for antibodies against the JC virus. As with other biologic therapeutics, the host immune system can react to treatment by producing antibodies against natalizumab that can cause an allergic reaction or neutralize its efficacy [35, 36]. Other drawbacks include the required administration by intravenous infusion and the development of hepatotoxicity with prolonged use.

6 Fingolimod (Gilenya®)

Description. Fingolimod (Fig. 2) is an analogue of sphingosine, a primary part of a class of cell membrane lipids called sphingolipids.

Efficacy. Fingolimod modestly reduces the relapse rate in MS [37, 38] and lowers the lesion burden [39].

Mechanism of Action. Fingolimod alters lymphocyte migration by binding to the sphingosine-1-phosphate receptor. This sequesters lymphocytes in the lymph nodes.

Drawbacks. There is an increased risk of life-threatening viral infection and risk of tumor development with fingolimod use. It can worsen severe MS relapses. Common side effects include headache, influenza, diarrhea, back pain, elevated liver enzymes, and cough.

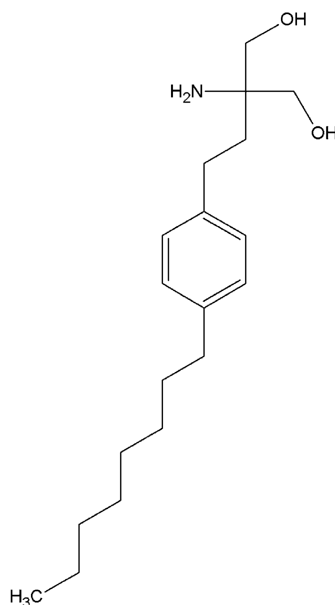

Fig. 2 Chemical structure of fingolimod. Structure generated using ACD/ChemSketch 12.01

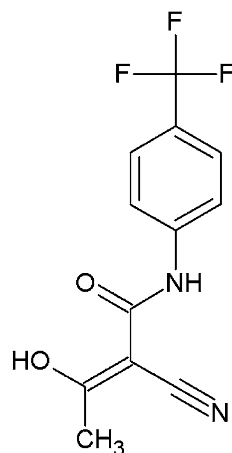

Fig. 3 Chemical structure of teriflunomide. Structure generated using ACD/ChemSketch 12.01

7 Teriflunomide (Aubagio® and Flucyamide)

Description. Teriflunomide (Fig. 3) is an active metabolite of the pyrimidine synthesis inhibitor leflunomide, which inhibits dihydroorotate dehydrogenase.

Efficacy. It appears to be beneficial in reducing the relapse rate in the relapsing-remitting stage of MS; however more trials are necessary for validation [40].

Mechanism of Action. Teriflunomide inhibits pyrimidine biosynthesis by blocking the enzyme dihydroorotate dehydrogenase. The subsequent reduction in pyrimidine

ribonucleotide uridine monophosphate inhibits rapidly dividing cells, including activated T cells. It may also disrupt the interaction of T cells with antigen-presenting cells.

Drawbacks. Teriflunomide may cause birth defects. Side effects with its use include diarrhea, nausea, and hair thinning. An increase in alanine aminotransferase in serum has also been reported indicating asymptomatic liver dysfunction.

8 Dimethyl Fumarate (Tecfidera™, Fumaric Acid, BG-12)

Description. Dimethyl fumarate (Fig. 4) is an activator of nuclear-related factor E2-related factor 2 (Nrf2) signaling.

Efficacy. Treatment with dimethyl fumarate reduces the relapse rate and the number of lesions in relapsing-remitting MS [41, 42]. Its effect on the progressive stage of MS has not been examined to date.

Mechanism of Action. Dimethyl fumarate is a known activator of the Nrf2 signaling pathway, which enhances expression of genes associated with the antioxidant response element. The gene products can protect cells from oxidative insults. Like many other molecular activators of Nrf2 signaling, dimethyl fumarate also has anti-inflammatory activity as demonstrated by inhibiting immunological expression of inflammatory mediators.

It is unclear whether enhanced production of protective gene products or anti-inflammatory activity of dimethyl fumarate is responsible for protection in relapsing-remitting MS. A reduction in lymphocyte numbers was observed in treated patients [41, 42]. This approach of attenuating the immune system peripherally parallels other disease-modifying drugs for the relapsing-remitting stage of MS. It is unknown whether dimethyl fumarate can regulate reactive microglia in the CNS. If so, this line of therapy may provide therapeutic protection in the progressive stage of MS or for other neurodegenerative diseases.

Drawbacks. Side effects of dimethyl fumarate use include flushing and gastrointestinal complaints. Its activity may be affected when taken orally by esterases in the small intestine which can cleave dimethyl fumarate to monomethyl fumarate.

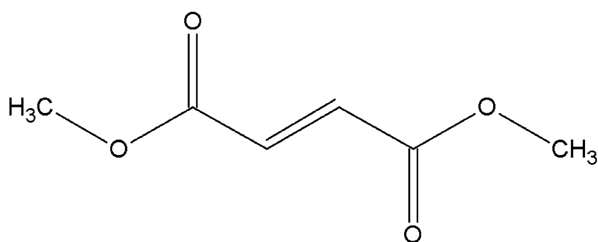

Fig. 4 Chemical structure of dimethyl fumarate. Structure generated using ACD/ChemSketch 12.01

9 Glucocorticoids

Description. Glucocorticoid receptor agonists are a long established and powerful class of anti-inflammatory drugs. They inhibit the expression of most inflammatory mediators. It also has immunosuppressive effects as well and is often used to address exacerbated relapses in MS. Pharmacologically active glucocorticoids include hydrocortisone, prednisone, prednisolone, methylprednisolone, dexamethasone (Fig. 5), betamethasone, fluticasone, and triamcinolone.

Efficacy. In relapsing-remitting MS, glucocorticoids are often prescribed to treat acute relapses. While they improve disability and promote lesion recovery, there is no effect on the risk of relapse [43–45]. Use of glucocorticoid therapy for the progressive stage of MS has provided some positive results [46–48], but a more controlled trial is warranted for validation.

Despite their proven utility in a number of peripheral inflammatory diseases, only one glucocorticoid clinical trial has been conducted for AD and no trials to date for PD or ALS. In the randomized controlled trial in AD patients, a low-dose regimen of prednisone was not found to be effective [49]. Prednisone, the sole glucocorticoid tested in a neurodegenerative disease clinical trial, is a prodrug that is converted to the active prednisolone form in the liver.

Mechanism of Action. Glucocorticoids diffuse into cells and bind to the intracellular glucocorticoid receptor forming a complex that translocates into the nucleus. There, it stimulates or represses expression of multiple gene targets by binding to glucocorticoid response elements. The glucocorticoid-glucocorticoid receptor complex blocks the synthesis of multiple inflammatory mediators by inhibiting immunological transcription factors (i.e., NF-kappaB and AP-1) and increases expression of anti-inflammatory enzymes (e.g., mitogen-activated protein kinase phosphatase-1).

Drawbacks. Glucocorticoids regulate multiple non-immunological genes, too. Prolonged use is associated with abnormal hypothalamic-pituitary-adrenal function (i.e., Cushing's syndrome), cardiovascular disease, osteoporosis, developmental problems, disturbed glucose metabolism, and mood disorders. There is also the potential problem of drug availability in the neural parenchyma. Penetration of glucocorticoids into the CNS is partially inhibited due to the p-glycoprotein efflux

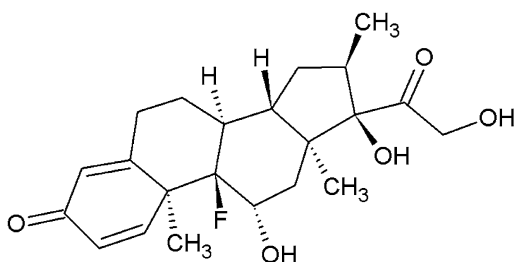

Fig. 5 Chemical structure of dexamethasone. Structure generated using ACD/ChemSketch 12.01

transporter expressed on the blood–brain barrier [50]. Glucocorticoids also bind the pregnane X receptor and increase p-glycoprotein activity [51]. This could augment blood–brain barrier resistance with continued usage.

In conclusion, the unsuccessful trial in AD by prednisone treatment may be due to inefficient access into the CNS. Increasing the dose is no solution due to severe metabolic side effect by glucocorticoids that include hyperglycemia, osteoporosis, growth impairment, and water retention abnormalities.

10 Nonsteroidal Anti-inflammatory Drugs (NSAIDs)

Description. Extracts from bark and leaves from willow trees were used to treat fever and inflammation more than a thousand years ago. The active component was later identified as salacin, the archetype for the class of drugs called nonsteroidal anti-inflammatory drugs (NSAIDs). The molecular target was later identified as prostaglandin-endoperoxide synthases (PTGS), more commonly called cyclooxygenases (COX). Constitutively expressed (COX-1 or PTGS1) and inducible (COX-2 or PTGS2) isoforms have been identified. These enzymes act on arachidonic acid—an unsaturated fatty acid released from cell membranes by phospholipases. COX-1 and COX-2 oxidize arachidonic acid forming prostaglandin H2, which can then be converted to a number of other prostaglandin isotypes or thromboxane by other tissue-specific enzymes. Early in the development of inflammation, there is an increase in the prostanoid class of fatty acids from enhanced COX-2 expression. These inflammatory products are critical in inflammation by interacting with selective G protein-coupled receptors (i.e., EP_{1-4}, DP, $FP_{A,B}$, IP, and TP) with effects including vasodilation, fever induction, leukocyte recruitment, and cytokine biosynthesis regulation. Over 20 nonselective COX inhibitors have been developed. They include acetylated (i.e., aspirin; Fig. 6) and non-acetylated (e.g., diflunisal) salicylates, para-aminophenol derivative (i.e., acetaminophen), propionic acids (e.g., ibuprofen), acetic acids (e.g., diclofenac), enolic acids (e.g., meloxicam), anthranilic acids (e.g., meclofenamate), and nonacidics (i.e., nabumetone). Selective COX-2 inhibitors include celecoxib, etoricoxib, and parecoxib.

Efficacy. In animal studies, COX inhibitors were initially found to extend survival in the transgenic mice expressing the mutant superoxide dismutase-1 gene found in

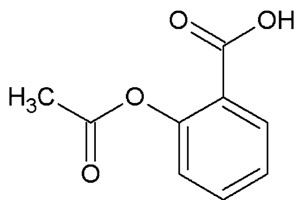

Fig. 6 Chemical structure of aspirin (acetylsalicylic acid). Structure generated using ACD/ChemSketch 12.01

some forms of familial ALS [52, 53]. However, a subsequent study using a larger sample size found no protective effect [54].

As opposed to their anti-inflammatory activity, some reports suggest COX inhibitors may also have immunostimulatory effects. By inhibiting COX-2, microglia activation and production of inflammatory mediators were enhanced in mice challenged with lipopolysaccharide or pro-inflammatory cytokines [55, 56]. Data from our laboratory suggest that ibuprofen moderately enhances lipopolysaccharide-stimulated IL-1β mRNA expression (Graber, unpublished observations). Furthermore, PGE2 and PGD2, products blocked by COX-2 inhibition, can be neuroprotective [57, 58]. In a model of excitotoxic neuronal death, COX inhibitors worsened kainic acid-mediated hippocampal neuron death [59].

Clinical trials for AD have been completed or are set to begin for celecoxib, ibuprofen, indomethacin, lornoxicam, naproxen, and rofecoxib. An intervention review showed that the rate of decline was not improved by either nonselective or COX-2-specific inhibitors [60]. One ALS trial with celecoxib showed no beneficial effect [61]. The unsuccessful trials in AD and ALS by COX inhibitors may be due to a combination of anti-inflammatory activity with counterproductive pro-inflammatory activity and enhanced neurotoxicity in the CNS.

Mechanism of Action. Oxidation of arachidonic acid, a fatty acid found in cell membranes, is enhanced during inflammation. The two main types of enzymes responsible are COX and 5-lipooxygenase. NSAIDs inhibit arachidonic acid from interacting with the COX enzyme by binding to a hydrophobic channel or acetylating a serine hydroxyl group near the active site. This decreases the levels of prostaglandin E2, prostacyclin, prostaglandin D2, and prostaglandin F2a produced during inflammation.

Drawbacks. Mucous production in the gastrointestinal tract is regulated by COX-1-mediated prostaglandins. Prolonged use of nonspecific COX inhibitors reduces mucous protection from gastric acids and can lead to ulcers. Selective COX-2 inhibitors circumnavigate these adverse gastrointestinal effects, but their prolonged use has been associated with cardiovascular dysfunction.

11 Minocycline (Dynacin®, Minocin®, Solodyn®, Minocycline, Minociclina, Minocyclinum, Minociclinum, and Borymycin)

Description. Minocycline (Fig. 7) is a semisynthetic tetracycline drug originally developed for its broad-spectrum antibacterial activity. It has also been purported to have anti-inflammatory effects and gain access into the CNS.

Efficacy. Minocycline's protective effects in animal models of neurodegenerative disease are inconsistent. In an MPTP toxicity model of Parkinson's disease, both protective [62, 63] and deleterious [64, 65] findings were found. Conflicting effects

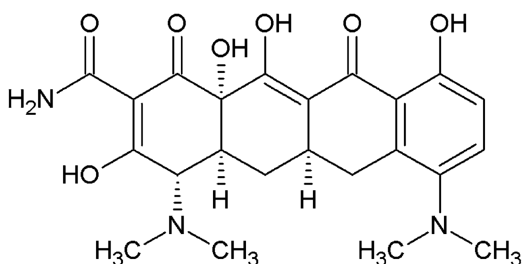

Fig. 7 Chemical structure of minocycline. Structure generated using ACD/ChemSketch 12.01

caused by minocycline were also found in transgenic mice expressing the mutant superoxide dismutase-1 gene found in some forms of familial ALS [54, 66].

Minocycline was tested in clinical trials for ALS and PD. The phase III clinical trial in ALS patients reported no improvement with minocycline and, in fact, showed an enhanced rate of deterioration [67]. The phase II clinical trial in PD patients found no improvement after 18 months of minocycline treatment [68].

Mechanism of Action. While minocycline is considered to have anti-inflammatory activity, it should be noted that these findings are inconsistent. An early study exploring its immunomodulatory potential in monocytes in vitro found minocycline enhanced IL-1β secretion [69]. A subsequent study revealed minocycline to enhance TNF-α secretion from monocytes stimulated with lipopolysaccharide, whereas minocycline suppressed TNF-α when monocytes were stimulated with phytohemagglutinin [70]. The unsuccessful trials in ALS and PD may be due to variable and condition-specific physiological anti-inflammatory activity by minocycline.

Drawbacks. Minocycline is well tolerated, but with some reports of dizziness and dermatological abnormalities such as rash and hyperpigmentation.

12 Thalidomide (Thalomid®, Distaval, Softenon, Sedoval, Talimol, Corronarobetin, Psychotablets, Theophilcholine, and Algosediv)

Description. Thalidomide (Fig. 8) was initially developed as a sedative with anti-emetic properties. It gained notoriety in the 1950s by causing serious birth defects after being prescribed to pregnant women to combat morning sickness. A physician in Israel continued to prescribe thalidomide in leprosy patients who were having trouble sleeping. Unexpectedly, the fever and painful skin nodules present in some patients, a condition called erythema nodosum leprosum, disappeared when treated with thalidomide. The beneficial effects were later thought to be due to anti-inflammatory properties. This lead to the revival of thalidomide, but with restrictions to avoid birth defects in pregnant women.

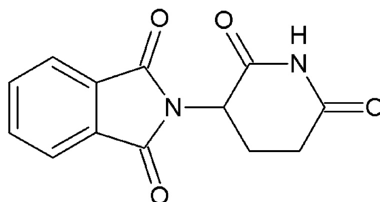

Fig. 8 Chemical structure of thalidomide. Structure generated using ACD/ChemSketch 12.01

Efficacy. Neuroprotective effects of thalidomide in the transgenic mice expressing the mutant superoxide dismutase-1 gene found in some forms of familial ALS were inconsistent [54, 71]. A clinical trial in ALS was conducted. No beneficial effect by thalidomide was found in a phase II trial [72].

Mechanism of Action. Thalidomide is considered to be a selective inhibitor of TNF-α expression. During an acute inflammatory state of erythema nodosum leprosum that sometimes occurs in leprosy patients, serum TNF-α levels have been reported to be variably increased and then suggestively decreased following thalidomide treatment [73, 74]. Cell culture experiments support thalidomide's inhibition of TNF-α expression with selective mRNA degradation as a likely mechanism [75, 76]. Evidence of thalidomide-mediated inhibition of TNF is equivocal, however. A more recent study examining erythema nodosum leprosum patients found no enhanced TNF-α, and thalidomide caused an increase in TNF-α serum levels along with clinical improvement [77]. Others have reported that thalidomide enhances TNF-α expression [78–82]. The mechanism and role of thalidomide in immunomodulation remains unclear. The unsuccessful trial in ALS may be due to a lack of physiological anti-inflammatory activity by thalidomide.

Drawbacks. A major risk with thalidomide is severe birth defects most commonly involving shortened limbs. Women who are or may become pregnant are excluded from thalidomide. Women prescribed thalidomide must remain abstinent or take two reliable methods of birth control. Since the mechanism for serious birth defects has not been fully elucidated, even males prescribed with thalidomide must restrict sexual contact and not donate sperm. Other adverse effects include deep vein thrombosis.

13 Identifying Novel Agents that Disrupt Neuroinflammation

The blood–brain/spinal barrier is a major obstacle in the development of drugs aimed at targeting microglia or infiltrated macrophages in the CNS. Many psychoactive drugs have been developed to penetrate into the CNS and augment neurotransmitter signaling. Physiochemical properties of these CNS-active drugs have been compared to other drugs known to not penetrate into the CNS [83–87]. This has led to physiochemical criteria that are favorable for entry into the CNS, which

should be helpful in developing anti-inflammatory agents to regulate parenchymal neuroinflammation. Chemical parameters that appear to be important include a molecular weight, numbers of hydrogen bond donors and acceptors, lipophilicity, and topographical polar surface area.

Anti-inflammatory agents can target a single inflammatory mediator or target an immunological transcription factor to regulate multiple inflammatory mediators. A solitary neurotoxic molecule which is responsible for parenchymal damage in all neuroinflammatory conditions has not been identified. It is likely that multiple inflammatory mediators work in concert to generate a neurotoxic environment. Neurons tend to be highly sensitive to free radical damage and excitotoxicity. Nitric oxide, superoxide anion, and glutamate can be produced by activated microglia. Cytokines such as interleukin-1beta and tumor necrosis factor-alpha may augment the effects by these neurotoxic molecules. These cytokines, in addition to chemokines like chemokine (C-C motif) ligand 2 (CCL2, MCP-1), may sustain neuroinflammation and recruit other microglia or infiltrating macrophages.

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is an important immunological transcription factor and an attractive therapeutic target. Novel anti-inflammatory agents that enter the CNS and block NF-κB signaling should be developed to test in models of neurodegenerative disease. Agents can target the NF-κB complex directly or indirectly through upstream signaling targets. Some promising indirect targets include glycogen synthase kinase-3β, nuclear-related factor E2-related factor 2, p38 kinase, and c-Jun N-terminal kinase.

14 Conclusion

Immunomodulatory drugs have been employed to treat a number of neurological illnesses. Agents that regulate the activity of the immune system peripherally or limit the influx of lymphocytes into the CNS have been most successful in relapsing-remitting MS. However, these same agents appear to be ineffective in the progressive stage of MS. Safe and reliable anti-inflammatory drugs that penetrate into and maintain activity within the CNS, especially those able to modify microglia activation, remain a promising therapeutic strategy for progressive MS, PD, ALS, and AD. Minocycline and thalidomide were unsuccessful to date, but their anti-inflammatory activity is not overwhelmingly convincing. Inhibitors to a single cytokine or inflammatory enzyme may not possess the effectiveness to alter disproportionate neuroinflammation. Developing new compounds that are CNS permeable and block key immune signaling molecules (e.g., NF-κB) that inhibit the expression of numerous inflammatory mediators is a promising approach. Ideally, these new drugs will not impede the targeted cells from their phagocytosis duties of clearing local debris (e.g., aberrant proteins, protein aggregates). An emphasis should be made to develop compounds with these properties as a primary or adjunctive treatment for neurodegenerative diseases and other neurological illnesses with a neuroinflammatory component.

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Therapeutic Potential of Cannabinoids

Guy A. Cabral and Melissa Jamerson

Abstract Cannabinoids alter the functional activities of immune cells and have potential to serve as agents for treatment of neuroinflammatory disorders. They readily access the brain, have low toxicity, and can target selective receptors on specific cell types. To date, two cognate receptors have been identified, the CB1R and the CB2R. The CB1R is expressed primarily in the CNS and testis, while the CB2R is found on immune cells, including microglia and other immunocytes that may be recruited into the CNS. Activation of the CB2R may prove therapeutically manageable in ablating neuropathogenic disorders such as Alzheimer's disease, multiple sclerosis, amyotrophic lateral sclerosis, Huntington's disease, HIV encephalitis, and closed head injury.

Keywords Alzheimer's disease • Amyotrophic lateral sclerosis • Cannabinoid • Cannabinoid receptor • Closed head injury • Endocannabinoid • HIV encephalitis • Huntington's disease • Multiple sclerosis • Neuroinflammation

List of Abbreviations

2-AG	2-Arachidonyl-glycerol
6-OHDA	6-Hydroxydopamine
Aβ	Amyloid-β
AD	Alzheimer's disease
AEA	Arachidonyl-2′-chloroethylamide
AIMs	Abnormal involuntary movements

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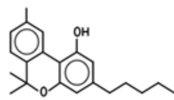
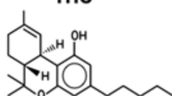
ALS	Amyotrophic lateral sclerosis
AMPA	α-Amino-3 hydroxy-s-methylisoxazole-4-propionic acid
APP	Amyloid-β precursor protein
BDNF	Brain-derived neurotrophic factor
$[Ca^{2+}]I$	Inducible calcium
CBD	Cannabidiol
CBN	Cannabinol
CFA	Complete Freund's adjuvant
CHI	Closed head injury
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CREAE	Chronic relapsing experimental autoimmune encephalomyelitis
DA	Dark agouti
Dtg	Double transgenic
EAE	Experimental autoimmune encephalomyelitis
EPSCs	Excitatory postsynaptic currents
FAAH	Fatty acid amide hydrolase
FALS	Familial amyotrophic lateral sclerosis
GP/GPe	Globus pallidus
HD	Huntington disease
HTT	Huntingtin gene
HU-211	Dexanabinol
huPBL	Human peripheral blood
ICAM-1	Intercellular adhesion molecule-1
iNO	Inducible nitric oxide
IPSCs	Inhibitory postsynaptic currents
JAK/STAT1	Janus kinase/signal transducers and activators of transcription
L-DOPA	3, 4-Dihydroxy-1-phenylalanine
LEW	Lewis
MAP	Mitogen-activated protein
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MPTP	1-methyl-1,2,3,6-tetrahydropyridine
NAPE-PLD	*N*-acyl phosphatidylethanolamine phospholipase D
NArPE	1-stearoyl, 2-docosahexaenoyl-sn-glycero-phosphoethanolamine-*N*-arachidonoyl
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NFTs	Neurofibrillary tangles
NK	Natural killer
NMDA	*N*-methyl-d-aspartate
NO	Nitric oxide
PET	Positron emission tomography
PD	Parkinson's disease
PGE2	Prostoglandin-E2
PLP	Proteolipid protein

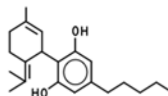
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PPMS	Primary progressive multiple sclerosis
SCID	Severe combined immunodeficient
SIV	Simian immunodeficiency virus
SNr	Substantia nigra pars reticulata
SOD1	Superoxide dismutase 1
tgHD	Transgenic Huntington's disease
THC	Delta-9-tetrahydrocannabinol
TMEV-IDD	Theiler's murine encephalomyelitis virus-induced demyelinating disease
VCAM-1	Vascular cell adhesion molecule-1
WIN	WIN 55,212-2

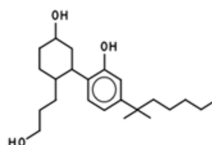
1 Introduction

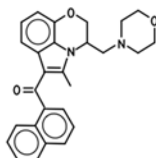
Cannabinoids are a category of compounds that includes phytocannabinoids, synthetic cannabinoids, and endocannabinoids (Fig. 1). Phytocannabinoids are terpenoid-like compounds derived from the marijuana plant, *Cannabis sativa*, which include Delta-9-tetrahydrocannabinol (THC), its major psychoactive component. THC also is immunomodulatory, a property that it shares with cannabidiol (CBD) and cannabinol (CBN). However, CBD and CBN are not psychoactive, a characteristic that renders them attractive candidates for therapeutic application. Synthetic cannabinoids that have been studied include CP-55940, HU-210, Win 55,212-2 (WIN), JWH-015, and arachidonyl-2′-chloroethylamide (ACEA). Endo-cannabinoids, or endogenous cannabinoids, are bioactive lipids that include anandamide (*N*-arachidonoylethanolamine, AEA) and 2-arachidonoyl glycerol (2-AG). Through the use of select radiolabeled cannabinoids, specific binding sites have been identified in mammalian brain and nonneuronal tissues that represent cannabinoid receptors. These have 7-transmembrane domains, are coupled to G inhibitory proteins, and are linked to signaling cascades that include adenylate cyclase and cAMP, mitogen-activated protein (MAP) kinase, and the regulation of intracellular calcium. While activation of the CB1R elicits psychotropic effects, activation of the CB2R results in the modulation of immune function in the absence of such effects. The CB2R is found in B lymphocytes, macrophages, monocytes, natural killer (NK) cells, and polymorphonuclear cells [1, 2], as well as the CNS during inflammatory states [3–6]. Additional cannabinoid receptors may exist, based on studies using CB1R knockout or CB1R/CB2R double-knockout mice [7–10]. Because activation of the CB2R dampens inflammatory responses in the absence of psychotropic effects, it has the potential to serve as a molecular target for attenuating inflammation linked to neuropathogenic disorders such as amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), closed head injury (CHI), HIV encephalitis (HIVE), Huntington's disease (HD), multiple sclerosis (MS), and Parkinson's disease (PD).

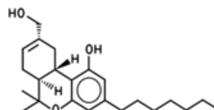


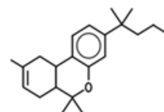


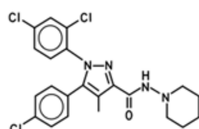


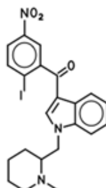


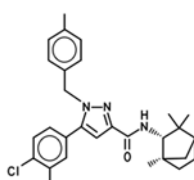


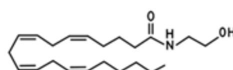












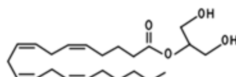


Fig. 1 Representative plant cannabinoids (phytocannabinoids), synthetic cannabinoids, and endogenous cannabinoids (endocannabinoids)

2 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a fatal chronic neuromuscular disease characterized pathologically by progressive degeneration of cortical motor neurons (upper motor neurons) and clinically by muscle wasting, weakness, and spasticity that progress to complete paralysis [11]. This adult-onset disease occurs in sporadic and familial (FALS) forms. The FALS form has been linked to mutations in the superoxide dismutase 1 (SOD1) gene that codes for a zinc- and copper-binding enzyme that neutralizes supercharged oxygen molecule (superoxide radical) by-products of normal cellular metabolism. Neuroinflammation is involved in ALS pathology and is mediated by proinflammatory cytokines, prostaglandins, and nitric oxide (NO). An animal model of FALS is the mutant SOD1 transgenic mouse. CBN

has been reported to delay onset of symptoms in this animal model [12]. Treatment of mice with nonselective cannabinoid receptor partial agonists has resulted in delay of disease progression and promoted prolonged survival in a mode linked to the CB2R [13]. Consistent with these observations, the CB2R agonist AM-1241 has been reported to contribute to prolonged survival of SOD1 mutant mice [13, 14].

Experimental ALS may be linked to an imbalance in levels of endocannabinoids. Levels of AEA and 2-AG, endocannabinoids that have been implicated in playing a neuroprotective role, were upregulated in the spinal cord of SOD1 transgenic mice [15]. Post-symptomatic treatment with WIN resulted in a delay in disease progression [16]. Examination of excitatory and inhibitory synaptic transmission in the striatum of SOD1 mice revealed a reduced frequency of glutamate-mediated spontaneous excitatory postsynaptic currents (EPSCs) and an increased frequency of GABA-mediated spontaneous inhibitory postsynaptic currents (IPSCs) [17], suggesting presynaptic defects in transmitter release. The sensitivity of CB1Rs controlling both glutamate and GABA transmission was potentiated in the ALS mice.

3 Alzheimer's Disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder that causes senile dementia. It has the presence of extracellular neuritic amyloid plaques and intracellular neurofibrillary tangles (NFTs) as defining neuropathological features. The amyloid plaques consist of extracellular aggregates of amyloid-β (Aβ) peptides [18–20] that often are surrounded by activated microglia and astrocytes. There are three major animal models of AD: a cholinergic deficit model in which the nonselective antagonists of muscarinic receptors scopolamine and atropine are used to mimic a cholinergic deficit, a brain Aβ infusion model in which diverse Aβ fragments are introduced by intracranial injection, and an Aβ precursor protein (APP) transgenic mouse model in which APP is expressed in the brain [21].

Intracerebroventricular administration of WIN to rats prevented Aβ-induced microglial activation, cognitive impairment, and loss of neuronal markers [4]. That prevention of neurodegeneration that occurred in AD by blockade of microglial activation [4] was supported by the observation that HU-210, WIN, and JWH-133 blocked Aβ-induced activation of cultured microglial cells. Chronic administration of WIN and JWH-133 was effective at reducing levels of COX-2 protein, tumor necrosis factor (TNF)-α mRNA, and cortical beta-amyloid in APP mice [22]. The PPAR-γ pathway was found to be crucial to WIN-induced neuroprotection [23]. WIN administration improved memory function and diminished elevated levels of hippocampal TNF-α and active caspase 3 and decreased the levels of nuclear NF-κB and the number of apoptotic (i.e., TUNEL-positive) neurons, suggesting a neuroprotective effect. Coadministration of the CB1R antagonist AM251 and the selective CB2R antagonist AR144528 led to complete abrogation of the WIN-induced effects indicating that the neuroprotective and anti-inflammatory actions against Aβ damage were linked to both the CB1R and CB2R.

The CB1R distribution in double transgenic (dtg) APP(swe)/PS1(DeltaE9) mice has been characterized [24]. It was shown that CB1R immunoreactivity in the CA1 and CA2/3 hippocampal regions was decreased in these mice. Reduced CB1R expression in the double transgenic mice was associated with astroglial proliferation and elevated expression of inducible nitric oxide (iNO) synthase and TNF-α. Chronic administration of the CB1R agonist ACEA at presymptomatic or at early symptomatic stages reduced the cognitive impairment observed in these transgenic mice [25]. ACEA-treated mice showed decreased astroglial response in the vicinity of Abeta plaques and decreased expression of the proinflammatory cytokine interferon (IFN)-γ in astrocytes.

However, the CB2R may also be involved in neuroprotection in AD. Selective stimulation of the CB2R by JWH-015 on cultured microglial cells resulted in suppression of IFN-γ-induced expression of CD40, a co-stimulatory protein found on antigen-presenting cells (i.e., B lymphocytes, monocytes, and dendritic cells) that activate T lymphocytes by binding to CD154 (CD40L) [26]. The CB2R-selective agonist also inhibited IFN-γ-induced phosphorylation of Janus kinase/signal transducers and activators of transcription (JAK/STAT1) and suppressed production of TNF-α and NO by microglia that was induced by IFN-γ or by Aβ peptide challenge in the presence of CD40 ligation. CBD also has been reported to modulate microglial cell function in vitro and to induce beneficial effects in an in vivo model of AD [27]. CBD decreased ATP-induced increase in intracellular calcium ($[Ca^{2+}]_i$) in cultured N13 microglial cells and in rat primary microglia. Thus, a variety of modalities that may, or may not, be linked to a cannabinoid receptor has been implicated in the moderation of experimental AD.

A limited number of studies have been conducted to assess effects of cannabinoids relative to neurodegeneration in human AD. Examination of normally aged and Alzheimer's human brains has revealed that, compared to normal brains, $[^3H]$CP-55940 binding was reduced in the hippocampal formation, caudate, substantia nigra, and globus pallidus [28]. The reduced $[^3H]$CP-55940 binding in areas of neuritic plaques and neurofibrillary tangles was associated with increasing age and with other forms of cortical pathology, suggesting that receptor losses were neither associated selectively with the pathology of AD nor with overall decrease in levels of cannabinoid receptor. On the other hand, senile plaques in AD patients have been shown to express the CB1R and CB2R together with markers of microglial activation, while CB1R-positive neurons were greatly reduced in areas of microglial activation [4]. The CB2R agonist JWH-015 was shown to induce the removal of native beta-amyloid from human frozen tissue sections [29], the effect involving the CB2R since the selective CB2R antagonist SR144528 prevented the JWH-015-induced plaque removal in situ. In addition, the CB2R and fatty acid amide hydrolase (FAAH) have been reported to be overexpressed selectively in neuritic plaque-associated glia in postmortem brains from patients with AD [30], suggesting that some elements of the endocannabinoid system serve as modulators of the inflammatory response associated with AD.

As in the case of other neuropathological processes, the role of endocannabinoids in neuroprotection has been examined. It has been suggested that these bioactive lipids stabilize lysosomes against Aβ-induced permeabilization and sustain cell survival by preventing Aβ-induced upregulation of the tumor suppressor protein, p53, and its interaction with the lysosomal membrane [31]. The CB1R was implicated in this stabilizing action. Consistent with these results, and through the use of a novel selective CB1R radioligand, it was demonstrated that CB1R density correlates inversely with Braak tau pathology, indicating an upregulation of binding of the CB1R radioligand in human brains [32]. Thus, enhanced endocannabinoid signaling, particularly around senile plaques, may exacerbate synaptic failure in AD. The balance in levels of endocannabinoids may be critical to the maintenance of neuroprotection in AD. Monoacylglycerol lipase has been reported to accumulate in CB1R-positive presynapses [33]. Subcellular fractionation revealed impaired monoacylglycerol lipase recruitment to biological membranes in postmortem Alzheimer's tissues, suggesting that disease progression slows the termination of 2-AG signaling. It has been suggested that $A\beta_{42}$-dependent impairment in brain AEA endocannabinoid mobilization contributes to cognitive dysfunction in AD [34]. Using liquid chromatography/mass spectrometry in the analysis of endocannabinoid-targeted lipidomics of postmortem brain samples from AD patients, it was demonstrated that the midfrontal and temporal cortex tissue from these patients contained, relative to control subjects, significantly lower levels of AEA and its precursor 1-stearoyl, 2-docosahexaenoyl-sn-glycero-phosphoethanolamine-N-arachidonoyl (NArPE) [34]. The levels of AEA and NArPE in midfrontal cortex correlated inversely with those of the neurotoxic amyloid peptide, amyloid beta-protein ($A\beta_{42}$).

4 Closed Head Injury

Trauma to the brain triggers a cascade that results in delayed edema, necrosis, and impaired function. Mediators such as cytokines that accumulate in the brain after injury play a role in the pathophysiology of closed head injury (CHI). Select cannabinoids, in their capacity as anti-inflammatory agents, have been reported to have a neuroprotective effect in CHI. HU-211 (dexanabinol), a noncompetitive *N-methyl-d-*aspartate (NMDA) receptor antagonist, was effective in improving motor function recovery in a model of CHI in rats [35]. The drug reduced BBB breakdown and attenuated cerebral edema possibly by dampening Ca^{2+} fluxes through NMDA receptor-mediated calcium channels and reducing depolarization-evoked Ca^{2+} fluxes [36]. HU-211 has been shown to be neuroprotective also in animal models of optic nerve crush, global ischemia, and focal ischemia [37]. HU-211 may be unique among putative neuroprotective agents in that it combines NMDA blocking and peroxy and hydroxy free radical scavenging properties that are toxic to neurons [38]. HU-211 also has been reported to inhibit TNF-α production [38]. In a randomized,

placebo-controlled, multicenter phase II clinical trial, patients treated with HU-211 achieved significantly better intracranial pressure/cerebral perfusion pressure control and exhibited a trend toward faster and better neurological outcome [39]. Endocannabinoids may play a role in neuroprotective effects in CHI. For example, 2-AG is released in mouse brain after CHI, and exogenous treatment of CHI with 2-AG exerts a neuroprotective effect mediated reportedly through the CB1R [40]. Additional studies have demonstrated that 2-AG decreases BBB permeability; inhibits the acute expression of TNF-α, IL-1β, and IL-6; and augments levels of endogenous antioxidants [41].

5 Human Immunodeficiency Virus Encephalitis

Human immunodeficiency virus encephalitis (HIVE) is characterized by progressive memory loss, intellectual deterioration, behavioral changes, and motor deficits. Brain damage is due primarily to the production and release of cytokines from immunocytes, neurotoxins such as glutamate and NO from activated monocytes and microglia, and HIV-specified gene products such as the transactivator protein Tat and the major envelope glycoprotein gp120 from infected monocytes and microglia. Two severe combined immunodeficiency (SCID) mouse models have been exploited to study HIV-induced neuropathogenesis. In the first model, second-trimester human fetal brain is inoculated into the anterior eye chamber, or fetal brain cells are injected into the interscapular fat pad [42, 43]. In the second model, human peripheral blood mononuclear cells, in concert with cell-free HIV, are injected intracerebrally into severe combined immunodeficient (SCID) mice [44]. Transgenic mouse models that express the complete HIV genome in neurons [45] and the HIV envelope glycoprotein gp120 in astrocytes [46] also have been developed. However, the simian immunodeficiency (SIV) model comes closest to replicating events that are associated with HIV infection of the human [47].

There have been a limited number of studies that has addressed the effects of cannabinoids in the context of models of HIV infection. THC has been reported to suppress immune function and to enhance HIV replication in a hybrid model in which human peripheral blood leukocytes were implanted into SCID (huPBL-SCID) mice [48]. Examination of brains of macaques with SIV-induced encephalitis has led to the suggestion that the endocannabinoid system participates in the development of HIV-induced encephalitis [49]. Expression of the CB2R was induced in perivascular macrophages, microglial nodules, and T lymphocytes, while the endogenous cannabinoid-degrading enzyme FAAH was overexpressed in perivascular astrocytes and astrocytic processes reaching cellular infiltrates. Recent studies suggest that chronic administration of THC does not increase viral load or aggravate morbidity in SIV disease [50]. It was suggested that overall mechanisms mediating the protective effect involved novel epigenomic regulatory mechanisms [51].

6 Huntington's Disease

Huntington's disease (HD) is an inherited neurodegenerative disorder characterized by cell dysfunction and death in the basal ganglia and cortex. The disease results in a major loss of the striatal GABAergic medium spiny neurons containing enkephalin and substance P that project principally to the globus pallidus (GP) and substantia nigra pars reticulata (SNr). The huntingtin gene (HTT or HD gene) is the IT15 gene that codes for the huntingtin protein, the mutation of which causes HD. R6 transgenic mice have served as a model of early pathogenic changes in HD [52].

There is accumulating evidence that modulation of the CB1R is a key pathogenic event in HD and that its activation may attenuate disease progression. Through the use of transgenic R6/2 mice, created by inserting exon 1 of the human IT15 mutant gene, and exhibiting 150 CAG repeats as well as signs of HD, a progressive decline of CB1R expression and abnormal sensitivity to CB1R were demonstrated [53]. In symptomatic R6/2 mice the levels of AEA, 2-AG, and palmitoylethanolamide (PEA) were decreased in the striatum, whereas few changes were observed in the hippocampus. Also, using a rat quinolinic acid model of striatal excitotoxicity in which it was shown that the neurotrophin brain-derived neurotrophic factor (BDNF) was involved in the pathophysiology of HD, a linkage of the upregulation of BDNF gene transcription to the CB1R was demonstrated [54]. Loss of enkephalin and CB1R in the external segment of the globus pallidus (GPe) and a loss of substance P and CB1R immune reactivity from the internal segment of the globus pallidus (GPi) have been observed [55]. In addition, using a double-mutant mouse model that expresses human mutant huntingtin exon 1 in a CBR1 null background, it was found that receptor deletion aggravated symptoms and pathology of HD [56]. It was postulated that the mutant huntingtin-dependent downregulation involved control of the CB1R gene promoter by repressor element 1 silencing transcription factor.

In addition, using [(18)F]MK-9470 and small animal positron emission tomography (PET), cerebral changes in CB1R binding in vivo in presymptomatic and early symptomatic rats of HD (tgHD) have been examined in relation to glucose metabolism, morphology, and behavioral testing for motor and cognitive function [57]. Early regional dysfunctions in endocannabinoid signaling involved the lateral globus pallidus and caudate-putamen were implicated. The effects of WIN on striatal synaptic transmission and on glutamate and GABA release in symptomatic R6/2 also have been investigated [58]. It was suggested that the balance between CB1Rs expressed by GABAergic and glutamatergic neurons and, thus, the net effect of CB1R stimulation was altered in HD mice.

7 Multiple Sclerosis

Multiple sclerosis (MS), also known as "disseminated sclerosis" or "encephalomyelitis disseminate," is a chronic, inflammatory demyelinating disease of the human CNS that primarily affects adults. It is characterized by degeneration of the myelin

sheath that covers axons in which T cells attack myelin resulting in stimulation of other immune cells to produce inflammatory factors. One model of MS is experimental autoimmune encephalomyelitis (EAE) that results from induction of primed myelin epitope-specific CD4+ T lymphocytes in mice immunized with myelin proteins or peptides in complete Freund's adjuvant (CFA) [59]. EAE also can be induced in inbred Lewis (LEW) and dark agouti (DA) rats using guinea pig myelin basic protein (MBP) emulsified in CFA [60]. DA strain rats also are highly susceptible to encephalomyelitis induced with proteolipid protein (PLP). Another model of MS is Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) characterized by Th1-mediated CNS demyelination and spastic hind limb paralysis [60].

THC has been reported to inhibit neurodegeneration in the EAE model and reduce the associated induced elevated level of glutamate in cerebrospinal fluid [61]. Studies using CB1R knockout mice suggest that signaling through the CB1R confers neuroprotection during EAE [62]. Changes in the status of the CB1R, as affected by the development of chronic relapsing experimental autoimmune encephalomyelitis (CREAE), have been reported to be region specific [63]. The CB1R exhibited downregulatory responses that were circumscribed to motor-related regions that were more marked during the acute and chronic phases of disease consistent with the efficacy of cannabinoid agonists in improving motor symptoms such as spasticity, tremor, and ataxia that are typical of MS. Control of spasticity as mediated by the CB1R also has been suggested from studies in which spasticity was induced in wild-type and CB1R-deficient mice following the development of relapsing EAE [64]. The CB1R has been implicated also in cannabinoid-mediated delay in progression of EAE in the rat [65]. Levels of AEA and 2-AG were decreased in motor-related regions such as the striatum and midbrain as well as in other brain regions. Studies employing a fetal mouse telencephalon aggregate cell culture model that allowed for comparison of tissue from CB1R knockout mice with their wild-type counterparts also have lent support for a role of the CB1R in neuroprotection in EAE [66]. WIN, acting preferentially through CB1R-mediated anti-inflammatory effects, had a positive effect in reducing neurological disability and improving motor coordination of EAE mice [67]. The CB2R also may play a role in experimental MS, especially as it relates to its inflammatory dimension. For example, it has been reported that the CB2R may be involved in the control of myeloid progenitor cell trafficking toward the inflamed spinal cord thereby contributing to microglial activation in EAE [68].

As in the case of other neuropathological processes impacted by cannabinoids, the accumulating data indicate involvement of the endocannabinoid system. Activation of the CB1R dampened the TNF-α-mediated potentiation of striatal spontaneous glutamate-mediated excitatory postsynaptic currents (EPSCs), believed to contribute to the inflammation-induced neurodegenerative damage observed in EAE mice [69]. Mice lacking FAAH, and thus expressing high brain levels of AEA, developed a less severe EAE. Also, exogenous administration of 2-AG ameliorated both acute and chronic EAE [70].

A protective role of cannabinoids has been obtained also using the TMEV-IDD model. Treatment with WIN, ACEA, and JWH-015 during established disease resulted in ablation of neurological deficits [71].A reduction in microglial activation, major histocompatibility complex (MHC) class II antigen expression, and number of CD4+ infiltrating T cells into the spinal cord was observed. WIN administration also resulted in amelioration of progression of symptoms of clinical disease in mice with preexisting TMEV-IDD [72]. Amelioration was associated with downregulation of virus and myelin epitope-specific Th1 effector functions (i.e., delayed-type hypersensitivity and IFN-γ production) and inhibition of CNS mRNA expression coding for proinflammatory cytokines. It has been proposed that WIN interferes with the progression of MS by downregulating adhesion molecules [73]. Administration of this cannabinoid at time of virus infection suppressed intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in brain endothelium and reduced perivascular CD4+ T-lymphocyte infiltration and microglial responses. The inhibition of brain adhesion molecules by WIN was attributed to an involvement of PPAR-γ receptors. WIN also has been reported to increase cyclooxygenase-2 (COX-2) expression and prostaglandin E2 (PGE2) release in endothelial cells following infection with Theiler's virus, an early event in the pathogenesis of TMEV-IDD [74]. Treatment with WIN resulted in upregulation of COX-2 protein and PGE2 release that was attributed to a mechanism independent of activation of the CB1R or CB2R.

Abnormalities in cerebrospinal fluid levels of endocannabinoids have been observed in patients with MS [75]. It has been observed using the TMEV-IDD model that neuronal cells responded to excitotoxic challenges by the production of endocannabinoids that exerted neuroprotective effects against excitotoxicity [76]. It has been suggested that AEA acts through the ERK1/2 and JNK pathways to downregulate IL-12p70 and IL-23 while upregulating IL-10. Downregulation of gene expression of IL-12p70 and IL-23 forming subunits of mRNAs in the spinal cord of TMEV-infected mice and ameliorated motor disturbances consistent with the existence of cross-talk between IL-12p70/IL-23 axis and IL-10 in microglial cells have been observed [77]. In addition, mice with established TMEV-IDD and treated with WIN exhibited a restoration of self-tolerance to a myelin self-antigen and amelioration of disease [78].

Studies using the passive variety of EAE, induced in LEW rats by adoptive transfer of myelin-reactive T lymphocytes, have indicated that WIN ameliorates clinical signs of disease and diminishes T-lymphocyte infiltration of the spinal cord [79]. The CB2R has been linked to playing a role in this process, although a receptor-independent mechanism, or a yet to be characterized novel cannabinoid receptor, also has been proposed. A number of studies have addressed the site of action at which cannabinoids are involved in mediating experimental MS. The cytokinetical and cellular events of axonal degeneration and demyelination following treatment with WIN have been examined [80]. Cannabinoid treatment rendered neurons less vulnerable than oligodendrocytes in EAE induced using MOG(1-125) in DA rats.

A relatively small number of studies have been conducted involving the functional relevance of cannabinoids in ameliorating MS in humans. An association has

been found between polymorphic markers at the gene encoding the CB1R (i.e., CNR1) gene and MS [81]. In a study in two case-control groups from Spain, genetic analysis of an AAT repeat microsatellite localized in the downstream region of the CNR1 gene suggested that the long (AAT) > or = (13) CNR1 genotypes could behave as risk factors for primary progressive multiple sclerosis (PPMS). The (AAT)n repeat of the CB1R gene was suggested as linked to disease progression in relapsing MS [82]. MS patients with the homozygous genotype for long AAT repeats in the CNR1 gene had more severe disease and higher risk of disease progression. In addition, the expression of the CB1R and CB2R and a key enzyme involved in synthesis of the endocannabinoid anandamide (i.e., *N*-acyl phosphatidylethanolamine phospholipase D [NAPE-PLD]) in autopsy brain samples from patients with MS has been examined [83]. These collective findings suggested a role for the endocannabinoid system in MS.

There is experimental evidence that the CB2R also may be involved in ablating effects of MS. It has been reported using a chronic model of MS in mice that clinical signs and axonal damage in the spinal cord are reduced by the AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor antagonist, NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione) [84] implicating AMPA as playing a direct role in excitotoxicity. HU-210 reduced the AMPA-induced excitotoxicity in vivo and in vitro in a mode attributed to activation of both the CB1R and CB2R. Direct suppression of CNS autoimmune inflammation in the EAE mouse model has been reported to be exerted through the CB1R on neurons and CB2R on autoreactive T cells [85]. The CB1R expression by neurons, but not T lymphocytes, was required for cannabinoid-mediated suppression of EAE, while expression of the CB2R by encephalitogenic T lymphocytes was shown to be critical for controlling inflammation associated with EAE. In addition, selective glial expression of the CB1R, CB2R, and FAAH has been reported as associated with MS, supporting a role for the endocannabinoid system in the pathogenesis and/or evolution of this disease [86].

In addition, the role of "indirect" agonists, compounds that reinforce endocannabinoid transmission divorced of psychotropic effects, has been examined in the context of animal models of MS [87]. Using the TMEV-IDD model, it was demonstrated that treatment with the selective AEA uptake inhibitor UCM707 during established disease resulted in significant improvement in motor function. Consistent with these observations, it has been reported that the endocannabinoid system is highly activated during CNS inflammation and that AEA protects neurons from inflammatory damage through a CB1R/CB2R-mediated rapid induction of microglial mitogen-activated protein kinase phosphatase-1 (mkp-1) that is associated with histone H3 phosphorylation of the mkp-1 gene sequence [88]. The cannabinoid system may share cellular mechanisms that affect brain disease progression [89]. For example, COX-2, CB2R, and P2X7-immunoreactivities are increased in activated microglia/macrophages of MS and ALS postmortem human spinal cord. It has been proposed that the increase of lesion-associated extracellular ATP contributes via P2X7 purinergic receptor activation to the release of IL-1β that, in turn, induces the production of COX-2 and downstream pathogenic mediators.

8 Parkinson's Disease

Parkinson's disease (PD) is characterized by the progressive loss of nigrostriatal dopamine neurons leading to motor disturbances and cognitive impairment. The therapeutic effects of THC have been tested in a 1-methyl-1,2,3,6-tetrahydropyridine (MPTP) marmoset model for PD [90]. THC improved activity and hand-eye coordination, but induced compound-related side effects. The role of WIN in protecting mouse nigrostriatal neurons from MPTD-induced neurotoxicity and neuroinflammation also has been investigated [91]. Activation of the CB2R resulted in protection against nigrostriatal degeneration by inhibiting microglial activation/infiltration. It has been indicated also that systemic administration of WIN ameliorates 3,4-dihydroxy-l-phenylalanine (L-DOPA)-induced abnormal involuntary movements (AIMs) in the 6-hydroxydopamine (6-OHDA) rat model of PD and reverses L-DOPA-induced PKA hyperactivity via a CB1R-mediated mechanism [92]. It was proposed that the results suggested that activation of CB1Rs, as well as reduction of striatal PKA hyperactivity, could be an effective strategy for the treatment of L-DOPA-induced dyskinesias [92]. Acute injections of L-DOPA or the CB1R antagonist rimonabant have been reported equally to improve contralateral forepaw stepping in rats with unilateral 6-OHDA lesions. The combined use of these compounds resulted in improved stepping more than when either drug was used alone [93].

9 Conclusions

A recurrent theme is that differential expression, or dysfunctional expression, of constituent elements of the endocannabinoid system has been linked to a variety of neuropathological processes. The endocannabinoid system is comprised of bioactive lipids such as AEA and 2-AG, cognate receptors, and enzymes that are involved in their biosynthesis and degradation. Select cannabinoids have been reported to ameliorate a variety of neuropathological processes in experimental animals. To date, two "cannabinoid" receptors have been identified, the CB1R and the CB2R, although GPR55 has been implicated as an additional "cannabinoid" receptor. The CB1R appears to be critical for the overall homeostatic balance and regulation of the CNS, and although data indicate that this receptor may attenuate cognitive impairment and degeneration in disorders such as AD, ALS, and MS, its potential usefulness as a molecular target is tempered by the recognition that its activation engenders psychotropic effects. On the other hand, the CB2R is not linked to psychotropic effects, and its activation results in the modulation of inflammatory immune responses. The CNS contains a diverse array of cell types that include neurons, oligodendrocytes, and glial cells. While astrocytes are the predominant glial cell type, microglial cells as resident macrophages of the brain function as immune effector and accessory cells. Microglial cells express phenotypic markers for macrophages as well as those for the CB1R and CB2R [94, 95]. Immune

responses from microglial cells, along with those from immune cells recruited into the CNS during neuropathological processes, result in the release of proinflammatory cytokines, chemokines, and other factors that secondarily activate other immune cells, contributing further to the expansion of inflammatory events. Thus, the CB2R has the potential to serve as a useful molecular target for attenuating untoward immune inflammatory responses and for compensating a dysregulated endocannabinoid response in a variety of neuropathological processes.

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