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INNATE IMMUNITY AND NEURODEGENERATIVE DISORDERS

Topic Editors
Roger A. Barker and Francesca Cicchetti

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INNATE IMMUNITY AND NEURODEGENERATIVE DISORDERS

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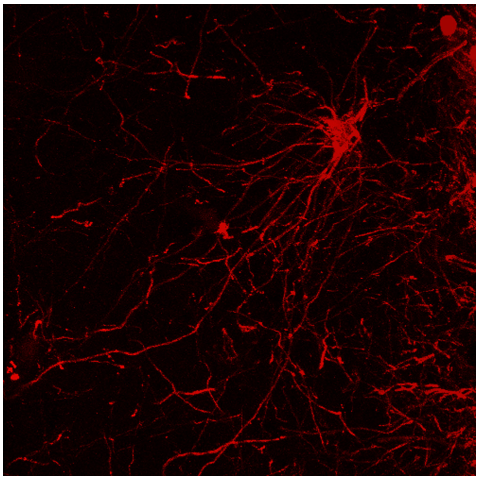

Astrocyte on the periphery of a fetal allograft in a patient with Huntington's disease.
Photo taken by Giulia Cisbani, PhD, Université Laval (Cicchetti's laboratory)

Inflammation of the brain in the context of neurodegenerative disorders is an area of intense debate and discussion, not least in terms of its pathogenic significance and the extent to which it drives disease processes and pathology. This inflammation can take several forms including innate responses recruiting microglia, humoral responses involving antibody, complement mediated processes and cellular T-cell activation, of which the role and extent of each may differ between diseases. Whilst some diseases have been more intensely linked to inflammation and long-term degeneration (e.g. MS), more traditional chronic neurodegenerative disorders have been thought of in terms of intrinsic neuronal pathology with a secondary innate response. However, it has been described that microglia activation is an early event of many degenerative disorders and evidence is accumulating that it may play a critical role in actually causing pathology and driving disease processes. If true, this would have major therapeutic implications, but what is the evidence that this is the case?

The initial observations by Patrick McGeer's group of post-mortem tissue from patients with Parkinson's disease revealed the presence of activated brain microglia and has thus lead to the hypothesis that chronic inflammation could participate to neuronal degenerative processes. The significance of these original observations has only been recently revisited, and the development of more powerful tools to study the brain immune response has certainly contributed to this field of research. Chronic inflammation in the brain can take many forms but of particular interest has been the resident microglia and the role they play in this process. In this context, microglia have often been thought to become activated only after the disease has begun and then to contribute minimally to the degenerative process. Emerging new concepts challenge this view by proposing that microglial senescence, for example, may release the disease process and/or accelerate it. In addition, microglia, once activated, can adopt different phenotypes which can be both pro-inflammatory and pro-repair and may impact not only on the healthy adult neuronal population but on those new neurons derived from neurogenic niches of the adult brain.

In this Research Topic, we attempt to explore this by first considering the innate immune responses in the brain and the methods by which they can be studied experimentally and in patients with various neurodegenerative disorders. This sets the scene for then discussing a range of different disorders including Alzheimer's, Parkinson's, Huntington's disease and amyotrophic lateral sclerosis. These papers seek to discuss the evidence for an innate immune response and whether this is beneficial or detrimental, as well as its therapeutic implications.

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EDITORIAL
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Neurodegenerative disorders: the Glia way forward

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Keywords: immunity, inflammation, neurodegenerative disorders, therapeutics, animal models

The realization that inflammation can affect the brain is not a new one, and many diseases of the CNS, such as multiple sclerosis (MS), have been shown to clearly respond to agents that target the immune system at one level or another. However, it is only more recently that the immune system has been shown to play a role in the normal development and homeostasis of the brain as well as contributing to disorders that have traditionally been thought of as being purely neurodegenerative in nature, such as Huntington's (HD) or Alzheimer's disease (AD). In this special issue of Frontiers in Neuropharmacology, we have sought to bring together experts in this new emerging area of neurobiology.

The best place to start in this special issue is with the paper by McGeer and McGeer (2011) as they lay out the history of the field and the skepticism that it has generated en route, and which still exists today. Indeed, many neurologists and neurobiologists still argue that any inflammatory or glial response in neurodegenerative disorders is simply a secondary phenomenon of no pathogenic or therapeutic relevance. However, evidence has accumulated in favor of it having a role and part of this relates to our ability to better image the microglial responses in patients with neurodegenerative disorders of the brain and Politis et al. (2012) explore this in their review on PET and TSPO radioligands.

A number of our papers summarize the role of microglia in ongoing CNS activities. Ekdahl (2012), for example, discusses how microglia may regulate adult neurogenesis in the healthy and diseased brain possibly through direct synaptic contacts. This is taken on by Streit and Xue (2012) who put forward the theory that the loss of the normal neuroprotective function of microglial, as a result of aging, leads to disease states, especially AD. Neher et al. (2012), on the other hand, discuss how microglia can directly phagocytose apparently healthy neurons as well as those in the diseased brain and by so doing are primary players in the cell loss seen in neurodegenerative disorders. This is explored in more detail by Phani et al. (2012) in Amyotrophic Lateral Sclerosis (ALS); Solito and Sastre (2012) in AD; and Huang and Halliday (2012) for Parkinson's disease (PD).

Finally in the first of our two reviews, we discuss how the astrocytic and microglial responses are different in a range of new experimental therapies for neurodegenerative disorders. These therapies include chronic deep brain stimulation, which induces a variable astro- and microglial response and in some cases even a collagenous band at the electrode tip. In contrast, growth factor infusions and gene therapies produce much less of a glial response whilst neural grafts vary in the intensity of response they provoke, in part as a function of disease state. The significance of these different responses (the type and magnitude) remains unknown, but what is emerging from these studies is the complex interplay that exists between glial cells and neurons and how astrocytes, in particular, can influence the activity of large neural and blood networks (Cicchetti and Barker, 2014). These new observations will likely help explain the variance seen in clinical trials using these agents in a range of neurodegenerative disorders.

In our second review we summarize the major findings of the papers that make up this special edition and how we can build on this work as we move forward into a new therapeutic era, which includes the use of a whole new range of immune modulating therapies (Barker and Cicchetti, 2012).

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History of innate immunity in neurodegenerative disorders

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The foundations of innate immunity in neurodegenerative disorders were first laid by Del Rio Hortega (1919). He identified and named microglia, recognizing them as cells of mesodermal origin. Van Furth in 1969 elaborated the monocyte phagocytic system with microglia as the brain representatives. Validation of these concepts did not occur until 1987 when HLA-DR was identified on activated microglia in a spectrum of neurological disorders. HLA-DR had already been established as a definitive marker of immunocompetent cells of mesodermal origin. It was soon determined that the observed inflammatory reaction was an innate immune response. A rapid expansion of the field took place as other markers of an innate immune response were found that were made by neurons, astrocytes, oligodendroglia, and endothelial cells. The molecules included complement proteins and their regulators, inflammatory cytokines, chemokines, acute phase reactants, prostaglandins, proteases, protease inhibitors, coagulation factors, fibrinolytic factors, anaphylatoxins, integrins, free radical generators, and other unidentified neurotoxins. The Nimmerjahn movies demonstrated that resting microglia were constantly active, sampling the surround, and responding rapidly to brain damage. Ways of reducing the neurotoxic innate immune response and stimulating a healing response continue to be sought as a means for ameliorating the pathology in a spectrum of chronic degenerative disorders.

Keywords: HLA-DR, Alzheimer disease, Parkinson disease, complement, neuroinflammation

BACKGROUND

Pio Del Rio Hortega, one of the greatest of all neuroscientists, established the basic foundation of neuroinflammation with his classic 1919 paper "El tercer elemento de los centros nerviosis" (Del Rio Hortega, 1919). He had developed an ammoniacal silver carbonate modification of the Golgi technique. With this new method he was able to identify small spidery cells which he named microglia. He recognized that they were of mesodermal origin, and that they migrated to the brain in late embryonic life. He also recognized their phagocytic capacity by examining their reaction to stab wounds. He categorized their morphology as resting, ameboid, and reactive. By further modifying his technique, he later recognized another type of glial cell which was of epithelial origin and had a sparse cytoplasm. He named these cells oligodendroglia, perhaps because of the difficulty he encountered in staining them.

These two glial cell types made up "the third element" which had baffled Ramon y Cajal. Cajal, Hortega's mentor and employer, had clearly identified astrocytes with his methodology, and knew there were still unclassified glial cells. He thought the cells identified by Hortega might be another astrocytic type that was resistant to staining. He objected to Hortega's desire to publish his results on oligodendroglia, and when Hortega went ahead, believing others would make the same discovery, Cajal dismissed him. Hortega's own account of these travails, which he never published, were obtained after his death, later appearing in part in Haymaker and Adams treatise on the histopathology of the nervous system (Haymaker and Adams, 1980a, pp. 484–485).

Hortega's travails were not limited to his falling out with Cajal. The Madrid laboratory he had set up after his departure from Cajal was bombed out in the Spanish civil war. He moved for a time to Paris, and then to Oxford, but was uncomfortable in these locations. As a Spaniard opposed to Franco, he emigrated to Buenos Aires in 1938 where, in 1945, he died of cancer.

His findings and their interpretation were still being actively challenged long after his death. Some scientists supported his conclusions, others did not. Wilder Penfield was the first prominent investigator to uphold Hortega. He described, in the first issue of the American Journal of Pathology, the various stages of microglia, going from resting to reactive (Penfield, 1925). Numerous others did differing experiments, interpreting microglia cells as being of epithelial origin with unknown function. A detailed account of the continuing controversy, which extended for over six decades, appears in Haymaker and Adams (1980b, Chapter VI). This unjustified controversy may help to explain why Hortega never received a Nobel prize for his epic work.

The next giant step was taken by Ralph van Furth. He elaborated the concept of a monocyte phagocytic system to explain the origin and function of resident phagocytes throughout the body. The Memoranda endorsing the concept, which was introduced at the Conference on Mononuclear Phagocytes held in Lieden in 1969, is available on the web Chttp://www.ncbi.nlm.nih.gov/pmc/articles/PMC2480884/pdf/bullwho00193-0157.pdf. He and his colleagues had studied labeled monocytes and their marrow precursors and followed their appearance as typical macrophages in various organs, including brain (Van Furth and Cohn, 1968). This concept replaced the vague theory of a reticuloendothelial system introduced by Aschoff (1924) which, despite its shortcomings, had become entrenched in medical teaching.

While van Furth's concept of a monocyte phagocytic system was widely accepted, its relationship to brain microglia was not.

Fujita in particular opposed the idea that microglia were the brain representatives of this system. He injected tritiated thymidine into chick embryos and identified labeled brain cells which he interpreted as glioblasts of subependymal origin (Fujita, 1965). He did later studies of human embryonic brain tissue and reached a similar conclusion, that brain microglia were of epithelial and not mesenchymal origin (Fujita, 1973; Fujita and Kitamura, 1973).

Oemichan et al. (1979) and Wood et al. (1979) also concluded that brain microglia could not be phagocytes of monocytic origin. The reason was that they could not detect the same surface antigens on microglial cells that they were able to detect on peripheral monocytes. The idea became established that inflammation of the brain did not involve microglia, and did not occur unless there was invasion of the brain by peripheral monocytes, which then became transformed into macrophages.

IDENTIFICATION OF HLA-DR POSITIVE MICROGLIA IN HUMAN BRAIN

The entry of our laboratory into the neuroinflammatory field was entirely serendipitous. Several researchers had suggested that AD might result from a herpes infection entering through the nasal passages because of involvement of the rhinencephalon (Ball, 1982). Our effort over some years to detect herpes virus in the CNS had failed, although we were able to detect it in some cases in the trigeminal nucleus, the source of herpes labialis (Walker et al., 1989). We sought the advice of local immunologists regarding what might be a broader indicator of a viral infection, and were counseled to look for HLA-DR since this class II glycoprotein would be responsible for presenting viral epitopes to T-cells. We were even presented with a gift of the HB-104 cell line, known to produce high levels of antibodies against HLA-DR.

The results astonished us. A plethora of cells was visible in AD tissue with a morphology that was completely unfamiliar to us. However we were able to confirm that they were microglia by showing them to the founder of our laboratory, Dr. William Gibson, who had been a student and biographer of Hortega (Prados and Gibson, 1946). The morphology was identical to that published by Del Rio Hortega (1919), permitting us to sense in a very small way the excitement he must have experienced.

As many frustrated investigators well know, peer reviewers, whether evaluating applications for research grants, or papers submitted to journals, are typically well versed in the dogmas of the day. Findings that radically conflict with established views are too often dismissed. Our data conflicted with two dogmas. The first was that microglia were of epithelial origin and were not immunocompetent cells. The second was that brain inflammation was not a characteristic of AD. A pathological journal rejected our initial manuscript, reasoning that the absence of a neuropathologist as an author explained the faulty conclusions. A peer reviewer for a renewal of our grant dismissed it with the comment "the hypothesis is ridiculous." Nevertheless, our first paper appeared as a brief report in Neuroscience Letters (McGeer et al., 1987).

This final validation of Hortega depended on the availability of HB-104, a powerful and highly specific monoclonal antibody. The field of immunohistochemistry had been opened by Cesar Milstein's work on the technique for producing monoclonal antibodies. He was a co-winner of the Nobel prize in 1984 for this accomplishment. Then, as now, the ability to visualize brain biochemistry through the eyes of immunohistochemistry depends on the strength and specificity of the antibodies employed. HB-104 was a particularly good antibody, permitting us to make a clear distinction, which had eluded other investigators, between HLA-DR positive microglia and GFAP positive astrocytes.

We were not the first to report class II staining of brain cells. Lampson and Hickey (1986) had reported class II activity in occasional cell bodies of human brain with the "morphologic appearance of microglia or astrocytes." Similarly deTribolet et al. (1984) reported HLA-DR positive cells in the white matter of normal brain which they interpreted as being astrocytes (deTribolet et al., 1984). Rogers et al. reported HLA-DR positive microglia and astrocytes in AD in an abstract at the Society of Neuroscience meeting in 1986 but, as in our case, formal publication was held up by disbelief in the findings. Their work finally appeared in 1988 (Luber-Narod and Rogers, 1988; Rogers et al., 1988).

These data led to a number of unanswered questions. What was the source of the inflammation in AD? Was it a special phenomenon or a general one that applied to many chronic neurological disorders? If it was a general phenomenon, was it localized to the brain or did it involve the peripheral immune system? Was it helpful or harmful to ongoing degenerative processes? What were the implications for treatment? Exploration of these questions led to the opening up of an important new field of neuroscience, namely neuroinflammation.

ESTABLISHMENT OF NEUROINFLAMMATION AS A DISTINCT FIELD OF NEUROSCIENCE RESEARCH

The question as to whether or not the presence of activated microglia in brain was a special one applying to AD, or a general phenomenon was quickly answered. HLA-DR activated microglia were observed in a variety of degenerative neurological conditions, including Parkinson disease (PD), Pick disease, ALS, Huntington disease, multiple sclerosis, AIDS encephalopathy, parkinsonism dementia of Guam, and the Shy–Drager syndrome (McGeer et al., 1988a). Their phagocytic function was easily demonstrated by melanin being observed within the HLA-DR positive microglia of the SN (McGeer et al., 1988b). Each of these diseases has a differing etiology, so the activated microglial response had to be the consequence of initiating factors in each condition and not the fundamental cause.

At this same time, the laboratory of Kreutzberg in Germany was carrying out work of a more fundamental nature. His team was utilizing the facial nerve axotomy model to examine the CNS reaction to a sterile lesion outside of the CNS. They found activated microglia enveloping damaged neurons of facial nerve cell bodies, with phagocytosis of dead cells taking place. They concluded that microglia might function as antigen presenting cells and thus be the effector cells responsible for recruitment of lymphocytes to the brain resulting in an inflammatory reaction. A review of their results appeared in the first volume of Glia (Streit et al., 1988) and a later review (Kreutzberg, 1996).

Further groundwork was laid by four key papers which appeared in a special issue of Glia in 1993 devoted to microglia. They were by Ling and Wong (1993) describing the origin and nature of microglia; Dickson et al. (1993) detailing cytokines and microglia in Alzheimer disease and AIDS; Banati et al. (1993)

on the cytotoxicity of microglia; and McGeer et al. (1993) on microglia in neurodegenerative diseases generally.

Since our laboratory and that of Joe Rogers, who had founded the Sun Health Research Institute, had identified HLA-DR activated microglia in AD, we joined forces for further investigation of the phenomenon, particularly the role of complement. The name complement was introduced by Paul Ehrlich in the 1890's to explain the heat labile factor in serum which helped antibodies to kill microorganisms. As a result, complement was considered to be a peripherally generated system for assisting the activity of antibodies. It was therefore believed that complement factors would only be found as an accompaniment to immunoglobulin antibodies.

Eikelenboom and Stam (1982) were the first to report the presence of complement factors in AD senile plaques. Previously it had been reported that immunoglobulins were associated with amyloid deposits, which was consistent with prevailing theory (Ishii and Haga, 1975). Our laboratory was able to detect the opsonizing components of the classical complement pathway in association with plaques, and the membrane attack complex in association with dystrophic neurites, consistent with bystander lysis occurring in AD (McGeer et al., 1989).

Working with Joseph Rogers, we were unable to detect immunoglobulins in AD tissue using a host of antibodies. Then Rogers explored the idea that beta amyloid protein itself might be an activator of complement. In a classic paper he and his colleagues demonstrated that immunoglobulin immunostaining did not colocalize with complement, and that beta amyloid protein and its N-terminal fragments bound to C1q directly, thus initiating the complement cascade independently of antibodies (Rogers et al., 1992). But what was the source of the complement proteins? It was soon determined from RNA studies that brain itself was the source (Johnson et al., 1992; Walker and McGeer, 1992). Meanwhile evolutionary studies were underway, establishing that the complement system could be traced back at least as far as horseshoe crabs and that it was the mainstay of innate immunity in most primitive organisms (Zhu et al., 2005). It far predated the antibody producing adaptive immune system which is an invention of higher vertebrates.

Two principles which had broad implications for the developing field of neuroinflammation emerged from this joint endeavor. The first was establishing that complement was part of the innate immune system and further that it had the potential of exacerbating the pathology through formation of the membrane attack complex. The second was showing that the inflammatory reaction in AD did exacerbate the pathology. We decided to explore whether patients on long term anti-inflammatory therapy were relatively spared from AD. We selected rheumatoid arthritic patients because onset of the disease typically appears at an earlier age than AD and involves aggressive anti-inflammatory therapy. The results showed an estimated sixfold sparing of AD in rheumatoid arthritic patients compared with age matched general populations (McGeer et al., 1990). Many subsequent epidemiological studies, in which consumption of NSAIDs in particular were targeted, confirmed these general findings (McGeer and McGeer, 1995).

Rogers was then motivated to establish a Neuroinflammation Working Group of 37 investigators to assemble the rapidly accumulating data on inflammation in AD. The conclusion of the group was that the data represented "a virtual textbook of inflammatory mediators." Included were complement proteins and their regulators, inflammatory cytokines, chemokines, acute phase reactants, prostaglandins, proteases, protease inhibitors, coagulation factors, fibrinolytic factors, integrins, anaphylatoxins, free radical generators, and other unidentified neurotoxins (Akiyama et al., 2000). All are products of the innate immune system of brain, with major contributions coming from neurons, astrocytes, microglia, and endothelial cells.

Parkinson disease was another chronic degenerative disorder where activated microglia were detected in association with the SN lesions (McGeer et al., 1988b). An accidental model of PD provided new insights into the consequences of chronic neuroinflammation. Langston et al. (1984) had identified a group of young drug users who suddenly developed a parkinsonian syndrome. The causative agent (MPP+) was a metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a contaminant in the street drug they were using. The condition was progressive, and autopsy studies on those who had died from this exposure years previously showed neuroinflammation of the SN similar to that observed in PD (Langston et al., 1999). We found a parallel situation in monkeys. They showed nigral degeneration and activated microglia in the SN 5.5–15 years after systemic exposure to MPTP (McGeer et al., 2003). These findings represent the clearest example of how neuroinflammation, once initiated, can persist, and cause continuing neurodegeneration.

Activated microglia are the pivotal cells. Their functioning *in vivo* has been remarkably demonstrated by the movies of Nimmerjahn et al. (2005). They developed mice transgenic for a green fluorescent protein in microglial cells and used two-photon microscopy through a window in the skull to observe their behavior. Microglial cells in the normal state were found not to be dormant, as implied by their traditional designation as resting, but were extremely active, continuously extending, and retracting their processes to sense their environment. When activated by a laser lesion of a capillary, they surrounded the lesion and phagocytosed the leaking blood.

The innate immune system is the body's first line of defense. As the Nimmerjahn movies demonstrate, it can act immediately. As shown in chronic degenerative diseases, as well as the MPTP model, it can maintain its activity indefinitely without significant engagement of the adaptive immune system. The adaptive immune system is slower to react but more powerful and specific in attacking targets. It depends upon appropriate presentation of epitopes to lymphatic organs so that lymphocytes can be cloned to attack targets where that epitope is exposed. There is a long list of diseases where the adaptive immune system directs self attack on healthy tissues. These conditions are known as autoimmune disorders. They differ from AD and other chronic neurodegenerative disorders where the adaptive immune system does not become significantly engaged. To distinguish between the two, we have suggested that such diseases be described as autotoxic disorders (McGeer and McGeer, 2000).

In theory, the self destruction in autotoxic disorders should be milder and more amenable to therapeutic intervention than the self destruction in autoimmune disorders. The question is how to ameliorate the autotoxic response? Anti-inflammatory and antioxidant approaches have been the most widely utilized to date. But another, and potentially more effective method may be possible. That is to transform microglia from the attack mode, which has been so well characterized, to a healing mode. Such a transformation might result in enhanced phagocytotic activity, coupled with a switch from expressing inflammatory cytokines such as IL-1 and TNF to expressing anti-inflammatory cytokines such as IL-4 and IL-10. In the process the beneficial effects of phagocytosis might be enhanced. For example, suppressing the CD-40/CD 40L interaction in transgenic mice enhances the phagocytic activity of microglia and increase Aβ clearance (Tan et al., 2002). Clearly there is much still to be learned. It can be said that, despite the huge expansion of activity that has taken place in recent years, it is still a field in its infancy.

SUMMARY

Our understanding of the innate immune system in brain commenced with recognition of a single marker, HLA-DR, on a single cell type, microglia, in a single disorder, Alzheimer disease. Twenty five years later, more than a thousand innate immune system markers have been identified which are associated with neurons, astrocytes, oligodendrocytes, and endothelial cells, as well as microglia.

They include, but are not limited to, complement proteins and their regulators, cytokines, chemokines, acute phase reactants, prostaglandins, proteases, protease inhibitors, coagulation factors, fibrinolytic factors, anaphylatoxins, integrins, and free radical generators. They are found in a spectrum of neurological diseases. Some stimulate inflammation, others inhibit it. Shifting the balance from a mode of attack to one of healing holds promise of having significant therapeutic benefit in a spectrum of degenerative diseases. Clearly there is much still to be learned. It can be said that, despite the huge expansion of activity that has taken place in the last 25 years, it is still a field in its infancy.

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Imaging of microglia in patients with neurodegenerative disorders

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Microglia constitute the main immune defense in the central nervous system. In response to neuronal injury, microglia become activated, acquire phagocytic properties, and release a wide range of pro-inflammatory mediators that are essential for the annihilation of the neuronal insult. Although the role of microglial activation in acute neuronal damage is well defined, the pathophysiological processes underlying destructive or protective role to neurons following chronic exposure to microglial activation is still a subject of debate. It is likely that chronic exposure induces detrimental effects by promoting neuronal death through the release of neurotoxic factors. Positron emission tomography (PET) imaging with the use of translocator protein (TSPO) radioligands provides an *in vivo* tool for tracking the progression and severity of neuroinflammation in neurodegenerative disease. TSPO expression is correlated to the extent of microglial activation and the measurement of TSPO uptake *in vivo* with PET is a useful indicator of active disease. Although understanding of the interaction between radioligands and TSPO is not completely clear, there is a wide interest in application of TSPO imaging in neurodegenerative disease. In this article, we aim to review the applications of *in vivo* microglia imaging in neurodegenerative disorders such as Parkinson's disease, Huntington's disease, Dementias, and Multiple Sclerosis.

Keywords: dementia, Huntington, microglia, multiple sclerosis, Parkinson, PET, PK11195

INTRODUCTION

Microglia account for approximately 10% of the adult brain cell population and represent the first and main form of immune defense in the central nervous system (CNS; Lawson et al., 1990; Kreutzberg, 1996). Upon CNS injury and disease, microglia become activated and they can be identified and distinguished from their resting phenotype based on a combination of morphological and immunophenotypic changes (Dheen et al., 2007; Ransohoff and Perry, 2009). Microglia initiate immune responses by enhancing the expression of toll-like receptors (TLR) and a wide range of pro-inflammatory mediators such as tumor necrosis factor-alpha (TNFα), interleukin (IL)-1 and IL-6 for the removal of the CNS threat (Suzumura et al., 1996; Hartlage-Rübsamen et al., 1999; Bsibsi et al., 2002; Floden et al., 2005). Microglia may also fulfill a neuroprotective role *via* the release of neurotrophic factors and promotion of neurogenesis for the restoration of normal physiology (Stadelmann et al., 2002). Hence, the acute inflammatory response is generally beneficial, as it tends to minimize injury and promotes tissue repair. However, chronic neuroinflammation is closely related to various neurodegenerative disorders such as Parkinson's disease (PD), Huntington's disease (HD), Dementias, and Multiple sclerosis (MS), although the consequences of sustained microglial activation in these diseases is unclear.

Activated microglia upregulate expression of the 18-kDa translocator protein (TSPO; Chen and Guilarte, 2008; Cosenza-Nashat et al., 2009; Scarf et al., 2009). TSPO are found in abundance throughout the body in peripheral organs (i.e., liver and adrenals), and hematogenous cells, but are present at very low levels in the normal healthy CNS (Banati, 2002). Functionally, TSPO has several biological functions including the control of cholesterol transport and neurosteroid synthesis (Papadopoulos et al., 2006), and may also be involved in the release of pro-inflammatory cytokines during inflammation (Choi et al., 2002; Wilms et al., 2003).

Enhanced TSPO expression can be detected *in vivo* by using positron emission tomography (PET) imaging with the selective TSPO radioligand ^{11}C-PK11195 (Benavides et al., 1988; Pike et al., 1993; Banati et al., 1999), with evidence that increases in ^{11}C-PK11195 binding potential (BP_{ND}) correspond to activation of microglia (Stephenson et al., 1995; Conway et al., 1998; Banati et al., 2000). Although TSPO is also expressed by reactive astrocytes, a ^{11}C-PK11195 PET study of patients with hippocampal sclerosis, a condition histopathologically characterized by marked astrogliosis, did not yield results that were significantly different to healthy normal controls (Banati et al., 1999). This is consistent with the view that reactive astrocytes, *in vivo*, do not significantly contribute to the ^{11}C-PK11195 signal. Therefore, in the absence of invading blood borne cells or severe focal leakage of blood-brain barrier, the increased PK11195 binding is likely to indicate the transition of microglia from a resting to an activated state, and is due to an increase in the number, rather than the affinity, of TSPO (Banati et al., 2000). Hence, the measurement of TSPO uptake using PET provides an *in vivo* tool to monitor progression and severity of neuroinflammation and is a useful indicator of active CNS disease. This article aims to review the use of PET imaging to promote the understanding of activated microglia in neurodegenerative disease.

PARKINSON'S DISEASE AND RELATED DISORDERS

Parkinson's disease is the second most common neurodegenerative disorder of the elderly and is associated with the motor symptoms of tremor, bradykinesia, and rigidity. It is characterized by the extended loss of dopaminergic neurons in the substantia nigra pars compacta, resulting in a deficiency of dopamine in the striatum (Braak et al., 2006), and the presence of alpha-synuclein (α-synuclein)-containing lewy bodies. PD is the most common of a group of parkinsonian movement disorders that also includes Multiple system atrophy (MSA), Corticobasal degeneration (CBD), and Progressive supranuclear palsy (PSP).

The presence of activated microglia close to dopaminergic neurons in post-mortem PD patient brains (McGeer et al., 1988a; Mogi et al., 1994; Langston et al., 1999; Imamura et al., 2003), and PD animal models (Czlonkowska et al., 1996; Kim et al., 2009) suggests a close relationship between neurodegeneration and neuroinflammation in PD. Numerous investigations have proposed a deleterious role of microglial activation in PD based on the vulnerability of dopaminergic neurons to various microglia-derived pro-inflammatory cytokines (Ferrari et al., 2006; Stone et al., 2009; De Lella Ezcurra et al., 2010), while α-synuclein can directly induce activation of microglia (Zhang et al., 2005). However, it seems that the plasticity of microglia must be considered with regards to their contribution in PD, and their role; whether beneficial or detrimental, it may depend on the stimuli present and the stage of disease (Li et al., 2007; Michelucci et al., 2009; Sanchez-Guajardo et al., 2010).

Further clues regarding the role of activated microglia has also come from *in vivo* PET imaging studies (**Table 1**). Significant microglial activation, as reflected by an increase in ^{11}C-PK11195 BP_{ND} was reported in the midbrain and putamen of PD patients when compared to controls, and was found to correlate positively with the motor severity of Parkinsonism (Ouchi et al., 2005; Bartels et al., 2010). These findings suggest that activated microglia has a pathogenic importance in the disease and indicate that the early introduction of a neuroprotective drug to suppress microglial activation could be favorable in PD. Additionally, PD patients exhibited significantly increased ^{11}C-PK11195 BP_{ND} in the basal ganglia, pons, and frontal and temporal cortical regions (Gerhard et al., 2006a). In this study, the increased microglial activation remained unchanged for 2 years, while the patients deteriorated clinically during this period. Hence, it is likely that microglia are activated early in PD, where they remain activated for longer periods and possibly drive progression of the disease (Gerhard et al., 2006a).

Multiple system atrophy is a sporadic neurodegenerative disorder involving a progressive akinetic-rigid syndrome, autonomic failure, and cerebellar dysfunction. It is associated by the appearance of abnormal glial cytoplasmic inclusions (GCI) containing (α-synuclein aggregates and neuronal loss within the nigrostriatal and olivopontocerebellar regions (Lantos and Papp, 1994). The presence of activated microglia is also a prominent feature of MSA (Schwarz et al., 1998). In an *in vivo* PET study of MSA patients, significant ^{11}C-PK11195 BP_{ND} was observed in the putamen, pallidum, pons, substantia nigra pars compacta, and dorsolateral prefrontal cortex, reflecting the known distribution of neuropathological changes in MSA (Gerhard et al., 2003). Although the role of microglia in MSA is inconclusive, microglial activation localization correlated significantly with the locations of GCIs in specific neuroanatomical systems affected in MSA (Ishizawa et al., 2004). A correlation between extent of

Table 1 | Positron emission tomography imaging studies assessing microglia in Parkinsonian disorders.

Study	Disorder	Subjects	PET technique	Main findings
Ouchi et al. (2005)	PD	10 Early PD patients, 10 NC	^{11}C-PK11195	^{11}C-PK11195 BP_{ND} in patients significantly higher than controls Midbrain ^{11}C-PK11195 BP_{ND} values correlated positively with motor disability
Gerhard et al. (2006a)	PD	18 PD patients, 11 NC	^{11}C-PK11195	Significantly increased ^{11}C-PK11195 BP_{ND} in pons, basal ganglia, and frontal and temporal cortical regions ^{11}C-PK11195 signal remained stable for 2 years in subset of patients
Bartels et al. (2010)	PD	14 PD patients, 8 NC	^{11}C-PK11195	Higher contralateral putamen and midbrain ^{11}C-PK11195 BP_{ND} in patients than controls
Gerhard et al. (2003)	MSA	5 MSA patients, 6 NC	^{11}C-PK11195	MSA patients showed significantly increased ^{11}C-PK11195 BP_{ND} in regions reflecting the known distribution of pathologic changes in MSA
Gerhard et al. (2006b)	PSP	4 PSP patients, 7 NC	^{11}C-PK11195	Significantly increased ^{11}C-PK11195 BP_{ND} in basal ganglia, midbrain, frontal lobe, and cerebellum of patients compared to controls Microglial activation remained stable as demonstrated in follow-up scans of two patients
Gerhard et al. (2004)	CBD	4 CBD patients, 5 NC	^{11}C-PK11195	CBD patients had significantly increased ^{11}C-PK11195 BP_{ND} in the cortical regions and basal ganglia that correspond to known distribution of pathological changes in CBD
Henkel et al. (2004)	CBD	1 CBD patient	^{11}C-PK11195	Marked asymmetric microglial activation in corresponding areas of basal ganglia and temporal and parietal cortices

BP_{ND}, binding potential; CBD, corticobasal degeneration; MSA, multiple system atrophy; NC, normal control; PD, Parkinson's disease; PSP, progressive supranuclear palsy.

microglial activation and dopaminergic neurodegeneration has also been reported (Stefanova et al., 2007).

Progressive supranuclear palsy is an adult-onset progressive neurodegenerative disease of unknown cause, characterized by PD-like symptoms such as postural instability and bradykinesia. The pathological hallmark of the disease is neurofibrillary tangles consisting of hyperphosphorylated tau, accompanied by neuronal loss in the thalamus, basal ganglia, and specific brainstem regions (Hauw et al., 1994). Several early studies including immunohistochemical investigations have confirmed the possible involvement of activated microglia in PSP (Kida et al., 1992; Komori et al., 1998; Ishizawa et al., 2000; Ishizawa and Dickson, 2001). ^{11}C-PK11195 PET have also reported significant levels of activated microglia in brain regions known to be affected by the disease process such as the midbrain, cerebellum, pons, frontal lobe, and basal ganglia (Gerhard et al., 2006b). Although these results were unable to support a direct causal contribution to neurodegeneration in PSP, they are at least suggestive of a role of microglia in the disease.

Corticobasal degeneration is a neurodegenerative disorder that affects both cortical and basal ganglial regions, with considerable clinical heterogeneity between patients. Typically, CBD features an asymmetric hypokinetic-rigid syndrome, coupled with alien limb phenomenon and cortical sensory impairment that is unresponsive to dopaminergic therapy (Rebeiz et al., 1968; Gibb et al., 1989). Information on the association of activated microglia in CBD is limited, and mainly coming from immunohistochemical-based assessments (Armstrong et al., 2000; Ishizawa and Dickson, 2001). However, more recent *in vivo* PET investigations have attempted to quantify microglial activation in CBD patients. Increased ^{11}C-PK11195 BP_{ND} was observed in regions such as the caudate nucleus, putamen, substantia nigra pars compacta, pons, and pre- and post central gyrus (Gerhard et al., 2004; Henkel et al., 2004) that correspond to the expected neuropathological changes seen in CBD (Ishizawa and Dickson, 2001; Dickson et al., 2002). These results indicate an involvement of activated microglia in pathogenesis of CBD.

HUNTINGTON'S DISEASE

Huntington's disease is an autosomal, dominant inherited progressive neurodegenerative disorder associated with motor, cognitive, and psychiatric symptoms. It is caused by an abnormal polyglutamine-repeat expansion on the IT15 gene that codes huntingtin, and involves the progressive loss of medium spiny dopaminergic receptor-bearing striatal GABA-ergic neurons (Vonsattel and DiFiglia, 1998). Although the role of chronic neuroinflammation in the HD pathogenesis is not fully understood, post-mortem assessments have reported high levels of activated microglia close to degenerating neurons (McGeer et al., 1988b; Messmer and Reynolds, 1998; Singhrao et al., 1999; Sapp et al., 2001). Upregulated inflammatory cytokines have also been detected in the striatum and plasma, indicative of an inflammatory component in HD (Dalrymple et al., 2007; Björkqvist et al., 2008).

In vivo imaging studies using ^{11}C-PK11195 PET have found increased microglial activation in both premanifest HD gene carriers and manifest HD patients when compared to healthy controls (**Table 2**; Pavese et al., 2006; Tai et al., 2007; Politis et al., 2008, 2011). In premanifest HD patients, significant increases in ^{11}C-PK11195 BP_{ND} in the striatum and hypothalamus was reported, which correlated inversely with neuronal dysfunction as measured by ^{11}C-Raclopride; a marker of dopaminergic D2/D3 receptor availability (Tai et al., 2007; Politis et al., 2008). Interestingly, microglial activation in the striatum, and regions related to cognitive function has been shown to predict the 5-year disease clinical onset in premanifest HD patients (Tai et al., 2007; Politis et al., 2011). These results imply that microglial activation is an early event in the HD disease course, with a possible pathogenic involvement that is associated with a subclinical progression of the disease.

In manifest HD patients, significant ^{11}C-PK11195 BP_{ND} in the striatum, hypothalamus, and various cortical regions was found, that correlated with greater disease burden and higher motor disability (Pavese et al., 2006; Politis et al., 2008, 2011). The cortical

Table 2 | Positron emission tomography imaging studies assessing microglia in Huntington's disease.

Study	Subjects	PET technique	Main findings
Pavese et al. (2006)	11 manifest HD patients, 10 NC	^{11}C-PK11195	Significantly increased ^{11}C-PK11195 BP_{ND} in patients than controls Increased ^{11}C-PK11195 uptake correlated positively with disease severity
Tai et al. (2007)	11 premanifest HD subjects, 10 NC	^{11}C-PK11195	Significantly higher striatal ^{11}C-PK11195 BP_{ND} that correlated inversely with D2 receptor availability Higher striatal uptake correlated with 5 year probability of clinical disease onset
Politis et al. (2008)	10 premanifest HD subjects, 9 manifest HD patients, 10 NC	^{11}C-PK11195	Significantly increased hypothalamic ^{11}C-PK11195 BP_{ND} in both premanifest and manifest subjects compared to controls Inverse correlation between increased hypothalamic ^{11}C-PK11195 BP_{ND} and D2 receptor availability
Politis et al. (2011)	8 premanifest HD subjects, 8 manifest HD patients, 16 NC	^{11}C-PK11195	In premanifest subjects, increased microglial activation in cognitive regions correlated with 5 year probability of clinical disease onset. In manifest HD patients, significantly increased ^{11}C-PK11195 BP_{ND} in globus pallidus, anterior prefrontal cortex, and limbic striatum

BP_{ND}, binding potential; HD, Huntington's disease; NC, normal control.

microglial activation is likely to indicate the involvement of cortical neurons in HD, a well-recognized phenomenon as the disease progresses. Collectively, these findings are consistent with the post-mortem studies (Messmer and Reynolds, 1998; Sapp et al., 2001) and suggest a detrimental microglial contribution to the ongoing neuronal degeneration in HD.

DEMENTIA

Dementias are a group of disorders that are expected to affect more than 100 million people by 2050 raising remarkable financial costs for healthcare (Wimo et al., 2003). AD is the most common cause of dementia and is the most common neurological disorder of the elderly. AD is characterized by the presence of amyloid plaques, neurofibrillary tangles, and activated microglia (for review, see Hardy and Selkoe, 2002). There is a plethora of evidence from post-mortem human AD studies (McGeer et al., 1988a; Venneti et al., 2009) and animal models (Frautschy et al., 1998; Stalder et al., 1999; Leung et al., 2011) reporting a high accumulation of activated microglia in close proximity with the amyloid plaques, and upregulated levels of pro-inflammatory cytokines (Akiyama et al., 2000; Eikelenboom et al., 2002).

Positron emission tomography enables a broad range of functional processes to assess the AD brain *in vivo* (**Table 3**). ^{11}C-PK11195 has been used to demonstrate increased levels of activated microglia in both AD animal models (Venneti et al., 2009) and AD patients (Cagnin et al., 2001; Edison et al., 2008; Yokokura et al., 2011). In AD patients, significant ^{11}C-PK11195 BP$_{ND}$ was consistently observed in the temporal, parietal, and occipital cortices, regions known to be affected by AD pathology (Cagnin et al., 2001; Edison et al., 2008; Yokokura et al., 2011). The increased activated microglia also inversely correlated with the patient Mini-Mental State Examination (MMSE) scores, which is compatible with a role of microglia in neuronal damage (Edison et al., 2008). Interestingly, elevated levels of activated microglia were also detected in patients with amnestic mild cognitive impairment (MCI; Okello et al., 2009a), although this was not observed in another study assessing MCI patients (Wiley et al., 2009). MCI could represent an early precursor stage of AD, since it was found that MCI patients with increased amyloid load were significantly more likely to clinically convert to AD within 3 years (Okello et al., 2009b). Therefore, microglial activation could be an early event in the AD pathogenesis that begins at the MCI stage.

Despite the evidence suggestive of a pathogenic role of activated microglia in AD, it is hypothesized that the accumulation of amyloid plaques is actually due to a failure in microglial clearance mechanisms that would normally remove the protein (Bornemann et al., 2001; DiCarlo et al., 2001; Napoli and Neumann, 2009). This indicates a beneficial, rather than detrimental role of microglia in AD. Notwithstanding the abundance of activated microglia close to senile plaques, they maybe inefficient in the clearance of amyloid, hence, resulting in aggregate formation (Bolmont et al., 2008). It has been shown that in the presence of pro-inflammatory cytokines, phagocytic functions of microglia are compromised (Koenigsknecht-Talboo and Landreth, 2005). Therefore, microglia may confer a dichotomous role in AD, where early microglial activation is possibly neuroprotective involving the removal of amyloid. However, chronic neuroinflammation may downregulate amyloid clearance mechanisms, thus, promoting aggregation and progression of disease.

Frontotemporal lobar degeneration (FTLD) which includes frontotemporal dementia is the name given to a group of pathologically, clinically, and genetically heterogeneous disorders involving focal atrophy of the frontal and temporal lobes, while unlike AD, with sparing of the parietal and occipital regions (Neary et al., 1998). Another important dissimilarity between AD and FTLD pathology is the absence of amyloid plaque formation (Paulus et al., 1993; Mirra and Hyman, 2002). Rather, the key histopathological features of FTLD, depending on subtype,

Table 3 | Positron emission tomography imaging studies assessing microglia in dementias.

Study	Disorder	Subjects	PET technique	Main findings
Cagnin et al. (2001)	AD	8 AD patients, 15 NC	^{11}C-PK11195	AD patients showed significantly increased regional ^{11}C-PK11195 BP$_{ND}$ in the entorhinal, temporoparietal, and cingulate cortex
Edison et al. (2008)	AD	13 AD patients, 10 NC	^{11}C-PK11195	Significant increased ^{11}C-PK11195 BP$_{ND}$ in the cortical regions Inverse correlation between increased cortical microglial activation and MMSE scores
Yokokura et al. (2011)	AD	11 AD patients, 10 NC	^{11}C-PK11195	Significantly increased ^{11}C-PK11195 uptake in the parietotemporal regions of patients than controls Inverse correlation between dementia scores and ^{11}C-PK11195 BP$_{ND}$ values
Wiley et al. (2009)	AD, MCI	6 mild-moderate AD patients, 6 MCI patients, 5 NC	^{11}C-PK11195	No significant differences in brain ^{11}C-PK11195 BP$_{ND}$ between subject groups
Okello et al. (2009a)	MCI	14 MCI patients, 10 NC	^{11}C-PK11195	5 of 13 MCI subjects had increased cortical ^{11}C-PK11195 BP$_{ND}$ compared to controls
Cagnin et al. (2004)	FTLD	5 FTLD patients, 8 NC	^{11}C-PK11195	Significantly increased ^{11}C-PK11195 BP$_{ND}$ in the frontotemporal regions

AD, Alzheimer's disease; BP$_{ND}$, binding potential; FTLD, frontotemporal lobar degeneration; MCI, mild cognitive impairment; MMSE, mini-mental state examination; NC, normal controls.

includes tau deposition (including Pick bodies) and ubiquitin-positive, tau-negative inclusions (Munoz et al., 2003; Uchihara et al., 2003). *In vivo* PET imaging of FTLD patients detected enhanced microglial activation in the expected frontotemporal regions (Cagnin et al., 2004). In the same study, significant ^{11}C-PK11195 BP_{ND} in the bilateral putamen is also consistent with previous neuropathological data showing the involvement of the basal ganglia in FTLD (Mirra and Hyman, 2002). These observations indicate the presence of an active microglial response that reflects progressive neuronal degeneration. Importantly, the detection of increased microglial activation in affected regions in FTLD suggests that microglial responses occur independently of amyloid deposition, and that neuronal loss alone is enough to induce activation (Cagnin et al., 2004). However, whether this applies to AD pathogenesis requires further investigation.

MULTIPLE SCLEROSIS

Multiple sclerosis is a disease characterized pathologically by inflammatory demyelination and axonal transection, and is the most common cause of non-traumatic disability in young adults (Compston and Coles, 2008).

The involvement of activated microglia has long been proposed in MS (Benveniste, 1997). Post-mortem investigations have detected activated microglia in the cortical GM of MS patients (De Groot et al., 2001; Peterson et al., 2001; Petzold et al., 2002), while histopathological studies have implicated microglia in lesion pathogenesis (for review, see Lassmann, 2008). An observed correlation between neuronal loss and microglial activation was reported in animal experimental MS (Rasmussen et al., 2007). Significant levels of activated microglia was also found in MS patients, especially in the progressive forms of disease that are associated with neurodegeneration (Kutzelnigg et al., 2005; Magliozzi et al., 2010), and selective ablation of parenchymal microglia was able to prevent demyelination and axonal damage (Heppner et al., 2005).

Pathological aspects of MS such as neuroinflammation, demyelination, and neurodegeneration may be explored *in vivo* with PET (for review, see Kiferle et al., 2011). PET with ^{11}C-PK11195 and other tracers has demonstrated inflammatory processes with microglial involvement in MS (**Figure 1**; **Table 4**). In animal experimental MS and human post-mortem brains it has been shown that ^{11}C-PK11195 uptake corresponds to the distribution pattern of activated microglia (Banati et al., 2000). It has also been demonstrated that there is increased ^{11}C-PK11195 BP_{ND} in areas of focal pathology identified by T1- and T2-weighted MRI (Vowinckel et al., 1997; Banati et al., 2000) and in gadolinium-enhanced T1-weighted MRI (Debruyne et al., 2003). Increased ^{11}C-PK11195 BP_{ND} was observed in normal-appearing gray and white anatomical structures (Banati et al., 2000; Debruyne et al., 2003; Versijpt et al., 2005). This is in line with the hypothesis that inflammatory processes initiated by microglia early in MS may constitute the real burden of disease, associated with invisible microglia-mediated damage that occur independently of relapses (Kesselring, 1990; Confavreux et al., 2000). A positive correlation has been suggested between ligand uptake and disease duration, disability, and brain atrophy (Banati et al., 2000; Debruyne et al., 2003; Versijpt et al., 2005), although the correlations were not consistently replicated across the different studies. However, recent data from our group found a significant association between high ^{11}C-PK11195 BP_{ND} in the cortical gray matter and disability in patients with secondary progressive MS, and with higher ^{11}C-PK11195 BP_{ND} in the secondary progressive group than the relapse-remitting MS group (Politis et al., in press). These findings are consistent with a detrimental role of microglia in MS. Enhanced microglial activation in MS has also been detected using the more recently developed TSPO tracers ^{11}C-vinpocetine and ^{11}C-PBR28 (Vas et al., 2008; Oh et al., 2011).

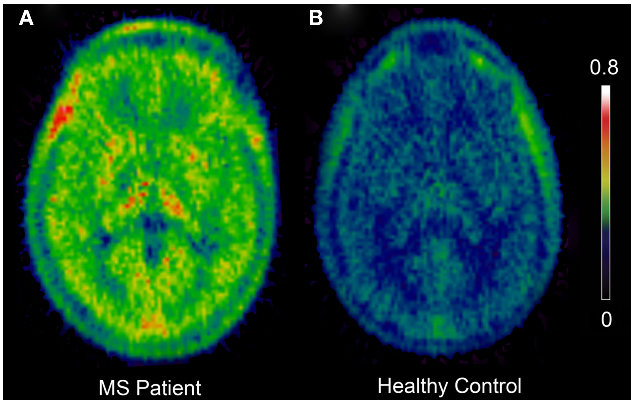

FIGURE 1 | Positron emission tomography images showing increased ^{11}C-PK11195 BP_{ND} in a Multiple Sclerosis patient (A) when compared to a healthy normal control (B). Color bar represents intensity of ^{11}C-PK11195 tracer binding (BP_{ND}). BP_{ND}, binding potential; MS, multiple sclerosis.

Microglial activation may contribute to the mechanism of axonal injury *via* the release of soluble factors that may either directly or indirectly cause neuronal dysfunction (Peterson et al., 2001; Barnett and Prineas, 2004; Dutta and Trapp, 2006; Zipp et al., 2006; Dal Bianco et al., 2008; Lassmann, 2008; Magliozzi et al., 2010), and consequently result in progressive increase in impairment and disability. However, activated microglia may also exert protective functions with MS through the release of neurotrophic factors (Stadelmann et al., 2002; Napoli and Neumann, 2009), and triggering of remyelination mechanisms (Li et al., 2005; Setzu et al., 2006). This suggests a possible dichromatic role of microglia in MS.

NEW TSPO LIGANDS

^{11}C-PK11195 was the first tracer to be consistently used for the study of activated microglia and neuroinflammation *in vivo*. However, limitations associated with the application of ^{11}C-PK11195 include a high level of non-specific binding (Petit-Taboué et al., 1991), and a poor signal to noise ratio, which complicates its quantification (Boutin et al., 2007). This has prompted the search for novel PET tracers (termed, second generation radioligands) with improved capacities to quantify TSPO expression.

Radioligands such as ^{11}C-PBR28, ^{11}C-DAA1106, ^{18}F-FEDAA1106, and ^{18}F-PBR111 have recently been developed to image TSPO *in vivo* (Gulyás et al., 2002; Ikoma et al., 2007; Fujita et al., 2008; Vas et al., 2008; Yasuno et al., 2008; Oh et al., 2011; for a review, see Chauveau et al., 2008). Published data using the second generation ligands ^{11}C-DAA1106 (Ikoma et al., 2007) and

Table 4 | Positron emission tomography imaging studies assessing microglia in multiple sclerosis.

Study	Subjects	PET technique	Main Findings
Vowinckel et al. (1997)	2 MS patients	^{11}C-PK11195	High ^{11}C-PK11195 BP_{ND} in MRI-defined active MS lesions
Banati et al. (2000)	12 MS patients (RR, SP, PP), 8 NC	^{11}C-PK11195	Increased global and focal (in active MS lesions) ^{11}C-PK11195 BP_{ND} in MS patients
Debruyne et al. (2003)	22 MS patients (RR, SP, PP), 7 NC	^{11}C-PK11195	Increased ^{11}C-PK11195 BP_{ND} in MRI-Gadolinium lesions. Higher uptake in T2 lesions during relapse Positive correlation between ^{11}C-PK11195 BP_{ND} and disease duration
Versijpt et al. (2005)	22 MS patients (RR, SP, PP), 8 NC	^{11}C-PK11195	Significant correlation between brain atrophy and both disease duration and severity For NAWM, ^{11}C-PK11195 BP_{ND} increased with amount of atrophy T2-lesional ^{11}C-PK11195 BP_{ND} values decreased according to increasing atrophy
Vas et al. (2008)	4 MS patients	^{11}C-PK11195, ^{11}C-vinpocetine	Regional uptake values increased in regions of brain damage for both tracers, but markedly higher for ^{11}C-vinpocetine than ^{11}C-PK11195
Oh et al. (2011)	11 MS patients, 7 NC	^{11}C-PBR28	High ^{11}C-PBR28 in MRI-gadolinium lesions in patients Increase in tracer uptake preceded appearance of gadolinium enhancement No difference in global ^{11}C-PBR28 uptake between patients and healthy controls
Politis et al. (in press)	16 MS patients (RR, SP), 8 NC	^{11}C-PK11195	Significant correlation between cortical GM ^{11}C-PK11195 BP_{ND} and disease severity Higher ^{11}C-PK11195 in SP than RR patients

BP_{ND}, binding potential; GM, gray matter; MRI, magnetic resonance image; MS, multiple sclerosis; NAWM, normal-appearing white matter; NC, normal control; PP, primary progressive multiple sclerosis; RR, relapse-remitting multiple sclerosis; SP, secondary progressive multiple sclerosis.

^{18}F-FEDAA1106 (Fujimura et al., 2006) in humans were promising, with both tracers showing significantly higher cerebral uptake than ^{11}C-PK11195. Furthermore, increased ^{11}C-DAA1106 binding was reported in AD patients (Yasuno et al., 2008) that were similar to the previous studies that used ^{11}C-PK11195 (Cagnin et al., 2001).

The only published study using the second generation radioligand ^{11}C-PBR28 found areas of focal increases in radiotracer binding in the brain of MS patients (Oh et al., 2011). Interestingly, the increased focal ^{11}C-PBR28 binding preceded the development of some gadolinium-enhancing lesions. Brain parenchymal ^{11}C-PBR28 binding in MS patients was positively correlated with the duration of the disease, however it was not significantly higher than that of healthy volunteers. Interpretation of these results is limited by the lack of characterization of the binding affinity pattern, which might have significantly affected the comparison between subjects.

It has been recently demonstrated that there are three different affinity patterns for second generation TSPO ligands in healthy volunteers as well as patients with MS, which was evident with all the ligands tested (^{11}C-PBR28; ^{11}C-PBR06; ^{18}F-PBR111; Owen et al., 2010). This presents a methodological problem, as differences in PET signal across subjects cannot be safely interpreted as differences in target density, but may reflect differences in the affinity pattern. A possible approach to solve this problem is based on the use of peripheral binding affinity, which can be characterized to classify subjects into one of the groups, as differences in affinity status between individuals have been shown to be present on peripheral cells as well (Owen et al., 2010).

Interestingly, the difference in binding patterns observed with second generation radioligands was not observed with ^{11}C-PK11195. Also, *in vitro* autoradiography data using ^{11}C-PK11195 suggest a receptor density (B_{MAX}) significantly higher than that found using second generation ligands. It could be speculated that ^{11}C-PK11195 and newer ligands bind to distinct sites within the TSPO molecule.

Although, data obtained from first generation studies have been promising and suggested that ^{11}C-PK11195 could be useful to image acute inflammatory lesions and microglial activation in MS, a conclusive demonstration of the potential of TSPO imaging for the application as disease biomarker, indicative of microglial activation in MS, is still lacking. Furthermore, despite second generation ligands constituting a potential improvement relative to ^{11}C-PK11195 at least from a methodological point of view, a clear advantage in their clinical application as disease biomarkers has not been demonstrated yet.

For these reasons, we aim to characterize a second generation TSPO PET radioligand *in vivo* in humans, and to evaluate its application as a disease biomarker in MS.

Among second generation TSPO tracers, ^{18}F-PBR111 presents different advantages, as there is low difference in its affinity for TSPO between high, medium, low affinity binders. Also, it could be potentially used in clinical applications as it is labeled with fluorine-18. Promising preclinical data, and ongoing studies in neurological patients, suggest it could be a good choice amongst second generation TSPO ligands to progress into studies in MS patients.

CONCLUSION

Inflammation coupled with the presence of activated microglia seems to be a common feature of a wide range of CNS diseases. However, despite a large number of research studies, the exact role of microglia in chronic neurodegenerative diseases remains uncertain. In line with the high plasticity of microglia that allows them to perform numerous CNS functions, microglia are likely to play a dichromatic role in disease, depending on signals present in their microenvironment and the duration of activation. While early microglial activation could represents a beneficial response (i.e., removal of CNS threat, promoting tissue repair and removal of misfolded protein), chronic exposure could induces detrimental effects by promoting neuronal death (i.e., through the sustained release of neurotoxic factors), thus, contributing to progression of disease. PET imaging with the use of TSPO radioligands provides a valuable tool that allows us to track the progression and severity of neuroinflammation in the living brain, and is a useful indicator of active CNS disease. Therefore, the early detection of microglia using PET could offer opportunities for pharmacological interventions to limit the potential disruptive effects of chronic microglial activation. Furthermore, with the development of newer TSPO tracers, the potential for PET imaging research to promote our understanding of activated microglia in CNS disease can only increase.

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Microglial activation – tuning and pruning adult neurogenesis

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New neurons are continuously generated in two adult brain regions: the subgranular zone of the hippocampus and the subependyma by the lateral ventricles, referred to as the neurogenic niches. During their development from neural stem cells to mature functionally integrated neurons numerous choices are made, such as proliferation or quiescence, cell survival or death, migration or establishment, growth or retraction of processes, synaptic assembly or pruning, or tuning of synaptic transmission. The process is altered by physiological stimuli as well as several brain diseases. Microglia are located within the neurogenic niches and have become interesting candidates for modulating neurogenesis in both the healthy and injured brain. They become activated by foreign antigens or changes in the brain homeostasis and transform this innate immunity into an adaptive immune response by recruiting systemic immune cells. Most studies report an acute decrease in the survival of new neurons following this classically activated microglia reaction. The long-term effects are more complex. In neurodegenerative diseases, microglial activation is more heterogeneous and the transformation from a pro- to an anti-inflammatory cytokine profile and the deactivation of microglia is not well defined. The diversity is reflected by numerous reports describing both beneficial and detrimental effects on neurogenesis, primarily on the proliferation, survival, and cell fate. However, relatively few studies have investigated alterations at later stages of neurogenesis including the functional integration. Though likely, it is not established how a fine-tuned cross-talk between microglia and adult-born neurons would work and how it changes upon microglia activation. This review will therefore launch three hypotheses for how microglia might direct synaptic integration of newborn neurons, currently a fast expanding research field.

Keywords: microglia, neurogenesis, synaptic pruning, synaptic transmission, adult-born neurons, subgranular zone, subependyma, neurogenic niche

FROM NEURAL STEM CELLS TO MATURE FUNCTIONALLY INTEGRATED NEURONS – CHOICES TO BE MADE

Neurogenesis persists in two adult brain regions: the subependyma of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus. In the subependymal layer, multipotential, self-renewing stem cells are the source of newly generated neurons migrating through the rostral migratory stream and incorporating into the olfactory bulb as interneurons (Seaberg and van der Kooy, 2002). New olfactory neurons are thought to participate in both long-term olfactory memory and predator avoidance (Sakamoto et al., 2011; Sultan et al., 2011). From the SGZ, neuronal and glial progenitors with limited self-renewal capacity migrate into the granule cell layer and develop primarily into granule cells, a few becomes interneurons (Seaberg and van der Kooy, 2002; Liu et al., 2003; Livneh and Mizrahi, 2011). The integration of new hippocampal neurons in the adult brain occurs over several months, with an initial tonic GABA-induced depolarization converting into an intermittent GABA-mediated hyperpolarization when the cells are 3 weeks of age (Ge et al., 2008). Mature excitatory synapses are visualized at about 2 months, while spine density increases in the new neurons up to 6 months of age (Zhao et al., 2006). Simultaneously, axons grow and establish either functional glutamatergic synapses with hilar interneurons, mossy cells and CA3 pyramidal neurons (Toni and Sultan, 2011) or occasionally GABAergic inhibitory synapses with granule cells (Liu et al., 2003). The continuous integration of adult-born hippocampal neurons is important for synaptic transmission and bidirectional plasticity in the dentate gyrus. It is suggested to account for an efficient integration of novel incoming information and in memory formation (Dupret et al., 2008; Massa et al., 2011; Sahay et al., 2011), such as pattern separation and pattern integration (Deng et al., 2010).

The development of adult-born neurons may also be described as a continuous decision process. Neural stem cells/progenitors will have to decide whether to proliferate or stay in quiescence. Almost immediately after birth some cells undergo apoptotic death, while others survive. The surviving cells will choose a neuronal or glial fate and thereafter either stay and establish contacts within the SGZ or subependyma, the so-called neurogenic niches, or migrate into other brain regions. The integration of new neurons includes both growth and retraction of dendrites and axons, assembly and pruning of excitatory and inhibitory synapses, and homeostatic tuning of the established synaptic transmission. It becomes evident that in order for the new neurons to make the most appropriate choices for the overall function of the surrounding network, they are dependant on environment cues at most likely all developmental stages.

THE NEUROGENIC NICHE

It is conceivable that the local circuitries within the adult neurogenic niches have region-specific instructive roles in directing neuronal production and stem cell maintenance. It has even been suggested that they may shield ongoing neurogenesis from possible external inhibitory influences (Riquelme et al., 2008). The neurogenic niches consists of several cell types and structures, including: (1) astrocytes that envelop and contact all cell types and structures in the niche, secret diffusible signals, and form a gap junction-dependant syncytium by which they propagate signals and may regulate activation and differentiation of stem cells. They also act as neural stem cells/precursors (Riquelme et al., 2008), (2) ependymal cells by the subependymal layer that regulate the absorption of ions, transport factors from the cerebral spinal fluid into the parenchyma, and act as a source of secreted pro-neurogenic factors like transforming growth factor (TGF)-α and basic fibroblast growth factor (Riquelme et al., 2008), (3) blood vessels that undergo a parallel angiogenesis within the niche and often use common factors with neurogenesis such as vascular endothelial growth factor, nitric oxide and erythropoietin (Riquelme et al., 2008), (4) meningeal projections, extracellular matrix (ECM) proteins, basal lamina, and perivascular cells and fibroblasts, which propagate or modulate signals from the blood vessels and cerebral spinal fluid as well as from surrounding brain parenchyma. Emerging evidence underscore the important interaction between the adult-born neurons and ECM proteins. Cell adhesion to its environment includes matrix components, extracellular proteolytic enzymes, integrins, and non-receptor tyrosine kinases, which influence both gene expression and post-transcriptional signaling cascades (Riquelme et al., 2008; Wojcik-Stanaszek et al., 2011), (5) interneurons and other neighboring mature neurons already highly integrated into the hippocampal network, which may modulate neurogenesis by activity- and signaling-dependent mechanisms (Markwardt et al., 2011; Masiulis et al., 2011; Toni and Sultan, 2011), (6) oligodendrocytes, which are numerous in the subependyma but few in the SGZ. Their interaction with the neural progenitor cells is, however, so far unclear (Morrens et al., 2012), and (7) myeloid cells including microglia and dendritic cells, which are located in close proximity to the newborn neurons, where their activity correlates with the neurogenic response (Ekdahl et al., 2003; Monje et al., 2003; Bulloch et al., 2008; Sierra et al., 2010).

MICROGLIAL ACTIVATION IN THE ADULT BRAIN

A microglia activation profile is constantly modulated by either initiating factors, such as pathogen- or damage-associated molecular patterns (Schratt et al., 2006; Aronica and Crino, 2011) or alterations in electromagnetic fields (Richerson et al., 2005), or intracellular transducing signals, or feed-forward loops amplifying resolution mechanisms, or feed-back loops as counter-regulators (Glass et al., 2010). During brain pathology, the microglial activation is often divided into two phenotypic profiles: the classical M1 and the alternative M2 activation/deactivation state (Gordon, 2003; Mosser, 2003; Michelucci et al., 2009). The classical M1 activation occurs as microglia encounter a foreign antigen. The microglia act as a first line of defense and participate in transforming the innate immunity into an adaptive immune response by recruiting systemic immune cells. The alternative and deactivated M2 phenotype, sometimes called "neuroprotective," is important when switching from a classical inflammatory response to a reduction of pro-inflammatory mediators, an increased production and release of anti-inflammatory cytokines, neurotrophic factors, and a production of cytoactive factors involved in repair and restructuring of the damaged ECM in the brain. However, these M2 phenotypes may also participate in chronic "neuroinflammation" in the brain (Colton, 2009). The heterogeneity becomes evident in for instance neurodegenerative diseases, where the transformation from a pro- to an anti-inflammatory cytokine profile and the deactivation of microglia is neither temporally nor spatially clearly defined. There are even suggestions for a dysfunctional microglia phenotype following long-lasting activation (Graeber, 2010).

In the healthy brain, microglia and/or perivascular cells have been suggested to form an "immunological blood–brain barrier" between the brain parenchyma and the vascular system. However upon activation, by for instance sterile inflammation following trauma, ischemia, or chemical damage, these cells may instead recruit blood-born neutrophils and macrophages and increase the amount of tissue damage (Perry, 2010; Rock et al., 2010; Graeber et al., 2011; Yirmiya and Goshen, 2011). Simultaneously, microglia seems to be able to protect neurons from systemic immune cells by direct engulfment of invading neutrophils (Neumann et al., 2008). Other circulating immune cells are, though, required to maintain and sense brain homeostasis through the choroid plexi, brain meninges and the cerebral blood fluid. For instance, recruitment of systemic circulating T cells specific to CNS antigens can promote the termination of a local neurotoxic inflammatory response together with microglia of a "neuroprotective" phenotype (Ziv et al., 2006; Shechter et al., 2009; Ron-Harel et al., 2011). Taken together, these findings suggest that the activation state of the microglia is closely intermingled with the alertness of systemic immune cells and that there may be a bidirectional cross-talk.

HETEROGENEOUS MICROGLIAL POPULATION WITHIN THE NEUROGENIC NICHES – REGION SPECIFICITY?

Microglial density and phenotype differ between regions of the healthy brain, which could implicate functional differences and sensitivity to the surrounding environment (Lawson et al., 1990; Olah et al., 2011). Also within the subependymal layer of the healthy adult rat brain, microglia constitute a heterogeneous cell population. Here, the vast majority of ionized calcium-binding adapter molecule 1 (Iba1)-immunostained microglia exhibit a ramified or surveying phenotype, followed by an intermediate form with shorter processes, less arborization, and larger soma (Thored et al., 2009). Few express an amoeboid or round morphology (**Figures 1A–D**). There are partly overlapping subpopulations of microglia expressing the phagocytic marker ED1, associated with a pro-inflammatory profile, as well as major histocompatibility complex (MHC) class II, insulin-like growth factor-1 (IGF-1), and triggering receptor expressed on myeloid cells-2, the three latter molecules being associated with alternative or anti-inflammatory microglia activation (Thored et al., 2009; Heldmann et al., 2011). Local proliferation of microglia occurs, but parts of the subependymal microglia population are also blood-born (Thored et al., 2009; Heldmann et al., 2011). Interestingly, following middle cerebral artery occlusion microglia develop different morphological phenotypes within the subependymal layer compared to the striatal peri-infarct area. The differences are evident at least 16 weeks post-injury, including fewer amoeboid and round microglia morphologies and an up-regulation of IGF-1 in the subependyma. This may imply a region-specific, permissive microglia population within the neurogenic niche (Thored et al., 2009). However, a region-specific depletion of parts of the microglia population (expressing CD11b/Mac-1) within the subependyma, early after an ischemic lesion in rats, did not alter the number of newly formed neuroblasts in the striatum or their migratory distance (Heldmann et al., 2011).

In the hippocampal SGZ of rodents housed in an enriched environment, increased number of Iba1+ microglia co-labeled with the anti-inflammatory factors IGF-1 and MHC class II, were found together with T cell recruitment and an increase in neurogenesis. Moreover, T cell deficient mice showed reduced neurogenesis, which could not be overcome by an enriched environment (Ziv et al., 2006). The findings suggest a beneficial role of SGZ microglia possibly instructed by CNS-specific T cells. Conversely, following seizures and in neurodegenerative models of Alzheimer's disease, an increased number of Iba1+ microglia expressing ED1 is evident both acutely and chronically, and primarily cytotoxic for the new neurons (Ekdahl et al., 2003; Bonde et al., 2006; Biscaro, Lindvall, Tesco, Ekdahl, Nitsch, unpublished observation). Taken together, microglia are capable of developing both pro- and anti-inflammatory activation profiles in neurogenic brain areas. Moreover, it is possible that under certain circumstances the temporal and spatial constitution of the activation may even be region-specific.

Apart from the heterogeneous morphology and plethora of immune mediators, the motility and dynamic configurations of the microglia are also likely to reflect different functional aspects. Microglial processes are highly mobile and continuously rebuilt. Two-photon imaging of neocortex has shown active microglia continually surveying their microenvironment even in the normal brain (Nimmerjahn et al., 2005). The motility of microglia and perhaps region-specific features within the neurogenic niches remains to be characterized.

MICROGLIA – BENEFICIAL AND DETRIMENTAL FOR NEUROGENESIS

In the last two decades many studies have implicated a central role for brain parenchymal microglia as well as systemic immune cells during the generation of adult-born neurons (Ekdahl et al., 2009; Whitney et al., 2009; Molina-Holgado and Molina-Holgado, 2010; Russo et al., 2011a). Among the first evidence for a beneficial role of microglia or microglia-released mediators are *in vitro* studies on neural stem cell cultures, where the formation of neuroblasts could be rescued by co-culturing with microglia or conditioned media from microglia (Aarum et al., 2003; Walton et al., 2006). This "pro-neurogenic" function of microglia has since then been further supported by a number of studies from the group of M. Schwartz. Originally, they demonstrated that microglia activated with cytokines related to T cell helper cells promoted neurogenesis *in vitro* (Butovsky et al., 2006) and that CNS-specific T helper cells regulate hippocampal neurogenesis *in vivo* (Ziv et al., 2006). Interestingly, mice devoided of T cells or both T and B cells showed impairments in several hippocampal-dependent spatial learning tests (Kipnis et al., 2004; Brynskikh et al., 2008; Ron-Harel et al., 2008), which could be improved by replenishing T cells or boosting T cell activation by agonist of self-reactive T cells (Kipnis et al., 2004). Even more intriguingly was the finding that cognitive tasks increased the number of T cells located within the meningeal spaces, where T cell depletion induced a pro-inflammatory phenotype of the myeloid cells (Derecki et al., 2010). Since neurogenesis is thought to be important for hippocampal plasticity and memory formation, the health of the systemic immune system could possibly directly or through the interaction with innate non-neuronal cell types, like microglia, continuously shape neuroplasticity in both the healthy and damaged brain. Another recent reports on the beneficial role of microglia for newly formed neurons comes from Bachstetter et al. (2011) who have studied the neuronal transmembrane chemokine fractalkine (FKN)/CX3CL1 and its microglia-expressed receptor, CX3CR1-pathway. Disruption of this pathway in young adult rodents decreased both survival and proliferation of hippocampal neural progenitor cells (Bachstetter et al., 2011). Unchallenged microglia have also been demonstrated to engulf apoptotic bodies of neural progenitors in the SGZ. During normal neurogenesis in the healthy brain, a large proportion of neural progenitors undergo apoptosis within their first days in life. By rapidly removing cell debri, the scavenging properties of microglia could have an important regulator role at the early stages of neurogenesis (Sierra et al., 2010). However, it is not fully clear whether the phagocytosis is beneficial for the surrounding newborn neurons by reducing pro-inflammatory mediators, or may be detrimental by further inducing apoptotic neuronal death (Magnus et al., 2001; Neher et al., 2011).

The first studies suggesting an acute detrimental role of activated microglia for neurogenesis were performed in rats following intraparenchymal lipopolysaccharide (LPS) injections (Ekdahl et al., 2003; Monje et al., 2003). LPS-induced microglial activation led to a dramatic decrease in the survival of newly formed hippocampal neurons. The pivotal role of microglial activation was further substantiated following both epileptic seizures and irradiation, where hippocampal neurogenesis could be rescued by inhibiting microglia activation through either administration of the anti-inflammatory agent minocycline or non-steroidal anti-inflammatory drugs (Ekdahl et al., 2003; Monje et al., 2003). Pro-inflammatory cytokines like interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF), released acutely by activated microglia, have been suggested as important mediators between the microglia and the new neurons (Monje et al., 2003;

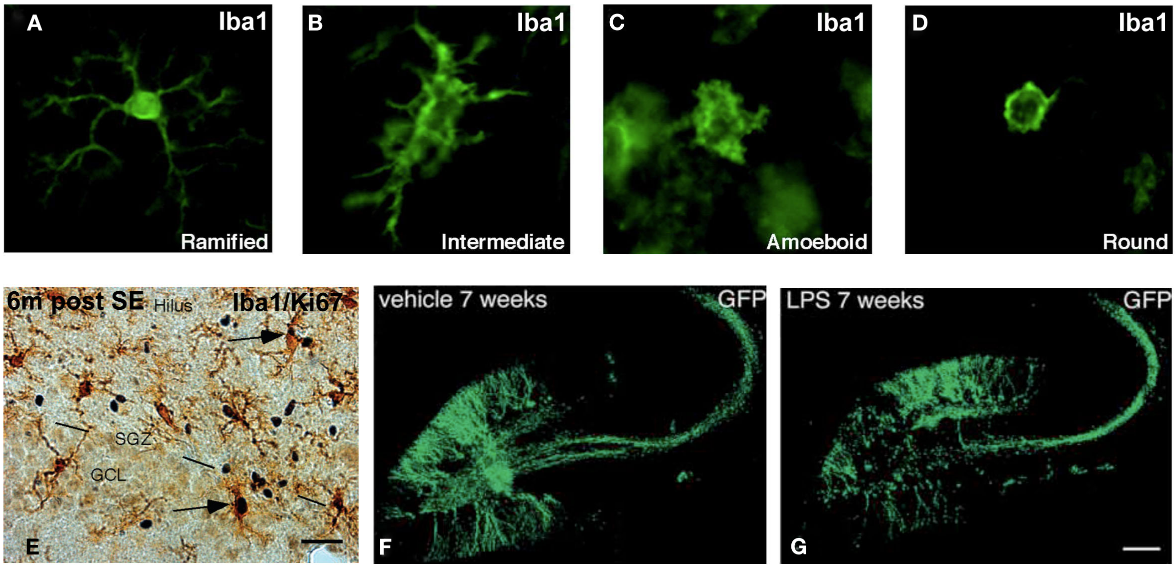

FIGURE 1 | Microglia and newly generated neurons in the neurogenic niche of the adult rat brain. (A–D) Photomicrographs showing four distinct phenotypes of Iba1+ microglia. **(E)** Image of Iba1+ microglia within the subgranular zone (SGZ) of the hippocampal dentate gyrus 6 months after electrically induced status epilepticus. Arrows mark microglia that express Ki-67, which implicate an ongoing proliferation. **(F,G)** Retroviral-GFP vector labeling of 7-week-old adult-born neurons in the hippocampus following vehicle or LPS-induced microglial activation. The cell bodies of the newly generated neurons are located in the SGZ and granule cell layer of the dentate gyrus. Their dendritic trees are visible throughout the molecular layer and their axons extend into the dentate hilus and the CA3 region. Modified from Bonde et al. (2006), Jakubs et al. (2008), and Thored et al. (2009).

Iosif et al., 2006; Yirmiya and Goshen, 2011). Also prostaglandins released by the microglia may negatively regulate different steps of neurogenesis. In support, mice lacking cyclooxygenase-1 lack both the acute LPS-induced decrease in proliferation, survival, and differentiation of new hippocampal neurons (Russo et al., 2011b).

When the initiating agent for microglia activation is no longer present and the acute pro-inflammatory phase is over, an anti-inflammatory cytokine profile may develop, as shown after LPS-stimulation (Cacci et al., 2008). This profile may be beneficial for the newly formed neurons, since for instance administration of anti-inflammatory cytokines like TGF-β, has provided beneficial effects on neurogenesis in the subependyma (Mathieu et al., 2010a). However, if the microglia are continuously activated, they may partly sustain their release of oxidative stressors, which could be neurotoxic (Glass et al., 2010; Nathan and Ding, 2010; Polazzi and Monti, 2010). The result may be disease-specific interaction between microglia and the newborn neurons (Graeber et al., 2011). Microglia are, though, important for terminating immune/immune-like responses, by, i.e., recruitment of systemic immune cells (Frank-Cannon et al., 2009; Shechter et al., 2009; Polazzi and Monti, 2010; Rivest, 2011; Ron-Harel et al., 2011), and may thereby protect new neurons and even recruit neural progenitors for repair (Czeh et al., 2010; Mathieu et al., 2010b). The correlation between seizure-induced microglial activation and neurogenesis is a typical example of this complexity. A severe seizure insult induces a strong transient increase in hippocampal neurogenesis (Parent et al., 1997), followed by a chronic decrease below control levels (Hattiangady and Shetty, 2010). In the acute phase, microglia activation is prominent within the SGZ and has been suggested to compromise the early survival of the new neurons (Ekdahl et al., 2003). Six months later, the microglial activation is decreased but still present (Bonde et al., 2006; **Figure 1E**). This may imply also a long-term negative correlation between the production of new neurons and the chronically activated microglia. However, the newly formed neurons generated directly after the seizure insult, that survived the acute post-seizure environment, are still present 6 months later. They have even been estimated to comprise about 10% of the total dentate granule cell layer. These adult-born neurons are surrounded by the chronically activated microglia, which may instead favor a possible supportive role of the chronically activated microglia (Bonde et al., 2006). Because the initial seizure insult is followed by additional spontaneous seizures throughout the life of the animals, there is a possibility for recurrent triggering of the microglial activation. Whether subpopulations of microglia with either detrimental or beneficial effect on the surrounding newborn neurons co-exist within the neurogenic niche is not known and may be directly depending on the number of spontaneous seizures. The initial severity of the seizure insult is directly correlated to both the number of activated microglia as well as the neurogenic response (Mohapel et al., 2004). In addition, parts of the newly generated neurons migrate aberrantly out into the dentate hilus, which may perhaps be due to the well-known seizure-induced death of hilar interneurons and the prominent population of activated hilar microglia. Collectively, these findings support a primarily beneficial interaction between microglia and new neurons in the intact brain. However, the cross-talk is complex and probably double-edged in pathological conditions, especially following long-term microglial activation.

THE ROLE OF MICROGLIA DURING FUNCTIONAL INTEGRATION OF ADULT-BORN NEURONS – AN EQUALIZER AMPLIFYING AND FILTERING SYNAPTIC SIGNALING?

Until now, most studies on the cross-talk between microglia and adult-born neurons have focused on the effect on the early stages of neurogenesis, such as proliferation, survival, and neuronal fate. The role of microglia at later stages of neurogenesis, i.e., during synaptic assembly, stability, and transmission is less characterized. Today, there is no direct evidence that microglia could regulate synaptic integration of adult-born neurons. However, there is some evidence and several suggestions that microglia regulate synaptic pruning and transmission in mature neurons, currently a fast expanding research area. Three main working hypotheses can be put forward for how microglia may also regulate synaptic integration of adult-born neurons: (1) involvement of microglia in synaptogenesis and pruning, (2) modulation of perisynaptic structures, and (3) spine structure and synaptic transmission.

MICROGLIA MAY BE INVOLVED IN SYNAPTOGENESIS AND PRUNING OF SYNAPSES ON ADULT-BORN NEURONS

This hypothesis is based on recent studies describing how microglia may be involved in synaptic elimination/stripping/pruning by phagocytic engulfment of synapses on mature neurons in the healthy brain (Paolicelli et al., 2011; Tremblay and Majewska, 2011). By electron microscopy and two-photon *in vivo* imaging of the primary visual cortex of juvenile mice during visual manipulations, subtle changes in the behavior of quiescent microglia were observed. This included geometric regulation of perisynaptic extracellular spaces, contact with subsets of structurally dynamic and transient dendritic spines, and phagocytic engulfment of intact synapses (Tremblay et al., 2010; Tremblay and Majewska, 2011). The findings were further substantiated by Paolicelli et al. (2011) proposing synaptic pruning by microglia during postnatal development in mice. Mice lacking microglia expressing the chemokine FKN receptor CX3CR1, exhibited a transient reduction in microglia number correlated with a delayed synaptic pruning. This resulted in an excess of dendritic spines and immature electrophysiological properties of CA1 pyramidal neurons at P13 and P16 in the CX3CR1 knockout mice (Paolicelli et al., 2011). The occurrence of synaptic pruning has also been correlated with the duration of the microglia-synaptic contacts. *In vivo* imaging studies of the ischemic brain, suggest more persistent contacts between microglia processes and dendritic spines and axon terminals, compared to the transient 4–5 min of normal contacts observed in the healthy brain. Following longer interactions in the pathological environment, these contacted synapses often disappeared (Wake et al., 2009; Kettenmann et al., 2011). In several brain pathologies, such as following axonal lesions or immune mediated-cortical lesions, pruning of synapses from the perikaryon and dendrites is evident. The suggested consequence of synaptic pruning during these conditions has been neuroprotection (Cullheim and Thams, 2007; Trapp et al., 2007; Kettenmann et al., 2011).

An interesting possible signaling pathway between the microglia and the synaptic structures on the new neurons is the complement cascade. Since the complements are involved in opsonization and cytolysis, they have become attractive possible candidates for executing synaptic pruning in both the healthy and injured brain. Mice deficient in the initiating protein in the classical complement cascade, C1q, exhibit large sustained defects in synapse elimination, excessive excitatory synapses and axon terminals in mature neurons (Stevens et al., 2007; Chu et al., 2010). Neural progenitor cells and immature neurons express the receptors for complement fragments C3a and C5a. C3a stimulates neuronal migration and differentiation, by modulating stromal cell-derived factor-1a-induced extracellular-signal-regulated kinases phosphorylation. Mice lacking C3 signaling (including C3a and C5a) have reduced basal neurogenesis as well as decreased survival of ischemia-generated new striatal neurons (Rahpeymai et al., 2006; Shinjyo et al., 2009). Also complement receptor 2 (Cr2) is expressed in adult neural progenitor cells from the dentate gyrus, though, Cr2 knockout mice exhibit increased hippocampal neurogenesis (Moriyama et al., 2011). Together these results suggest a fundamental role of the complements for neurogenesis, including proper synaptic pruning and integration of newly formed neurons. However, in a mouse model of glaucoma, unwanted synapses may be tagged by complement for elimination, which suggests that complement-mediated synaptic pruning can become aberrantly reactivated in neurodegenerative disease (Stevens et al., 2007). In human temporal lobe epilepsy with hippocampal sclerosis, the complements factors are expressed in both astrocytes, mature neurons and microglia, but particularly in microglia (Aronica et al., 2007). Whether it is really the microglia that eliminate the complement-tagged neuronal synaptic terminals on either mature or newly formed neurons, in the intact and the damaged brain, is not yet shown (Perry and O'Connor, 2008; Tremblay and Majewska, 2011). Another suggested pathway between microglia and newborn neurons is the MHC class I and its related receptors, which is expressed in both microglia and in neurospheres and the subependymal layer (Popa et al., 2011). MHC class I molecules have been shown to influence both the strength and pattern of synaptic elimination (Cullheim and Thams, 2007; Kettenmann et al., 2011).

MICROGLIA MAY MODULATE THE PERISYNAPTIC STRUCTURE OF SYNAPSES ON ADULT-BORN NEURONS

Microglia might affect synaptic transmission though proteolytic modification of the perisynaptic environment (Tremblay and Majewska, 2011). This would include inactivation, degradation, and/or activation of the ECM, leading to compartmentalization of proteases, ions, cytokines, and neurotransmitters within individual synapses. One interesting possible pathway is through the matrix metalloproteases (MMP), such as MMP-9, shown already to be involved in the migration and differentiation of adult neural progenitor cells in the subependymal layer (Barkho et al., 2008) Another candidate is via a disintegrin and metalloproteases (ADAMs), implicated in dopamine-induced release of epidermal growth factor from stem cells in the subventricular zone (O'Keeffe and Barker, 2011). Proteolytic remodeling of the ECM by MMPs may convert trophic factors to their biologically active forms (i.e., vascular endothelial growth factor and TGF-β) and thereby influence synaptic integration. ECM ligands, such as integrin receptors, provide outside-in signals for cells to sense their microenvironment. This leads to modulation of receptor tyrosine kinases, which closely cooperate with growth factors (Wojcik-Stanaszek et al., 2011).

MICROGLIA MAY MODULATE THE STRUCTURE OF DENDRITIC SPINES AND SYNAPTIC TRANSMISSION IN ADULT-BORN NEURONS

Another mechanism by which microglia may regulate neuronal activity is through remodeling of dendritic spine morphology, including size, shape, and motility (Tremblay and Majewska, 2011), as well as synaptic adhesion and transmission. The dendritic structure is closely associated with its synaptic activity and activity-driven changes in synaptic efficacy may modulate spine morphology due to alterations in the underlying actin cytoskeleton (Fortin et al., 2011).

There are also several immune mediators involved in synaptic remodeling of mature neurons. For instance, in cultured hippocampal neurons, TNF receptor-associated protein 1 knockdown modulated the morphology of dendritic spines (Kubota et al., 2009). TNF deficient mice displayed also smaller dendritic trees in the hippocampus (McCoy and Tansey, 2008). Furthermore, mice lacking IL-1β receptor had reduced dendritic spine size (Goshen et al., 2009) and IL-6 over-expression in cerebellar granule cells caused impairments in granule cell adhesion, migration, and increased formation of excitatory synapses on granule cells (Wei et al., 2011). MicroRNAs, which may control the expression of hundreds of genes (Schratt et al., 2006), were recently suggested to be interacting with immune mediators. Knocking down miR-132 in PC12 cells resulted in an increased expression of especially pro-inflammatory molecules. Interestingly, retroviral knockdown of miR-132 impaired the integration of newborn neurons in the adult hippocampus. (Luikart et al., 2011). However, whether or not the structural synaptic changes in either mature or newly formed neurons directly depend on cytokines released by the microglia is not clear.

When synapses have been established, microglia might fine-tune the synaptic strength to ensure proper synaptic transmission and plasticity (Beique et al., 2011). Mice lacking CX3CR1 show alterations in both hippocampal long-term potentiation (LTP) and long-term depression (LTD), supporting a microglia-driven neuronal plasticity during development and in mature neurons (Bachstetter et al., 2011; Maggi et al., 2011; Paolicelli et al., 2011). In addition, LPS-activated microglia in acute mice hippocampal slices enhance the frequencies of excitatory postsynaptic currents in mature neurons. This effect was abolished by applying purinergic antagonists, especially against the P2Y1 receptor, which is only expressed on interneurons and astrocytes in the hippocampus. By producing ATP, microglia may thereby act on astrocytes through purinergic signaling, which could amplify the ATP production, release glutamate, and act on mature neuronal glutamatergic receptors (Tremblay et al., 2011). Moreover, prolonged changes in electrical activity may lead to uniform adjustments in the strength of all synapses, called homeostatic synaptic scaling/tuning (Stellwagen and Malenka, 2006). Synaptic scaling in response to prolonged blockade of neuronal activity *in vitro* can be mediated by TNF-α from glial cells (astrocytes and/or microglia; Stellwagen and Malenka, 2006). Also, TNF-α may increase the expression of AMPA receptors on synapses (Pickering et al., 2005), resulting in excitatory synaptic scaling, and decrease GABAA receptor expression (McCoy and Tansey, 2008). When adult-born hippocampal neurons integrate into a pathological environment, hosting either seizure- (Jakubs et al., 2006) or LPS-induced microglial activation (Jakubs et al., 2008; **Figures 1F,G**), they possess an increased inhibitory synaptic drive onto their afferent synapses, compared to new neurons formed in healthy conditions. Conversely, in a less severe seizure-environment, without a prominent microglial activation, this increase is lacking (Wood et al., 2011). How or whether it is really the microglial activation that account for these changes observed in the synaptic transmission of newborn neurons needs further investigations, though, it is a tempting speculation.

Microglia may also regulate synaptic transmission in newly formed neurons by directly targeting synaptic adhesion molecules. Seizure-induced microglial activation is associated with structural changes in dendrite spine formation (Murphy et al., 2011), as well as altered expression of synaptic adhesion molecules and scaffolding proteins on newly formed hippocampal neurons (Jackson, Chugh, Nilsson, Karlström, Lindvall, Ekdahl, unpublished observation). In support, several adhesion molecules have been shown to modulate synaptic integration of newborn neurons. One of the most studied adhesion molecules is the polysialated neural cell adhesion molecule (NCAM), which is specifically expressed on new neurons (Gascon et al., 2010). Another interesting pathway is the N-cadherin/beta-catenin/neurogenin signaling cascade, which has been shown to modulate adhesion, neuronal differentiation, and neurite outgrowth in neurospheres (Chen et al., 2006). Also the adhesion molecules neuroligin 3 and 4 are involved in presynaptic differentiation and synapse formation in human induced pluripotent stem cell-derived neurons (Kim et al., 2011). The adhesion molecules may be regulated by immune mediators released by microglia, such as TNF. A recent report described reduced expression of the N-cadherin by TNF receptor-associated protein 1 knockdown in cultured hippocampal neurons (Kubota et al., 2009).

CONCLUDING REMARKS

Various brain pathologies and physiological stimulations modify neurogenesis (Zhao et al., 2008). Two of the strongest modulators include epileptic seizures involving the SGZ of the dentate gyrus (Bengzon et al., 1997; Parent et al., 1997; Jakubs et al., 2006) and ischemia close to the subependymal layer (Arvidsson et al., 2002). Understanding the signaling pathways between the new neurons and the environment within the neurogenic niches in pathological conditions, may yield new targets for therapeutic interventions. Several drugs are currently studied in order to increase the neuroprotective functions of microglia or to shift the microglial phenotype toward neuroprotection (Polazzi and Monti, 2010). However, microglia in particular may exhibit strong compensatory mechanisms upon external modulation and quickly rearrange their proliferation capacity and phenotypic characteristics. The heterogeneity of the microglial activation makes predictions of the outcome from immune-modulating therapies very difficult (Rivest, 2011). Future studies on the impact of more subtle tunings of the cross-talk between microglia and the newly formed neurons may perhaps ease these interventions.

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Alzheimer's disease, neuroprotection, and CNS immunosenescence

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This review is focused on discussing in some detail possible neuroprotective functions of microglial cells. We strive to explain how loss of these essential microglial functions might contribute toward the development of characteristic neuropathological features that characterize Alzheimer's disease. The conceptual framework guiding our thinking is provided by the hypothesis that microglial senescence accounts for impaired neuronal protection and consequent neurodegeneration.

Keywords: Alzheimer's disease, CNS immunosenescence, impaired neuronal protection, microglial cells, microglial senescence, neurodegeneration, neuropathological features, neuroprotection

INTRODUCTION

The role of CNS microglial cells in the development of Alzheimer's disease(AD) has been the subject of considerable interest since McGeer's initial description of reactive microglia in human AD brain in 1987 (McGeer et al., 1987). Most of the numerous studies that followed corroborated McGeer's findings and collectively over the years they coalesced into the amyloid cascade-neuroinflammation hypothesis, which claims that neurodegenerative changes in AD (neurofibrillary degeneration) are the result of an uncontrolled, chronic intracerebral inflammatory reaction triggered by the accumulation/aggregation of amyloid-beta (Aβ) protein in plaques. Today, the neuroinflammation hypothesis is difficult to uphold given that clinical trials with anti-inflammatory drugs and strategies to remove Aβ from the brain have met with disappointing results and have yielded little in the way of effective treatments for humans. In this paper, we briefly describe our own vision of what may be the role of microglia in AD pathogenesis, which is different in that our primary focus is not on a single protein (Aβ) as the cause of AD, but instead relies on the incontrovertible fact that the incidence of sporadic AD is strongly correlated with aging. Our theory, which we call the microglial dysfunction hypothesis, states that neurodegeneration in AD is the result of an aging-related, gradually progressive breakdown of innate CNS immunity and loss of neuroprotection, i.e., microglial cell senescence. In contrast to normal aging this deterioration of innate CNS immunity appears to be accelerated in AD. The difference in perspective between the dysfunction hypothesis and the neuroinflammation theory is due in large part to our conviction that microglia are entirely beneficial cells, whose single-most important function is to provide neuronal protection at all times in the normal and injured CNS. In the following, we shall discuss succinctly several microglial neuroprotective functions and how senescent deterioration of these could contribute to development of neurodegenerative changes.

THE BRAIN'S INNATE IMMUNE SYSTEM IS NEUROPROTECTIVE

Even though doubts about the very existence of microglia were expressed in textbooks until the early 1990s (Graeber, 2010), the notion that microglia are the key cellular elements comprising the brain's innate immune system (Graeber and Streit, 1990; Streit and Kincaid-Colton, 1995) is now widely recognized. A common view that has been expressed many times is that together with peripheral macrophages, such as Kupffer cells of the liver, Langerhans cells of the epidermis, or alveolar macrophages in the lung, microglia are another member of the mononuclear phagocyte system, i.e., a tissue-specific macrophage. While there is little doubt regarding validity of a mononuclear lineage relationship, microglia in the CNS do not maintain a macrophage state constitutively or continuously. In the normal adult brain, microglia display a well-differentiated, dendritic morphology similar to that of other brain cells. They exhibit multiple, finely branched cytoplasmic processes with which they constantly explore the CNS microenvironment searching for disturbances that may require their quick response (Nimmerjahn et al., 2005; Tremblay et al., 2010). These ramified microglia are sensors of pathology (Kreutzberg, 1996; Stence et al., 2001; Petersen and Dailey, 2004; Davalos et al., 2005) and they do not exhibit macrophage morphology, nor do they express the typical macrophage marker, CD68 (recognized by ED1 antibody in the rat). Even activated microglia with hypertrophic morphology express the CD68 antigen only after having engaged in phagocytic activity (Graeber et al., 1998). It is therefore only under conditions of tissue/cell necrosis when debris must be phagocytized that microglia show expression of CD68 and can therefore be considered brain macrophages. The currently popular idea of differentiating functionally distinct macrophage phenotypes classified as M1 (cytotoxic), M2 (reparative), and a third, "deactivated" form might thus apply merely to those few microglial cells that at any given time are in a macrophage state during CNS injury or disease. To generalize and assume that the M1/M2 classification applies to the microglial population at large represents a rather indiscriminate extrapolation of *in vitro* studies performed with peripheral macrophages (Colton, 2009; Michelucci et al., 2009; Moon et al., 2011), and it could be misleading to assume that resting and activated microglia within the brain microenvironment function in the same manner as peripheral (professional) macrophages that have been cultured and manipulated *in vitro* with cytokines. There is currently no way of reliably identifying functionally distinct microglial phenotypes in brain tissue *in situ* and without this capability no real progress on functional involvement of putative M1 or M2 microglial subtypes in the cellular pathogenesis of AD can be made. In part because of this we are assuming that all microglia are potentially beneficial cells and our view of microglial involvement in AD is rooted firmly in that assumption. It is known that the CNS parenchyma represents a unique compartment that is segregated to a large extent from the rest of the body by the blood brain barrier (BBB), and that it represents an immunologically subdued environment (Ford et al., 1995; Perry et al., 1995; Carson et al., 1998; Hoek et al., 2000; Cardona et al., 2006), although not necessarily an "immunologically privileged site" (Galea et al., 2007). There are no constitutively active (professional) macrophages in the brain parenchyma under normal conditions. Our definition of a microglial cell is that of a cell type of the mononuclear cell lineage that has evolved to be highly adapted and specialized for residing within the unique CNS microenvironment. As such its immunological potential is limited largely to phagocytosis while its neuroprotective capabilities are maximized, and one might thus view microglia as a hybrid between a moderately immunocompetent mononuclear cell and a powerful neuroprotective glial cell.

We view the neuroprotective capabilities of microglia in the broadest possible meaning of the term, namely, anything that microglia do that is beneficial for and/or conducive toward proper neuronal functioning. One obvious beneficial function of microglia is simply their ability to transform into macrophages that can clear out debris and process waste when/if the need arises. These microglia-derived macrophages are not necessarily cytotoxic because much of the debris generated within the brain, for example after trauma or is chemia, is sterile (Chen and Nunez, 2010) and would thus not require elaboration of cytotoxins to kill microorganisms. Direct infection of the CNS with microorganisms is largely prevented by the natural barriers of skull, meninges, and BBB, but if these fail and when microorganisms do gain access to the parenchyma, they are likely to be eliminated quickly and efficiently by microglial cells and probably without too much collateral damage, as there are multiple mechanisms involved in post-infection neuropathology other than microglial neurotoxicity (Nau and Bruck, 2002; Mariani and Kielian, 2009; Ribes et al., 2011). One condition that deserves special mention is infection with the human immunodeficiency virus-1 (HIV-1), where microglial cells themselves are the target of the infectious agent (Michaels et al., 1988). It is therefore reasonable to assume that the functionality of HIV-infected microglia is compromised, although in ways that are not yet entirely understood. From our perspective, compromised microglial function includes primarily an impaired ability to provide neuroprotection, and it is perhaps because of this that patients with HIV/AIDS frequently suffer from the consequences of neurodegeneration, a.k.a. HIV-associated dementia (Anthony and Bell, 2008). It seems obvious that compromised innate immunity within the CNS due to HIV infection is what accounts at least in part for the high incidence of opportunistic CNS infections in HIV/AIDS patients, notably toxoplasmosis (Mariani and Kielian, 2009).

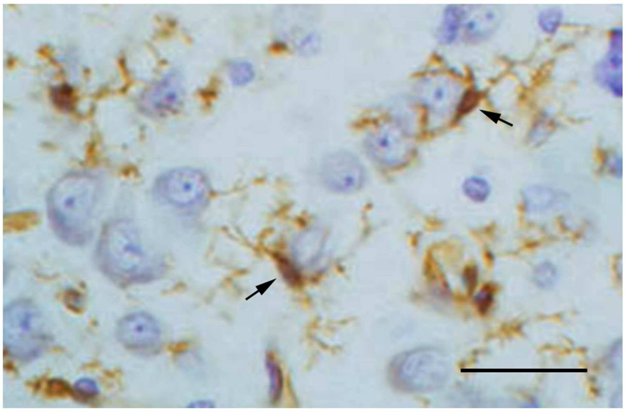

FIGURE 1 | Lectin staining of microglia (brown) in the normal cerebral cortex of a rabbit shows two microglial cells (arrows) extending their processes around cortical neurons. This close spatial relationship suggests ongoing interactions between resting microglia and neurons. Neurons are stained with cresyl violet. Scale bar: 50 μm.

A second and very direct neuroprotective function provided by microglia is the production and secretion of neurotrophic factors, notably BDNF (Elkabes et al., 1996; Batchelor et al., 1999; Suzuki et al., 2001; Nakajima et al., 2002; Coull et al., 2005) and NGF (Mallat et al., 1989; Heese et al., 1997; Frade and Barde, 1998), but also others like TGF-β (Kiefer et al., 1993; Lehrmann et al., 1998), bFGF (Araujo and Cotman, 1992), and GDNF (Batchelor et al., 1999; Suzuki et al., 2001). Production of neurotrophins by microglia is usually increased after injury/during recovery when the cells become activated and neurons require more neurotrophic support than under normal conditions. However, even under normal conditions microglia are likely to sustain neuronal functioning during periods of high activity or sub-pathological stress when smaller doses of neurotrophic factors may be required. Although it is difficult to demonstrate directly that this type of specific microglial-neuronal interaction actually occurs, histological images showing some neurons covered tightly by microglial processes suggests that this could be the case (**Figure 1**). Different neuronal populations require different neurotrophic factors and quite possibly microglia have the ability to sense which factors and how much of them may be needed in any particular circumstance. Their ability to migrate and home in on neurons in need is essential for facilitating such targeted neuroprotection.

A third way in which microglia can be neuroprotective is by diverting noxious and potentially harmful substances away from neurons. It has long been known that astrocytes take up excess glutamate from the extracellular space under conditions of heightened neuronal activity in an effort to minimize glutamate-mediated excitotoxicity. During the past 10 years it has become clear that astrocytes are not alone in this effort and that activated microglial cells also participate in the removal of glutamate by upregulating primarily glutamate transporter-1 (GLT-1) *in vivo* following traumatic lesions as well as *in vitro* after stimulation with LPS, TNF-α, or neuronal conditioned medium (Lopez-Redondo et al., 2000; Nakajima et al., 2001, 2008; van Landeghem et al., 2001). Interestingly, glutamate uptake by microglia is coupled to enhanced glutathione synthesis by microglia, perhaps reflecting an effort by the cells to protect themselves from damage (Persson et al., 2006). These studies underscore the extensive crosstalk that takes place continuously between microglia and neurons. In addition to glutamate, a second substance with considerable and non-specific damage potential is free, redox-active iron, which may be present either as ferric (Fe^{+3}) or ferrous (Fe^{+2}) ions. We propose that the sequestration of free iron in the CNS by ferritin in microglia constitutes an important neuroprotective mechanism that becomes most relevant when there is a breach in the BBB and the possibility exists of free iron entering the brain parenchyma. The evidence for this mechanism of microglial neuroprotection is as follows: we and others have previously reported that many of the dystrophic (senescent) microglia in human brain are positive for the iron storage protein, ferritin (Simmons et al., 2007; Lopes et al., 2008), suggesting that the sequestration and concomitant accidental escape of free iron atoms can contribute to microglial senescence by increasing oxidative stress within these cells. This has raised the interesting and novel possibility that microglia rather than neurons may be primary victims of oxidative damage (Dringen, 2005; Lopes et al., 2008; Nakanishi and Wu, 2009), representing somewhat of a paradigm shift since in the past microglia have been seen primarily as a source of free radicals that endanger neuronal survival (Colton and Gilbert, 1987; Boje and Arora, 1992; Chao et al., 1992). It seems plausible then to think that microglia can protect neurons by taking the brunt of at least some oxidative stress and deflecting it away, which makes sense since microglia are relatively expendable and possess renewal capacity from within the CNS (mitosis) or from bone-marrow derived precursor cells. One might draw the analogy to the game of chess where pawns are sacrificed to save the more valuable pieces. Compatible with this idea of microglial self-sacrifice is evidence from animal experiments showing that activated microglia in the axotomized facial nucleus are negative for ferritin (unpublished) and that therefore ferritin expression in microglia is not necessarily linked to microglial activation. Importantly, transection of the facial nerve leaves the BBB undisturbed, but conversely, when a brain lesion does result in a breach of the BBB some microglia do become ferritin-positive and even dystrophic (Xue et al., 2010). Thus, neuroprotective microglia are like the HazMat Team in the CNS which will put itself in harm's way to protect neurons.

A final aspect of microglial neuroprotection within the scope of this discussion concerns their emerging role in the regulation of the plasticity of neuronal circuits, that is, their involvement in the pruning/elimination and maintenance of synaptic connections. This aspect of the microglial functional repertoire was recognized for the first time more than 40 years ago by Blinzinger and Kreutzberg (1968), yet it remained largely unexplored until quite recently when a number of laboratories started to reexamine and delve deeper into this phenomenon. It now appears that microglial regulation of neuronal connectivity is important during development, as well as in the normal and injured adult brain (Wake et al., 2009; Tremblay et al., 2010; Paolicelli et al., 2011). The stripping of synapses from axotomized motoneurons (Blinzinger and Kreutzberg, 1968) perhaps best illustrates a direct neuroprotective effect of such cellular action in that displacement of synapses from the surface of injured motoneurons may prevent afferent excitation, which is hardly needed at a time when motoneurons are working to regenerate their severed axons (Streit, 1993). In fact, tight microglial ensheathment of injured motoneurons instantaneously fulfills multiple goals of neuroprotection: while preventing unnecessary excitation through synaptic displacement, it also puts microglia into very close proximity to the neuronal cell soma to facilitate targeted delivery of neurotrophic factors and, in addition, it perfectly prepositions microglia for rapid phagocytosis should a neuron fail to survive as a result of having been axotomized. Elimination of synapses during development (Paolicelli et al., 2011) or during altered sensory processing (Tremblay et al., 2010) is neuroprotective insofar as it facilitates the proper formation and rearrangement of synaptic connections and thus optimizes neuronal functioning.

NEUROINFLAMMATION AND THE SIGNIFICANCE OF MICROGLIAL ACTIVATION IN AD

Countless studies in laboratory animals involving experimental CNS lesions have shown that microglia become activated rapidly in response to neuronal injury (Kreutzberg, 1996; Kettenmann et al., 2011). This prompt cellular reaction to CNS tissue injury (glial activation), which can occur within seconds after injury (Nimmerjahn et al., 2005), constitutes acute neuroinflammation. The primary purpose of such acute inflammation, being limited in its range to the immediate vicinity of the lesion, is to initiate wound healing and to restore homeostasis as soon as possible, and microglial activation subsides as healing occurs. Excess microglia generated during the activation response *via* mitosis of resident cells undergo programmed cell death which reduces cell numbers back to baseline (Gehrmann and Banati, 1995; Jones et al., 1997; Conde and Streit, 2006), constituting a form of physiological cell death perhaps similar to what happens in normal ontogenetic development. One seemingly trivial insight to be gained from these lesion studies is that they clearly establish the cause-and-effect relationship between injury and subsequent microglial activation underscoring the fundamental definition of inflammation, i.e., the cellular response to injury. In the context of AD and the amyloid cascade-neuroinflammation theory a cause-and-effect relationship is anything but trivial. Although deposition of the Aβ protein is seen by many as a trigger for microglial activation in AD and has thus given rise to a large body of literature resulting from sundry experimental approaches, there is no consensus to date whether or not Aβ actually stimulates microglial activation. Case in point, we and others have observed that some human brains with substantial Aβ loads reveal a notable lack of microglial activation (Itagaki et al., 1989; Ohgami et al., 1991; Streit et al., 2009; **Figure 2**). Similarly, the supposition that microglial cells are involved in the clearance of Aβ has not been proven. Generally speaking, studies performed *in vitro* with cultured microglia

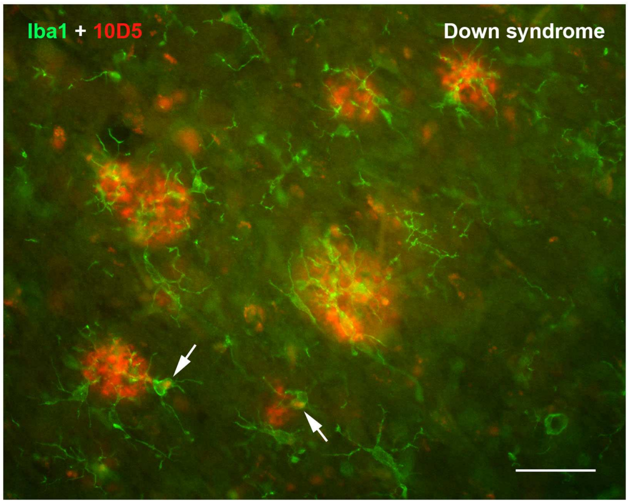

FIGURE 2 | Double immunofluorescent staining for microglia (Iba1) and Aβ protein (10D5) in the cerebral cortex of a human with Down syndrome reveals apparently normal, non-activated microglia in and around Aβ deposits. Microglia are ramified and show no evidence of having internalized any Aβ protein. Two microglial cells show presence of intracellular, autofluorescent lipofuscin (arrows). Scale bar: 50 μm.

provide the only convincing evidence that microglia can phagocytize Aβ protein, but these stand in stark contrast to most studies in Aβ-overexpressing animal models (which do not) and certainly to studies in human brain which reveal a lack of Aβ phagocytosis by microglia. Thus, the relationship between Aβ, microglial activation, and/or neuroinflammation remains enigmatic and perplexing even after more than 20 years of intense research. If one also considers the alleged causal connection between amyloid deposits and neurofibrillary degeneration the picture gets blurred even more as this connection seems to grow weaker and evidence is mounting against rather than in favor of it.

Yet another issue that complicates our understanding of neuroinflammation in AD is found in the fact that primary infections outside of the CNS influence the state of microglial cells. In particular, sepsis, which is more commonly found in elderly patients due to compromised immune function, will induce microglial activation within the brain (Lemstra et al., 2007; Streit et al., 2009). It is likely that there are other types of systemic pathology that may do so as well (Mattiace et al., 1990). Nearly all prior studies that have reported microglial activation and/or upregulation of inflammatory cytokines in the CNS of AD subjects have not made a distinction between cases that were free of peripheral infectious disease and those that were not, and it is therefore quite possible that neuroinflammatory changes reported could have been the result of peripheral diseases. Studies are underway in our laboratory to further investigate this possibility.

MICROGLIAL SENESCENCE AND NEURODEGENERATION – CONNECTING THE DOTS

The discovery of dystrophic microglia in human brain represents a critical step in conceiving the microglial dysfunction hypothesis because it raises the possibility that microglia are subject to senescence and degeneration (Streit et al., 2004). Dystrophic microglia were first identified in the aged human brain as cells displaying abnormal morphological features, such as shortened, gnarled, beaded, or fragmented cytoplasmic processes, as well as loss of fine ramifications and formation of spheroidal swellings. Because they are present in greater numbers in aged vs. young humans microglial dystrophy is thought to reflect degenerative changes related to cell senescence. Our subsequent demonstration of a close spatial and temporal relationship between neurofibrillary degeneration (tau pathology) and microglial dystrophy in subjects with either AD or Down syndrome has provided another crucial piece in the puzzle consolidating the dysfunction theory by linking microglial senescence and neurodegeneration (Streit et al., 2009; Xue and Streit, 2011). Additional support for this link is derived also from the well-known fact that aged rodents typically do not develop neurofibrillary degeneration, and that microglial degeneration has been undetectable in uninjured rodent brain (Streit and Xue, 2010). Clearly, the next major step in advancing this line of thinking would be to induce microglial dystrophy experimentally and determine if it is accompanied by neurodegenerative changes. Current efforts in our laboratory are directed toward that goal.

The morphological abnormalities that characterize dystrophic microglia have been described in detail before (Streit et al., 2004, 2009; Xue and Streit, 2011), suffice it to say here that the most advanced and striking change involves fragmentation of the cells' cytoplasm, which is termed cytorrhexis (**Figure 3**). Cytorrhexis reflects obvious degeneration of cell structure and it appears to be the end result of a progression from beading of processes to subsequent fragmentation not unlike what has been observed during axonal degeneration (Kerschensteiner et al., 2005; Coleman and Freeman, 2010). We view microglial cytorrhexis as a form of accidental cell death with as of yet unknown causes, although much points toward undue oxidative stress as a likely etiologic factor. Our prior work has shown that cytorrhexis does not involve detectable nuclear fragmentation further supporting the idea of an accidental rather than an apoptotic mechanism (Fendrick et al., 2007).

With regard to the aforementioned neuroprotective roles of microglia, there are a number of interesting points to consider in terms of how deterioration of these neuroprotective functions could be critically important in the development of aging-related neurodegenerative diseases. Phagocytosis of debris by microglia is an essential cellular activity that ensures maintenance of a clean, debris-free brain parenchyma, and is conducive toward facilitating optimal interneuronal electrochemical signaling. A substantial decline in microglial phagocytosis due to cell senescence and/or degeneration would almost certainly contribute to NDD development (Neumann et al., 2009). In addition, impaired phagocytosis could be a factor contributing to reduced clearance of amyloid deposits, a possibility that has been raised in previous reports (Fiala et al., 2005; Streit et al., 2008; Njie et al., 2012). Areas of the human brain showing advanced tau pathology are characterized by widespread presence of cytorrhectic microglia but also reveal a conspicuous absence of phagocytic activity. This very likely reflects the fact that fragmented microglia cells no longer are capable of performing phagocytosis, which explains why areas of tau pathology are littered with uncleared microglial debris. Although not yet shown, it would hardly come as a surprise to find out that the ability

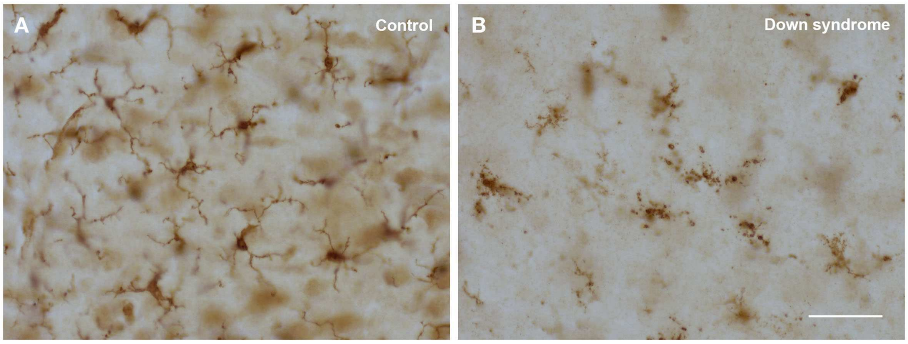

FIGURE 3 | Comparison of normal (ramified) and degenerating (dystrophic) microglia using Iba1 immunostaining in human cerebral cortex. (A) 22-year-old male non-demented subject reveals cells with normal morphology; **(B)** 48-year-old female subject with Down syndrome shows cells displaying obvious cytoplasmic fragmentation. Scale bar: 50 μm.

of cytorrhectic microglia to produce sufficient amounts of neurotrophic factors is much reduced. The possibility also exists that dying microglial cells could elaborate toxic factors, which might be a way to reconcile the dysfunction and neuroinflammation ideas.

For quite some time now the presence of increased brain iron and/or a disturbance in iron metabolism has been seen as a major factor contributing directly to free radical mediated neuronal degeneration (Jellinger et al., 1990; Dexter et al., 1991; Youdim and Riederer, 1993; Lynch et al., 2000; Berg et al., 2001; Zecca et al., 2004; Smith et al., 2010). Here again, microglial degeneration offers an opportunity to deepen our understanding of the importance of brain iron in the pathogenesis of neurodegenerative diseases. As levels of brain iron increase with aging, perhaps due in part to presence of microbleeds, the burden for resident microglial cells in sequestering free iron through ferritin expression becomes progressively higher. At the same time the risk for microglia to develop degenerative changes through iron-mediated oxidative stress is heightened, thus resulting in an ever increasing number of dystrophic and dysfunctional microglial cells with impaired ability to provide neuroprotection. Observations in humans showing that a large proportion of ferritin-positive microglia are dystrophic and that these dystrophic cells accumulate in advanced lesions (senile plaques) substantiate this line of thinking (Kaneko et al., 1989; Lopes et al., 2008; Xue and Streit, 2011).

As mentioned, there is an increasing number of recent studies that suggest significant involvement of microglia in synaptic plasticity (Tremblay et al., 2011). If microglia are indeed the electricians of the brain and important for maintaining synaptic integrity of neuronal circuits (Graeber, 2010), then their deterioration in the AD brain could certainly play a direct role in the loss of synapses which represents a hallmark feature of the disease (DeKosky and Scheff, 1990; Terry et al., 1991; Lassmann et al., 1993). Naturally, if the dysfunction hypothesis is correct and microglial degeneration contributes to neurodegeneration, the loss of synapses secondary to neuronal degeneration would also fit into this scenario. Some studies have suggested a role for microglia in synapse elimination as well as in synaptogenesis during CNS development (Bessis et al., 2007), and these functions are likely to be performed much more effectively by young microglial cells in the developing brain than by senescent ones in the aged CNS.

CONCLUSION

As the brain's innate immune system one might be inclined to think of microglia primarily as immunological defenders that fight invading microorganisms. However, in so doing one underestimates their importance as supportive and neuroprotective glial cells essential for helping to maintain neuronal functioning in the normal CNS and especially their crucial involvement in CNS repair and regeneration during injury and disease. We believe that microglial neuroprotection constitutes a most important aspect of the cells' biological significance because it defines a single common denominator that contributes to improved understanding of CNS development, normal adult brain function, as well mechanisms of injury, disease, and repair. Specifically, with regard to aging-related neurodegenerative diseases, which represent one of the greatest challenges for biomedical science in this day and age, we think that the concept of CNS immunosenescence has considerable potential for advancing progress in terms of new and improved approaches toward treatment and prevention.

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Primary phagocytosis of neurons by inflamed microglia: potential roles in neurodegeneration

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Microglial phagocytosis of dead or dying neurons can be beneficial by preventing the release of damaging and/or pro-inflammatory intracellular components. However, there is now evidence that under certain conditions, such as inflammation, microglia can also phagocytose viable neurons, thus executing their death. Such phagocytic cell death may result from exposure of phosphatidylserine (PS) or other eat-me signals on otherwise viable neurons as a result of physiological activation or sub-toxic insult, and neuronal phagocytosis by activated microglia. In this review, we discuss the mechanisms of phagocytic cell death and its potential roles in Alzheimer's Disease, Parkinson's Disease, and Frontotemporal Dementia.

Keywords: microglia, phagocytosis, inflammation, neurodegeneration, phosphatidylserine, MFG-E8, lactadherin, reactive oxygen and nitrogen species

INTRODUCTION

Phagocytosis of host cells is generally thought to be *secondary* to the target cell dying by some means such as apoptosis (Savill et al., 2002; Ravichandran, 2003). However, phagocytosis can execute cell death of viable cells, and we shall refer to this form of cell death as "*primary* phagocytosis," with the defining characteristic that inhibition of phagocytosis prevents cell death. Examples of primary phagocytosis outside the brain include macrophage phagocytosis of "aged" erythrocytes (Föller et al., 2008; Lee et al., 2011) and activated neutrophils (Lagasse and Weissman, 1994; Jitkaew et al., 2009; Stowell et al., 2009; Bratton and Henson, 2011). In *C. elegans*, primary phagocytosis has been shown to contribute to programmed cell death of neuronal precursors during development (Hoeppner et al., 2001; Reddien et al., 2001), the elimination of cells subjected to sub-toxic insults (Neukomm et al., 2011) or simply as a result of phosphatidylserine (PS) exposure on the surface of cells (Darland-Ransom et al., 2008). In this review, we will briefly discuss the mechanisms of phagocytosis of cells, current evidence for primary phagocytosis in the central nervous system (CNS) and the resulting implications for neurodegenerative disease.

MECHANISMS OF PHAGOCYTOSIS OF HOST CELLS

The process of phagocytosis is normally initiated by the release of attractive signals from the target cell (referred to as "come-get-me" signals) leading to chemotaxis of a nearby macrophage. Upon reaching the target cell, the macrophage recognizes cell-surface signals on the target cell ("eat-me" signals), which then induce its uptake. The best characterized "eat-me" signal is the cell-surface exposure of phosphatidylserine (PS; Fadok et al., 1992; Martin et al., 1995), although display of proteins such as calreticulin has also been implicated (Gardai et al., 2005). In healthy cells, PS is found exclusively on the inner leaflet of the plasma membrane, because the aminophospholipid translocase removes PS from the outer leaflet. However, a second enzyme, the phospholipid scramblase, can cause PS exposure by randomizing phospholipid distribution between the inner and outer leaflets. PS exposure may occur as a result of: (i) apoptosis (by unknown mechanisms), (ii) necrosis (due to plasma membrane rupture), (iii) calcium elevation (which stimulates the phospholipid scramblase and inhibits the aminophospholipid translocase), (iv) ATP depletion (which inhibits the aminophospholipid translocase), (v) oxidative stress (which inhibits the aminophospholipid translocase and stimulates the scramblase), and/or (vi) fusion of intracellular vesicles with the plasma membrane (Bratton et al., 1997; Gleiss et al., 2002; Tyurina et al., 2007).

While PS display has generally been regarded as an early sign of apoptotic cell death, it is now clear that PS exposure can be reversible and independent of apoptosis (Dias-Baruffi et al., 2003; Mackenzie et al., 2005; Tyurina et al., 2007; Jitkaew et al., 2009; Neher et al., 2011), and therefore may lead to the phagocytosis of viable host cells in the presence of macrophages. For example, galectin-1 induces PS exposure on the surface of neutrophils, which is fully reversible when galectin-1 is removed and does not lead to cell death. However, when macrophages are present at the time of PS exposure these cells are phagocytosed and thus killed (Dias-Baruffi et al., 2003; Stowell et al., 2009). Whether exposure of PS by itself is sufficient for recognition and removal is not entirely clear: PS exposure may be sufficient for some cells (Fadok et al., 2001), while others may require PS oxidation or other co-stimulatory signals to induce phagocytosis (Borisenko et al., 2003, 2004). Furthermore, some healthy cells actively protect themselves from phagocytic removal by displaying signals on their surface,

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Abbreviations: AD, Alzheimer's disease; DAP12, DNAX adaptor protein-12; FTD, Frontotemporal dementia; MerTK, Mer receptor tyrosine kinase; MFG-E8, milk fat globule EGF-like factor 8; MS, multiple sclerosis; PD, Parkinson's disease; PS, phosphatidylserine; Tim4, T-cell immunoglobulin- and mucin-domain-containing molecule-4; TLR, Toll-like receptor; TREM2, triggering receptor expressed by myeloid cells-2; VR, vitronectin receptor.

which inhibit phagocytosis ("don't-eat-me" signals, such as CD200 or CD47; see below).

A range of receptors on the macrophage can mediate recognition of PS on target cells, implying redundancy on first glance. However, not all receptors are expressed by a macrophage at any one time, but rather are dependent on its activation state, e.g., classically activated/pro-inflammatory vs. alternatively activated/anti-inflammatory macrophages. For example, resting peritoneal mouse macrophages express the PS receptors Tim4 (T-cell immunoglobulin- and mucin-domain-containing molecule-4; Kobayashi et al., 2007; Miyanishi et al., 2007), and alternatively activated human monocyte-derived macrophages express stabilin-1 (Park et al., 2009) and stabilin-2 (Park et al., 2008). In contrast, inflammatory activated (thioglycollate-elicited) peritoneal macrophages upregulate the PS-binding protein MFG-E8 (Milk fat globule EGF-like factor 8, also known as lactadherin, SED1; Hanayama et al., 2002) and the Mer receptor tyrosine kinase (MerTK), which recognizes PS or other eat-me signals through bridging molecules Gas6, protein S, galectin-3, tubby, and Tulp1 (Scott et al., 2001; Seitz et al., 2007; Shao et al., 2009; Caberoy et al., 2011). At the same time, activated (thioglycollate-elicited) macrophages downregulate expression of other phagocytic proteins such as Tim4 (Miyanishi et al., 2007). Interestingly, it therefore appears from the literature that resting macrophages express receptors that bind PS directly (i.e., Tim4, stabilin-1/2), whereas activated/pro-inflammatory macrophages utilize phagocytic pathways that recruit bridging proteins (e.g., MFG-E8, Gas6), which bind both PS on the target cell and a receptor on the macrophage [e.g., vitronectin receptor (VR), MerTK].

Microglia are resident brain macrophages, which continuously and actively survey their microenvironment (Nimmerjahn et al., 2005). Microglia represent a distinct population of tissue macrophages as they arise at least in part from primitive myeloid progenitors during early embryonic stages and are thought to be maintained by self-replication throughout life in the healthy brain (Ginhoux et al., 2010). However, under specific conditions, such as brain injury and inflammation, peripheral monocytes can be recruited to the brain. This occurs for example after stroke, but whether peripheral monocytes modify the pathology of chronic neurodegenerative diseases is disputed. In Alzheimer's disease (AD) for example, recent evidence indicates that peripheral monocytes do not invade the brain, but contribute to removal of amyloid-β (Aβ) in the perivascular environment (Mildner et al., 2011). While it is beyond the scope of this review to discuss this issue in detail, it appears that invading peripheral monocytes may mediate effects distinct from resident microglia (Prinz et al., 2011). Nevertheless, microglia share a number of similarities with peripheral macrophages, including their recognition of both exogenous "non-self" ligands (e.g., lipopolysaccharide, LPS) and endogenous "self" ligands (such as Aβ, HSP60, and HMGB1) through pattern recognition receptors (such as Toll-like receptor-2 and -4; Kawai and Akira, 2010). Recognition of these ligands results in inflammation and associated neurodegeneration due to inflammatory activation of microglia, which also renders the microglia highly phagocytic (Babcock et al., 2006; Boivin et al., 2007; Hanisch and Kettenmann, 2007; Neher et al., 2011; Neniskyte et al., 2011). Other macrophage populations in the brain, with phenotypes distinct from microglia, include those associated with the perivascular space, the circumventricular organs, the choroid plexus, and the meninges (Ransohoff and Perry, 2009).

Although data on microglia is limited, they appear to express similar phagocytic receptors and bridging proteins as peripheral macrophages. For example, primary microglia in culture express MFG-E8 (Neher et al., 2011) and MerTK (Grommes et al., 2008), possibly consistent with a partially activated microglial phenotype induced by cell-isolation procedures (Streit et al., 1999) as expression levels of these two proteins are low in homogenates of the naïve brain (Binder et al., 2008; Fuller and Van Eldik, 2008). In support of this notion, it has been reported that stimulation of microglia with the TLR4 ligand LPS increases MerTK expression in culture, and MerTK and its ligand Gas6 are also upregulated after a demyelinating insult in the brain (Binder et al., 2008). The expression of PS receptors in resting microglia has not been investigated in detail, but the mRNA levels of Tim4 appear to be low in brain homogenates (Miyanishi et al., 2007; Tanaka et al., 2009). Importantly, in the CNS, surrounding astrocytes may support microglial phagocytic function by producing bridging proteins such as MFG-E8 (Boddaert et al., 2007; Cahoy et al., 2008; Kranich et al., 2010) and protein S (Stitt et al., 1995), thus enabling efficient clearance of PS-exposing neurons.

A different phagocytic receptor, triggering receptor expressed by myeloid cells-2 (TREM2), mediates microglial clearance of debris and apoptotic neurons *in vitro* after activation by TREM2 ligands expressed on neuronal cells. Accordingly, knockdown of TREM2 impairs phagocytic function of microglia and increases the generation of pro-inflammatory cytokines *in vitro* (Takahashi et al., 2005). The function of TREM2 and its signaling partner DNAX adaptor protein-12 (DAP12) are essential for CNS immune homeostasis as loss-of-function mutations cause Nasu–Hakola disease (also known as polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy, PLOSL), which presents with inflammation and neurodegeneration (Neumann and Takahashi, 2007). This supports the idea that microglial phagocytosis of dead and dying cells (rather than viable cells) can be protective and anti-inflammatory. The only identified TREM2 agonist is the endogenous "self" ligand HSP60, which upon binding to TREM2 strongly stimulates microglial phagocytosis (Stefano et al., 2009). Interestingly, HSP60 is also a ligand for TLR4, and TLR4 activation by HSP60 can cause microglial activation and inflammatory neurodegeneration *in vitro* (Lehnardt et al., 2008). Thus, TLR4 activation by HSP60 may contribute to the inflammation and neurodegeneration seen in Nasu–Hakola disease, where the anti-inflammatory signaling via HSP60 and TREM2 would be missing.

Wang and Neumann (2010) identified Siglec-11 as a microglial receptor, which binds polysialylated proteins on the surface of neurons (in particular neuronal cell adhesion molecule, NCAM) resulting in inhibition of inflammation and phagocytosis. Transfection of mouse microglia with human Siglec-11 reduced the spontaneous phagocytosis of neurites and neuronal cell bodies occurring in neuronal–microglial co-cultures, and this was dependent on the presence of polysialylated proteins on the surface of neurons. Thus polysialylation can act as a "don't-eat-me" signal for neurons *in vitro*.

Several other molecules can downregulate microglial activity. CD200 and CD47 are both expressed on the surface of neurons, and are known to act as "don't-eat-me-signals." The CD200 receptor (OX2) is found on microglia (Wright et al., 2000), and CD200 suppresses brain inflammation in experimental autoimmune encephalomyelitis and after facial nerve transection (Hoek et al., 2000). CD47 expression on cells and myelin sheaths inhibits phagocytosis by microglia via a CD47 receptor, SIRPα (Gitik et al., 2011).

Another signaling pathway that has been shown to modulate microglial activity is the CX3CL1 (fractalkine)/CX3CR1 pathway. CX3CL1 is expressed by neurons, while its receptor is predominantly expressed by microglia in the brain (Harrison et al., 1998). Inflammatory microglial activity can be suppressed by the neuronal chemokine CX3CL1 (also known as fractalkine) via the microglial chemokine receptor CX3CR1 as shown in neuron–microglia co-cultures (Zujovic et al., 2000) and in CX3CR1-deficient mice after systemic injection of LPS, in the MPTP model of Parkinson's Disease (PD), as well as in a transgenic model of amyotrophic lateral sclerosis (Cardona et al., 2006). Furthermore, CX3CR1 knockout restricts natural killer cell recruitment in experimental autoimmune encephalomyelitis thereby worsening disease outcome (Huang et al., 2006). However, in other circumstances fractalkine/fractalkine receptor knockout has been shown to improve neuropathology. For example, CX3CR1 deficiency resulted in improved outcome after spinal cord injury possibly due to enhanced recruitment of bone marrow derived macrophages, which also displayed reduced release of inflammatory mediators (Donnelly et al., 2011). Further, it has been reported that CX3CL1 deficient mice displayed smaller infarcts (about 30%) 24 h after middle cerebral artery occlusion (MCAo; Soriano et al., 2002), although the mechanisms of this effect were not analyzed in this study. However, a different group showed an even more pronounced reduction of infarct size (more than 50%), reduced blood brain barrier damage, leukocyte infiltration, neuronal death, and levels of IL-1β (Denes et al., 2008) between 1 and 3 days after MCAo. Importantly, a recent study analyzed the influence of the fractalkine/fractalkine receptor pathway after permanent MCAo (Cipriani et al., 2011). Similar to the two studies described above, they found reduced infarct size in both CX3CL1 and CX3CR1 knockout mice 24 h after the insult. Interestingly, *icv* infusion of recombinant CX3CL1 in rats also reduced infarct size and this effect persisted for up to 56 days. When analyzing the *in vitro* responses of wildtype and CX3CL1 knockout microglia to medium from oxygen–glucose deprived neurons, the authors found that microglial phagocytic activity was suppressed only in wildtype, but not in CX3CL1 knockout microglia. In the same experiment, the release of TNF-α was reduced in CX3CL1 knockout but not in wildtype microglia demonstrating a changed microglial response resulting from fractalkine knockout.

Fractalkine is normally displayed on the cell surface of neurons, but its release is induced by stress such as nerve injury or excitotoxicity, when it may suppress microglial inflammation but can also act as a chemokine for leukocyte infiltration as well as microglial recruitment. Additionally, soluble fractalkine may also promote microglial phagocytosis of neuronal debris by stimulating microglial production and release of MFG-E8 (Harrison et al., 1998; Cook et al., 2010; Fuhrmann et al., 2010; Noda et al., 2011) and induces upregulation of microglial integrin β_5 expression, which is one of the subunits of the receptor for MFG-E8, the VR (Leonardi-Essmann et al., 2005). Interpretation of experiments in CX3CL1 or CX3CR1 knockout animals are therefore difficult as the outcome may be due to any of the above mechanisms or combinations thereof. However, from the literature described above, it appears that suppression of leukocyte recruitment and microglial inflammation may dominate the outcome.

EVIDENCE FOR PRIMARY PHAGOCYTOSIS IN THE CNS

Activation of microglial phagocytosis is generally considered to be beneficial via removal of pathogens or potentially pro-inflammatory debris and apoptotic cells (Neumann et al., 2009). However, we and others have shown that microglia can also phagocytose viable synapses and neurons. For example, during development microglia may be involved in synaptic pruning, i.e., elimination of synapses, and mice lacking the fractalkine receptor, CX3CR1, show higher densities of spines and functional synapses during early postnatal development, which the authors attributed to temporarily reduced microglial density (Paolicelli et al., 2011). Furthermore, microglia kill developing neurons in cerebellar organotypic slices leading to an increase in the number of fully differentiated Purkinje cell clusters (Marín-Teva et al., 2004). Similarly, two phagocytosis-related proteins, CD11b and DAP12, appear to mediate developmental neuronal death in the hippocampus *in vivo* (Wakselman et al., 2008). In animals with a loss-of-function mutation in DAP12 as well as by inhibition of the complement receptor 3 subunit CD11b, neuronal death was reduced at postnatal day 1 by about 25%. Both in cerebellar slices and in the hippocampus, this effect appeared to be mediated through the release of reactive oxygen species, although it is unclear whether phagocytosis is required for this developmental death. These data are reminiscent of findings in the nematode, where knockdown of phagocytic genes was shown to result in the survival of neuronal precursor cells that would otherwise die during development (Hoeppner et al., 2001; Reddien et al., 2001) indicating a potential role for primary phagocytosis of immature neurons.

Importantly, we and others have found that stressed but viable cells can reversibly expose PS *in vitro* (Tyurina et al., 2007; Jitkaew et al., 2009; Kim et al., 2010; Neher et al., 2011) and therefore macrophages may potentially phagocytose viable cells. In particular, we have shown that microglia activated with LPS (a bacterial TLR4 agonist), lipoteichoic acid (LTA, a bacterial TLR2 agonist), or nanomolar concentrations of Aβ (an endogenous TLR2/4 agonist) phagocytose viable neurons *in vitro* (**Figure 1**). We found that microglial activation with these ligands greatly increases their phagocytic activity, and video imaging of the inflamed glial-neuronal cultures showed highly mobile microglia phagocytosing large numbers of neurons appearing morphologically healthy (Neher et al., 2011). Mechanistically, we showed that inflammatory activated microglia release reactive oxygen and nitrogen species, which induce reversible PS exposure on neurons and neuronal phagocytosis by microglia (Neher et al., 2011; Neniskyte et al.,

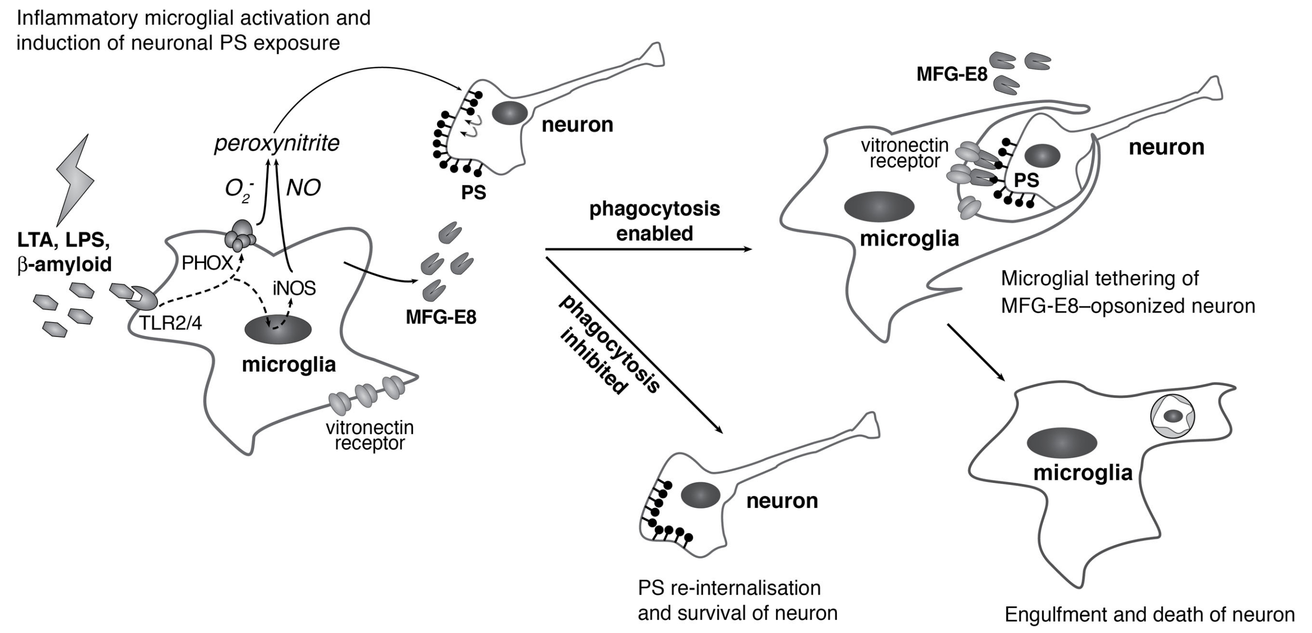

FIGURE 1 | Suggested mechanism of primary phagocytosis in inflammatory neurodegeneration. Inflammatory microglial activation with lipopolysaccharide (LPS), lipoteichoic acid (LTA), or amyloid-β (Aβ) through Toll-like receptors-2/4 induces expression of inducible nitric oxide synthase (iNOS) and assembly of the phagocytic NADPH oxidase (PHOX). iNOS and PHOX produce nitric oxide (NO) and superoxide (O_2^-), respectively, which react to form low concentrations of peroxynitrite. These sublethal levels of peroxynitrite (while not causing neuronal death) are sufficient to cause neuronal exposure of phosphatidylserine (PS), which is recognized by the bridging protein MFG-E8 (milk fat globule EGF-like factor 8). MFG-E8 also binds to the microglial vitronectin receptor (the heterodimeric $\alpha_v\beta_{3/5}$ integrin), thereby causing engulfment of the PS-exposing neuron. If phagocytosis is blocked, however, neurons are able to re-internalize PS, thus evading phagocytosis and death.

2011). Specifically, when inflammatory activated microglia were co-cultured with neurons but physically separated by a transwell membrane, neurons showed increased PS exposure but no signs of cell death. When microglia were then allowed to interact with these neurons, the neurons were phagocytosed and thus killed. However, if the activated microglia were removed from the transwell co-culture, the neuronal PS exposure was fully reversed and neurons remained viable for as long as they were cultured. This implied that inflamed microglia release soluble mediators that cause reversible PS exposure on neurons. We identified these soluble mediators to be reactive oxygen or nitrogen species, probably peroxynitrite, based on the findings that the reversible PS exposure and neuronal loss in glial-neuronal cultures is prevented by inhibitors of the NADPH oxidase (PHOX) or nitric oxide synthases (NOS), or addition of superoxide dismutase or a peroxynitrite scavenger. Furthermore, addition of 5–10 μM peroxynitrite alone caused neuronal loss in the presence of microglia, but reversible PS exposure and no neuronal loss or death in the absence of microglia (Neher et al., 2011).

We further identified a pathway consisting of neuronal PS exposure, recognition of PS by the bridging molecule MFG-E8, and MFG-E8 binding to the VR to be essential for neuronal loss. Again, blocking different components of the PS/MFG-E8/VR pathway (proteins blocking the exposed PS, antibodies to MFG-E8, VR antagonists) rescued neurons, leaving apparently healthy cells behind (Neher et al., 2011; Neniskyte et al., 2011; Fricker et al., 2012). If the neurons had been killed first and then eaten, then blocking phagocytosis would leave dead neurons. Instead, blocking phagocytosis in LPS or LTA-treated cultures prevented essentially all neuronal loss and death, leaving neurons with intact plasma and mitochondrial membrane potentials, and these neurons remained alive for as long as untreated cultures (Neher et al., 2011).

More recently we have found that LPS-induced neuronal loss is absent in glial-neuronal cultures from *Mfge8* knockout mice, but can be reconstituted by adding purified MFG-E8 to these cultures (Fricker et al., 2012). The absence or presence of MFG-E8 had no apparent effect on microglial inflammation, and this is very strong evidence that the inflammatory neuronal death is mediated by the MFG-E8 pathway of phagocytosis. Further, we have shown that LPS (5 μg and 1 μg, respectively) injection into the striatum of rats and mice *in vivo* causes strong microglial inflammation and loss of 20–30% of neurons, which is reduced by approximately 50% through co-injection of a VR inhibitor and in *Mfge8* knockout mice (Fricker et al., 2012).

Similarly, McArthur et al. (2010) recently reported that a microglial cell-line (BV2), when activated with LPS or Aβ phagocytosed viable neuron-like cells (PC12). Although a rescue of neurons by blocking phagocytosis was not shown in this study, these data further support the idea that inflammatory activated microglia may phagocytose stressed but viable neurons, thereby executing neuronal death. Altogether, this suggests the possibility that inflammatory neuronal loss in human pathologies may be prevented by inhibitors of specific phagocytic pathways.

POTENTIAL ROLES OF PRIMARY PHAGOCYTOSIS IN NEURODEGENERATIVE DISEASES

Alzheimer's disease is characterized by extracellular plaques of which the main constituent is Aβ. These plaques are associated with inflammatory activated microglia, and there is evidence that inflammation may contribute to the disease (Griffin et al., 1998; Combs, 2009). Some studies have indicated a beneficial role of microglia in the disease process, which may be due to phagocytic removal of Aβ, although it is possible that this effect is mostly mediated by invading (CCR2+) peripheral monocytes rather than resident microglia (Simard et al., 2006; El Khoury et al., 2007). However, it has recently become clear that these data may have been confounded by the fact that many studies investigating monocyte recruitment to the brain used whole-body irradiation, which may prompt invasion of peripheral monocytes. By shielding the brain, a recent study found that peripheral monocytes did not invade the brain and that amyloid clearance was mediated by perivascular myeloid cells (Mildner et al., 2011).

In humans, AD is also characterized by extensive loss of neurons and synapses by means that are not entirely clear. In contrast, in most animal models of AD neuronal loss is limited, restricted to specific brain regions and negatively correlated to plaque load. However, interestingly in these models neuronal loss occurs predominantly in the hippocampus, the area affected most severely in the human condition (Calhoun et al., 1998; Fuhrmann et al., 2010; Rupp et al., 2011).

High concentrations of Aβ (μM) can induce direct toxicity to neurons, but low concentrations (nM, which may be more relevant to AD) induce neuronal loss via inflammatory activation of glia *in vitro* (Maezawa et al., 2011). We found that nanomolar concentrations of Aβ caused microglia to phagocytose viable neurons and synapses in culture, and if we blocked this phagocytosis then all neuronal loss and death was prevented (Neniskyte et al., 2011). This suggests the possibility that microglial phagocytosis of synapses and neurons contributes to AD.

In line with this hypothesis, Aβ can induce PS exposure on neurons (Mohmmad Abdul and Butterfield, 2005), and there appears to be an increase in PS-exposed neurons in AD and mild cognitive impairment, as evidenced by PS-exposing synaptosomes isolated from patients' brains (Bader Lange et al., 2008, 2010). We have found that the PS-binding bridging protein MFG-E8 and its VR mediate inflammatory neuronal loss by primary phagocytosis *in vitro* (Neher et al., 2011; Neniskyte et al., 2011; Fricker et al., 2012). Levels of MFG-E8 are reduced in the brains of AD mice and patients with AD (Boddaert et al., 2007; Fuller and Van Eldik, 2008) possibly due to phagocytosis and degradation, while integrin β_3, a subunit of the MFG-E8 recognizing VR is strongly upregulated on reactive microglia in AD (Akiyama et al., 1991). However, it has also been reported that MFG-E8 can interact directly with Aβ (Boddaert et al., 2007), and it therefore remains to be determined whether in AD MFG-E8 mediates phagocytosis of Aβ or of PS-exposing synapses and/or neurons.

Microglia have been shown to contribute to neuronal loss in an Alzheimer's mouse model (Fuhrmann et al., 2010). Two-photon imaging of neuronal loss in the brain of living triple transgenic APP/PS1/tau AD mice revealed an involvement of microglia in neuron elimination. Microglial number and migration velocity was increased prior to loss of neurons and knockout of the microglial chemokine receptor CX3CR1, which is critical for microglial chemotaxis, prevented this neuron loss (Fuhrmann et al., 2010). However, testing whether phagocytosis of neurons in AD is primary or secondary to neuronal death by other means *in vivo* is challenging. Primary phagocytosis (unlike apoptosis or necrosis) leaves no cell corpse to diagnose the cause of death. Furthermore, most mouse models expressing mutant APP and presenilins lack significant neuronal loss unless they also include mutant tau, and we currently lack suitable phagocytosis inhibitors that cross the blood–brain barrier. However, in principle, mouse models deficient in phagocytic genes could be used to test for primary phagocytosis.

More support for an involvement of phagocytosis of cells in AD comes from recent genome wide association studies. These analyses revealed that variants in a number of phagocytosis-related genes promote late onset AD, including ApoE, ApoJ (clusterin), ABCA7, and CR1 (Jun et al., 2010; Morgan, 2011). ApoE genotype has the largest effect on AD incidence, and is known to modulate phagocytosis of apoptotic cells *in vitro* and *in vivo* (Grainger et al., 2004). Clusterin expression is upregulated by exposure to phosphatidylserine and it facilitates the uptake of cellular debris through a pathway mediated by the LDL receptor-related protein (LRP), megalin, and other yet undefined endocytic receptors (Bach et al., 2001; Bartl et al., 2001). Optimal ligand-induced signaling through LRP requires the presence of another protein related to increased AD risk, namely ABCA7. After stimulation, ABCA7 is transported to the cell membrane, localizes to the phagocytic cup and enhances the clearance of apoptotic cells *in vitro* and *in vivo* (Jehle et al., 2006). Altogether, accumulating evidence suggests that microglial phagocytosis of neurons may have a role in AD. Since impaired phagocytosis of cells can lead to inflammation, it remains to be determined whether phagocytosis may contribute to AD pathology through primary phagocytosis of viable synapses/neurons or through inflammation resulting from impaired phagocytosis of dying cells.

Parkinson's disease is characterized by motor dysfunction, resulting from progressive loss of neurons, particularly dopaminergic neurons of the pars compacta region of the substantia nigra (SN). The causes of neuronal loss may include: α-synuclein inclusions (which may be directly toxic to neurons), mitochondrial dysfunction, and glial inflammation (Tansey et al., 2007). A role for glial inflammation is suggested by the findings of: (i) inflamed glia and pro-inflammatory cytokines in the SN of patients and animal models, (ii) pro-inflammatory agents, e.g., LPS, causing loss of dopaminergic neurons in culture and *in vivo*, and (iii) anti-inflammatory drugs being protective in patients and animal models (McGeer et al., 1988; Herrera et al., 2005; Block et al., 2007; Tansey et al., 2007). We have found that LPS injection into rodent striatum causes microglial inflammation and neuronal loss reminiscent of the dopaminergic neuronal loss in LPS-induced PD. Using this model, we found that neuronal loss and death is reduced in *Mfge8* knockout mice or by co-injection of phagocytosis inhibitors (Fricker et al., 2012), suggesting that primary phagocytosis may contribute to inflammatory neuronal loss as it may occur in PD.

Substantia nigra neurons are black because they contain neuromelanin. Neuromelanin granules are found within activated microglia of the SN in PD patients, suggesting microglial phagocytosis of neurons (McGeer et al., 1988; Banati et al., 1998), even though there is little evidence of neuronal apoptosis in PD (Banati et al., 1998). Furthermore, addition of human neuromelanin causes microglial activation and dopaminergic neuronal loss in culture and *in vivo*, and this neuronal loss is prevented if a microglial phagocytosis receptor (Mac-1/CR3) is genetically deleted (Zhang et al., 2011b). Thus the neuromelanin-induced neuronal loss could be due to primary phagocytosis, however these results were interpreted in terms of a Mac-1 requirement for phagocytosis of neuromelanin (Zhang et al., 2011b). Interestingly, Aβ-induced neuronal loss is also dependent on Mac-1 (Zhang et al., 2011a), suggesting either that Mac-1 is an Aβ receptor or that neuronal loss is occurring by primary phagocytosis.

Importantly, it has been reported that in the 6-hydroxydopamine model of PD microglia become activated before any significant decrease in the number of dopaminergic neurons. Phagocytic microglia are found attached to morphologically intact neurons with normal chromatin distribution, suggesting that microglia may engulf pre-apoptotic neurons precluding the possibility of their recovery (Marinova-Mutafchieva et al., 2009), in accordance with a role for primary phagocytosis in PD.

Frontotemporal lobar degeneration (FTD) has a strong genetic component, and one of the major causes is inactivating mutations in the progranulin gene (Baker et al., 2006; Cruts et al., 2006). Progranulin deficiency leads to exaggerated microglial response after insult as well as to age-dependent accumulation of activated microglia (Ahmed et al., 2007; Yin et al., 2010a,b). Importantly, progranulin was recently found to inhibit phagocytosis of apoptotic/PS-exposed cells in culture and *in vivo*, and it was suggested that neuronal loss in FTD was due to primary phagocytosis that progranulin normally suppressed (Kao et al., 2011). Strikingly, polymorphisms in the progranulin gene are also associated with AD, PD, and amyotrophic lateral sclerosis (Brouwers et al., 2007, 2008; Sleegers et al., 2008). Therefore it is possible that premature neuronal phagocytosis as observed in the FTD model may also contribute to pathogenesis of other neurodegenerative diseases.

CONCLUSION

We have recently found that inflammatory activated microglia can execute neuronal death through phagocytosis. This primary phagocytosis is a potential mechanism for neuronal loss in neurodegenerative diseases that present with inflammation and microglial activation, and we have reviewed evidence here suggestive of such a mechanism. Primary phagocytosis may have been overlooked as a form of cell death as it is difficult to assess whether a neuron was viable prior to its phagocytosis and because the general assumption has always been that a cell must die before it is phagocytosed. The only way to determine whether phagocytosis mediates cell death is by comparing neuronal survival in the presence and absence of phagocytosis. It will be important to test the contribution of primary phagocytosis to neuronal death using models of neurodegenerative disease in mice that are deficient in phagocytic components.

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The role of the innate immune system in ALS

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Amyotrophic lateral sclerosis (ALS) is a fatal, adult-onset neurodegenerative disease that is characterized by the death of upper and lower motor neurons. Recent studies have made it clear that although motor neurons are the primary targets of the degenerative process, other cell types play key roles in the death of motor neurons. Most notably, cells of the immune system, including astrocytes and microglia have come under increasing scrutiny, after multiple lines of evidence have shown these cells to be deleterious to motor neurons. Both *in vitro* and *in vivo* experiments have shown that astrocytes and microglia containing mutated SOD1 are harmful to motor neurons. Several studies on ALS and other neurodegenerative diseases have revealed that reactive astrocytes and microglia are capable of releasing pro-inflammatory factors such as cytokines and chemokines, which are harmful to neighboring neurons. In addition, it is believed that diseased astrocytes can specifically kill motor neurons through the release of toxic factors. Furthermore, in an animal model of the disease, it has been shown that the reduction of SOD1 in microglia may be able to slow the progression of ALS symptoms. Although the exact pathways of motor neuron death in ALS have yet to be elucidated, studies have suggested that they die through aBax-dependent signaling pathway. Mounting evidence suggests that neuroinflammation plays an important role in the degeneration of motor neurons. Based on these findings, anti-inflammatory compounds are currently being tested for their potential to reduce disease severity; however, these studies are only in the preliminary stages. While we understand that astrocytes and microglia play a role in the death of motor neurons in ALS, much work needs to be done to fully understand ALS pathology and the role the immune system plays in disease onset and progression.

Keywords: amyotrophic lateral sclerosis, astrocyte, microglia, motor neuron, innate immune system

INTRODUCTION

Amyotrophic Lateral Sclerosis (ALS) is a fatal adult-onset neurodegenerative disorder that is characterized clinically by muscle weakness and wasting. Pathologically, it is identified by the degeneration of upper and lower motor neurons (Boillee et al., 2006a). Generally, death results from respiratory failure due to paralysis of the respiratory muscles. The incidence of ALS is reported to be between 1.5 and 3 per 100,000 in Europe and North America (Wijesekera and Leigh, 2009; Hardiman et al., 2011). ALS onset peaks between the ages of 50–75 and declines thereafter. These factors suggest that ALS may not be a disease of aging, but rather that age may be one of a multitude of contributing factors (Hardiman et al., 2011). Thus far, there are no effective treatments and no cure for ALS.

Amyotrophic lateral sclerosis is essentially a sporadic disorder, with greater than 90% of the cases originating from an unknown cause. However, approximately 10% of the cases are considered familial (FALS) and are generally inherited as a dominant disorder (Ince et al., 2011) due to mutations in a number of seemingly disparate genes. These include, but are not limited to, mutations in genes such as TAR DNA binding protein (TDP)-43 and Fused in Sarcoma (FUS) (Sreedharan et al., 2008; Kwiatkowski et al., 2009; Vance et al., 2009). Despite major enthusiasm by the research community about the recent discoveries of mutations in FUS and TDP-43, the best characterized form of FALS still remains that which is linked to mutations in the copper-zinc superoxide dismutase gene (SOD1) and which accounts for 10–20% of all FALS cases (Andersen, 2001). Myriad mutations in SOD1 all seem to give rise to an ALS phenotype via a toxic gain-of-function mechanism, which is clinically almost indistinguishable from sporadic cases. Histopathologically, however, recent studies have found TDP-43 to be a reliable marker for differentiating sALS from SOD1-related fALS. These studies have shown that all of the cases of sporadic ALS and SOD1-negative fALS had neural and glial inclusions which were immunoreactive for both ubiquitin and TDP-43, whereas fALS cases with mutations in SOD1 were universally absent of TDP-43 immunoreactivity (MacKenzie et al., 2007; Tan et al., 2007).

More recently, studies have identified an expanded GGGGCC hexanucleotide repeat in a non-coding region of C9ORF72. This expansion has been shown to be linked to chromosome 9p-linked FTD and ALS. Interestingly, this same repeat expansion was also identified in the majority of families with a combined FTD/ALS phenotype and TDP-43 pathology. Extended studies have identified the C9ORF72 expansion as the most common genetic abnormality in fALS (23.5%; DeJesus-Hernandez et al., 2011).

A number of studies have pointed to oxidative stress, protein aggregation, or mitochondrial dysfunction as mechanisms of mutant SOD1 toxicity. In addition, SOD1 toxicity has been shown to be linked to changes in the immune system, which has also been shown to contribute to the motor neuron pathology seen in ALS; however, the exact nature of the pathogenic mechanisms provoking motor neuron degeneration in mutant SOD1-linked ALS remains elusive (Ince et al., 2011). Interestingly, the repeat expansion in C9ORF72 results in the formation of nuclear RNA foci not seen in SOD1-linked pathology, suggesting multiple disease mechanisms (DeJesus-Hernandez et al., 2011).

While the discovery of the C9ORF72 repeat expansion has opened new avenues of ALS research, the abundance and well-studied nature of SOD1-mutant animals make them the *de facto* models of choice to study ALS pathology for the time being. Although imperfect, these models allow for a great deal of insight into potential mechanisms involved in ALS pathology, with the hope that the mechanisms elucidated through the use of these models may also provide understanding of sALS and non-SOD1-mediated fALS.

INFLAMMATION AND NEURODEGENERATION

Although ALS is a disease primarily affecting upper and lower motor neurons, it is increasingly recognized that the entire pathogenic process of ALS is not restricted to a set of cell-autonomous deleterious mechanisms taking place within motor neurons. Instead, it is now believed that non-cell autonomous mechanisms, such as neuroinflammation may also contribute to the disease process.

Germane to this issue is the fact that the immune system has been found to be altered in sporadic ALS. Studies have shown immunological differences in the blood of ALS patients compared to healthy controls. These include increased levels of CD4+ cells, and reduced CD8+ T-lymphocytes (Mantovani et al., 2009). Interestingly, blood samples analyzed from patients at an earlier and less severe stage of the disease also show altered expression of immune cells, such as significant reductions in CD4+CD25+ T-regulatory (T-reg) cells as well as CD14+ monocytes (Mantovani et al., 2009). Additionally, T-reg cells have been shown to play significant roles as neuroprotectants responsible for modulating the neuroinflammatory response in mouse models of neurodegeneration (Kipnis et al., 2004). It is therefore possible to hypothesize that the reduction of T-reg cells in the blood of sporadic ALS patients might represent a recruitment of these cells from the periphery into the CNS in order to activate resident innate immune cells such as microglia, as well as anti-inflammatory cytokines such as interleukin-10 and transforming growth factor-β in an effort to protect the area most affected by the early effects of ALS degeneration (Kipnis et al., 2004; Mantovani et al., 2009).

Markers for resident innate immune cells have also been found to be altered in the brains of ALS patients as well as in animal models of ALS. For instance, immunostaining for glial fibrillary acid protein (GFAP), a common marker for astrocytes, is markedly increased in all forms of ALS in the precentral gyrus of human samples (Kawamata et al., 1992). In addition, staining for leukocyte common antigen (LCA), lymphocyte function associate molecule-1 (LFA-1), and complement receptors CR3 (CD11b) and CR4 (CD11c) are increased, supporting the idea that microglia and macrophages are activated in the areas of ALS degeneration, such as the motor cortex, brainstem, and corticospinal tract (Kawamata et al., 1992; Papadimitriou et al., 2010). Remarkably, it is believed that the early site of pathological changes in ALS is the neuromuscular junction, and while this particular site of the lower motor neuron pathway has been extensively studied, very little information exists about the immune response at that level. Nonetheless, the data reviewed above provide compelling evidence that a robust neuroinflammatory response is part of the neuropathological changes that characterize ALS. However, none of the aforementioned studies address the actual role, if any, of neuroinflammation in ALS. Investigations in other neurodegenerative disorders have shown that neuroinflammation may exert dual effects, that it can be protective or harmful. In order to distinguish between these two possibilities, efforts have generally concentrated on studying animal models of ALS.

The blood brain barrier renders the brain as immune-privileged, therefore, the brain contains resident astrocytes and microglia that perform the duties of immune surveillance and act as an innate response system. Reactive astrocytes are most commonly recruited to the site of an injury, such as a spinal cord injury or ischemic events. Their main functions are to compact inflammatory cells, and to re-establish the blood brain barrier (Okada et al., 2006) they may also be involved in clearing CNS debris, such as amyloid plaques (Papadimitriou et al., 2010). Aside from astrocytes, the CNS immune system also consists of ramified resting microglial cells, whose processes play an active role in maintaining the homeostasis of the neural environment. Once activated by the presence of antigens and during times of distress, such as is the case during neurodegeneration, microglia, like other immune cells, become activated taking on an ameboid morphology. Once activated, microglia can secrete a myriad of neurotrophic factors and cytokines (Napoli and Neumann, 2009), and engage in the clearance of pathogens and debris through phagocytosis. However, unregulated activation of microglial activation may be harmful to neurons through the release of potentially harmful neurotoxins including quinolinic acid, reactive oxygen and nitrogen species (ROS; RNS), and pro-inflammatory cytokines. Although a large number of studies have shown that microglia and, to a lesser extent, astrocytes are activated in the affected areas of the CNS in ALS, thus far little attention has been paid to the determination of the actual phenotype of these cells. For instance, since activated resident immune cells can display either a pro-inflammatory or anti-inflammatory phenotype, sometimes referred to as M1 or M2, it will be critical to determine which phenotype they exhibit as a whole, and if possible as individual cells. It is also important to determine whether such profiles differ among the various affected structures of the CNS and stages of the disease. Aside from these outstanding questions, it is also critical to elucidate whether these inflammatory cells play a role in the pathogenesis of ALS. The use of animal models is greatly aiding in the attempts to answer the questions raised by the findings described above.

The most commonly studied animal models of ALS are rats and mice expressing mutant SOD1 (mSOD1). Although, as indicated above, SOD 1 mutations only account for a small percentage of human cases, animal models using mSOD1 are used with the hope that the mechanisms leading the disease are shared across cases, and knowledge gained from these models can be used to understand the development of the disease in humans. Notably, evidence for increases in astrocytes and microglia have also been found in transgenic mice expressing different forms of mutant SOD1 (mSOD1), supporting human pathology studies (Fischer et al., 2004). Specifically, GFAP and CD11b staining showed evidence for significant increases in reactive astrocytes and microglia in the motor regions of transgenic mice containing mSOD1 (Fischer et al., 2004). Although both astrocytosis and microgliosis have been observed in rodent models, the timing of events seems to be unclear. Some studies using mSOD1 mice have suggested astrocytosis to be an early stage event, with microgliosis as a late stage reaction (Yang et al., 2011). However, others have suggested that microglial response occurs at a much earlier stage of the disease (Sanagi et al., 2010). Nevertheless, it does appear that microglial activation starts after motor neuron degeneration, and initially tries to protect motor neurons from degeneration (Fischer et al., 2004; Henkel et al., 2009).

IMMUNE CELL MEDIATED NON-CELL AUTONOMY IN ALS

Motor neuron death and degeneration has been implicated in causing the debilitating symptoms characteristic of ALS, however, mounting evidence indicates that motor neuron death is, at least in part, non-cell autonomous. *In vivo* studies have shown that mSOD1 expressed only in motor neurons of transgenic mice is either not sufficient to cause neurodegeneration or causes only a mild ALS-like phenotype (Jaarsma et al., 2008). Support for non-cell autonomy comes from studies showing that when mSOD1 expression was reduced in microglia and macrophages in transgenic mice (Boillee et al., 2006b; Wang et al., 2009), motor neuron degeneration was decreased. Aside from microglia, evidence has shown that astrocytes may also play a role in motor neuron death. Early studies using transgenic mice expressing mSOD1 in astrocytes suggested the need for motor neurons to be impaired for a degenerative phenotype (Gong et al., 2000), however more recent studies have concluded that astrocytes expressing mSOD1 released toxic factor(s) which were sufficient to cause motor neuron degeneration in an *in vitro* model of ALS (Nagai et al., 2007).

THE ROLE OF ASTROCYTES IN ALS NON-CELL AUTONOMY

Astrocytes are one of the main cell types responsible for the clearance of potentially excitotoxic glutamate from the synaptic space, and as mentioned earlier, play a distinct role when they become activated by the immune system. Quite interestingly, glutamate handling has been reported to be modified in both sporadic and familial ALS (Boillee et al., 2006a). Studies have found that brain and spinal cord samples from ALS patients showed altered glutamate transport (Rothstein et al., 1992) resulting from changes in the glutamate transporter EAAT2 (Maragakis et al., 2004). Evidence suggests that EAAT2 is significantly decreased in the motor cortex and spinal cords of ALS patients (Fray et al., 1998), as well as in the spinal cords of mSOD1 transgenic mice (Bruijn et al., 1997) and rats (Howland et al., 2002). It should come as no coincidence then, that astrocytes were quickly targeted for their role in the ALS disease processes.

More interestingly in terms of inflammation, samples analyzed from the brains and spinal cords of ALS patients were found to contain greater numbers of activated or reactive astrocytes, a clear sign of an immune response (Sta et al., 2011). Of note, and useful for research purposes, increased reactive astrocyte expression has been found both in ALS patients and in animal models of ALS (Sta et al., 2011).

Although the main function of astrocytes in the synaptic space is the maintenance of a homeostatic environment for neurons though activities such as the clearance of neurotransmitters, reactive astrocytes gain properties that resting astrocytes do not exhibit. It has been shown that during and after injury, astrocytes have the ability to ameliorate symptoms by physically isolating the injured area through the formation of a glial scar, as well as release neurotrophins and growth factors such as IGF-1 which have been deemed beneficial to injured cells (Dong and Benveniste, 2001). Astrocytes have also been found to release NGF and induce additional sprouting both *in vitro* and *in vivo*, a key step in neuronal recovery (Chalmers et al., 1996; Wu et al., 1998).

Even though astrocytes do have the potential to be beneficial to neurons, the majority of evidence suggests that astrocytes actually contribute to neuronal degeneration during disease, with ALS being no exception. Interestingly, NGF release, which has generally been regarded as a positive attribute of reactive astrocytes, has been shown to directly lead to motor neuron apoptosis through the p75 pathway (Pehar et al., 2004). Notably, mSOD1-containing astrocytes have been found to be even more toxic to their environments than reactive astrocytes alone. Studies have found that reducing mSOD1 expressing astrocyte levels in an *in vivo* model of ALS decreased motor neuron degeneration and correspondingly increased the life span of the affected animals (Lepore et al., 2008; Barbeito et al., 2010). Although the exact mechanisms for astrocyte-mediated motor neuron toxicity is not known, several lines of evidence have pointed to the activation and release of toxic or harmful products such as pro-inflammatory cytokine oxidative stressors. These include, but are not limited to prostaglandins, leukotrienes, and RNS (Hensley et al., 2006a,b). In addition, *in vitro* models of ALS have shown that astrocytes containing mSOD1 release toxic factors that are preferentially toxic to motor neurons. Furthermore, it was noted that even wild-type motor neurons die in the presence of mSOD1-containing astrocytes. Moreover, wild-type motor neurons die when grown in media conditioned by mSOD1 containing astrocytes. These *in vitro* studies have suggested the pro-apoptotic Bax pathway as another mechanism through which astrocytes mediate motor neuron cell death (Nagai et al., 2007).

THE ROLE OF MICROGLIA IN ALS

Microglial cells perform the duties of immune surveillance in the CNS, and once activated, such as is the case during neurodegeneration, these cells secrete cytokines and neurotrophins in order to help restore homeostasis to the neuronal environment (Napoli and Neumann, 2009). However, just as astrocytes may play a deleterious role in ALS, microglia have also been found to exacerbate neuronal injury. *In vivo* studies have shown that microglial cells increase their expression and release of pro-inflammatory cytokines, such as TNF-α and IL-1β in a mouse model of ALS. Additionally, activated microglia have been shown to promote the generation of reactive oxidative species, causing more harm than good to motor neurons (Henkel et al., 2009).

In the case of ALS, a number of studies have looked at mSOD1 expression in microglia, and its effect on ALS disease progression and motor neuron degeneration. Recent studies have found that diminishing mSOD1 levels in microglia in a mouse model of ALS did not alter the age of onset of paralysis, however it was able to significantly slow the disease progression (Boillee et al., 2006b). Yet, the lack of effects on onset must be regarded with caution, as one cannot exclude that the reduction in mSOD1 in microglia was only at the time when some actual motor neuron degeneration has already occurred. Indeed, the technology used in this study to reduce mSOD1 expression was driven by the microglial specific promoter CD11b which only acquires significant driving forces in response to pathological stimuli such as motor neuron degeneration. Accordingly, it would not be surprising that the disease phenotype in these mice has only been modified after the onset of motor neuron degeneration. It has also been found that mSOD1 containing microglia produce and release greater levels of pro-inflammatory cytokines and free radicals than their wild-type counterparts (Henkel et al., 2009). While the presence of mSOD1 in microglia has been shown to be deleterious, new evidence has begun to pinpoint the mechanisms that may control ALS-related microglial toxicity. Specifically, increases in the pro-inflammatory cytokines and prostaglandin E_2 have been reported to be present in ALS patients (Papadimitriou et al., 2010). Additionally, *in vitro* data suggested that conditioned media obtained from activated microglia is sufficient to cause neuronal degeneration through the concurrent stimulation of TNF-α and NMDA receptors. These pathways are thought to up-regulate inducible nitric oxide synthase activity, thus causing oxidative stress which leads to cell death. Of note is the fact that activation of either the TNF-α or NMDA receptor alone was not found to cause cell death, and the blockade of either of these receptor pathways was found to alleviate cell death caused by microglial-conditioned media, suggesting that both of these receptors need to be activated for MN death to occur (Moisse and Strong, 2006).

Although there is compelling evidence supporting the notion that microglial activation could be sufficient to kill motor neurons in ALS, *in vivo* studies have cast doubt on this theory by showing that repopulating microglial-deficient mice with mSOD-containing microglia failed to cause motor neuron death. These studies have yet to be repeated (Boillee et al., 2006a,b). In addition, studies done in mSOD1 mice absent in TNF-α, a potent inflammatory signaling molecule, did not show any decrease in motor neuron death (Gowing et al., 2006). These data are perhaps suggestive of alternate inflammatory pathways, and may implicate inflammatory mechanisms as coincidental, but not causative.

When viewed together, mounting *in vitro* and *in vivo* evidence have given credence to the toxic effects of astrocytes and microglia on motor neurons and have suggested multiple mechanisms that may be involved. In addition to these, it must be noted that mSOD1 containing motor neurons are particularly vulnerable to Fas ligand and NO-triggered cell death (Moisse and Strong, 2006). Therefore, it is thought that the combined effects of mSOD1 in motor neurons as well as in neighboring astrocytes and microglia are what ultimately result in cell death and degeneration in ALS. While the discovery of the expansion repeat in C9ORF72 is too recent to know its relationship with the inflammatory system, it would be conceivable that the immune system response to such a widespread mutation would be equal to or greater than what has already been observed in other forms of ALS.

ANTI-INFLAMMATORY THERAPEUTICS IN ALS

With inflammation potentially playing a significant role in ALS pathogenesis and progression, it is logical to attempt anti-inflammatory drugs for use as therapeutics in combating ALS. Using SOD1 mice, a number of groups have attempted to administer COX2 inhibitors such as cyclosporine, thalidomide, and lenalidomide to COX2-deficient mice in order to attenuate the negative effects of inflammation. Interestingly, the use of these drugs in mSOD1 animal models was sufficient to prolong lifespan (Karlsson et al., 2004; Kiaei et al., 2006). Cyclosporine is currently in clinical trials. Initial double-blind, placebo-controlled clinical trials suggest that men may benefit from cyclosporine administration when given within 18 months of initial diagnosis (Appel et al., 1988). Based on these results, the group of Karlsson et al. are currently running Phase II clinical trials.

Another approach to anti-inflammatory therapy was the use of the drug Copaxone, which has most often been used to treat patients diagnosed with multiple sclerosis. The active ingredient, glatiramer acetate, was used both in animal models as well as clinical trials. While there was initial excitement for the drug, which increased survival in mSOD1 mouse models of ALS (Angelov et al., 2003), these results could not be replicated (Turner and Talbot, 2008). Clinical trials with glatiramer acetate were also unsuccessful (Meininger et al., 2009), suggesting the mouse strain or the delivery mechanism in the original study may have played a greater role than initially indicated.

Perhaps the most well-known of the anti-inflammatory compounds used against ALS is minocycline, a second-generation tetracycline which has been shown to have anti-inflammatory properties (Yrjanheikki et al., 1999). The use of minocycline was shown to delay motor neuron degeneration as well as increase survival in several different mSOD1 mouse models of ALS (Kriz et al., 2002; Zhu et al., 2002). These studies found that minocycline may have had a direct impact on motor neurons by decreasing apoptosis through a reduction in cytochrome *c* release (Zhu et al., 2002; Moisse and Strong, 2006). Furthermore, it may have decreased the level of microglial activation and proliferation in mSOD1 mouse models of ALS (Kriz et al., 2002; Moisse and Strong, 2006). Given these promising results, minocycline was moved into clinical trials, and thus far, no observable difference was found between placebo and drug cohorts in phase III trials (Barbeito et al., 2010).

It should be noted however, that the use of anti-inflammatory drugs in animal models does not lead to direct knowledge of drug dosing and further study may be needed in order to find correct dosing levels, and potentially more importantly, dosing schedules. The use of animal models allows researchers to administer therapeutics before, during, after, or any combination therein relative to symptom onset. Unfortunately, patients being treated for neurodegenerative disorders do not have the luxury of being pre-treated for ALS symptoms before they arise, thus vastly complicating any therapeutic regimen.

CONCLUSION

Recent lines of evidence have given strong support to the role of astrocytes and microglia in ALS disease pathology. Tissue samples from ALS patients have been found to contain increased inflammatory by-products, and animal models of ALS have corroborated these increases. There is some debate as to whether inflammation in ALS could potentially be protective, however most studies have found the activation of microglia and astrocytes to be more harmful than beneficial. These harmful effects may come through the up-regulation and increased release of pro-inflammatory cytokines and reactive oxidative species, as well as through decreased uptake of excitotoxic neurotransmitters such as glutamate from the synaptic space. One major unanswered question, however, is whether an external event causes neuroinflammatory activation leading to ALS, or whether an external event leads to ALS causing neuroinflammation. In either case, both ALS pathology and neuroinflammation seem to act concurrently to exacerbate the symptoms of neurodegeneration. Future therapies to combat motor neuron death may involve a combination of approaches which recognize the role of multiple mechanisms and cell types in the progression of ALS.

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Microglia function in Alzheimer's disease

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Contrary to early views, we now know that systemic inflammatory/immune responses transmit to the brain. The microglia, the resident "macrophages" of the brain's innate immune system, are most responsive, and increasing evidence suggests that they enter a hyper-reactive state in neurodegenerative conditions and aging. As sustained over-production of microglial pro-inflammatory mediators is neurotoxic, this raises great concern that systemic inflammation (that also escalates with aging) exacerbates or possibly triggers, neurological diseases (Alzheimer's, prion, motoneuron disease). It is known that inflammation has an essential role in the progression of Alzheimer's disease (AD), since amyloid-β (Aβ) is able to activate microglia, initiating an inflammatory response, which could have different consequences for neuronal survival. On one hand, microglia may delay the progression of AD by contributing to the clearance of Aβ, since they phagocyte Aβ and release enzymes responsible for Aβ degradation. Microglia also secrete growth factors and anti-inflammatory cytokines, which are neuroprotective. In addition, microglia removal of damaged cells is a very important step in the restoration of the normal brain environment, as if left such cells can become potent inflammatory stimuli, resulting in yet further tissue damage. On the other hand, as we age microglia become steadily less efficient at these processes, tending to become over-activated in response to stimulation and instigating too potent a reaction, which may cause neuronal damage in its own right. Therefore, it is critical to understand the state of activation of microglia in different AD stages to be able to determine the effect of potential anti-inflammatory therapies. We discuss here recent evidence supporting both the beneficial or detrimental performance of microglia in AD, and the attempt to find molecules/biomarkers for early diagnosis or therapeutic interventions.

Keywords: microglia, amyloid-β, Alzheimer's disease, inflammation, NSAIDs, annexin A1, immunity

INTRODUCTION

For many years, the central nervous system (CNS) was considered to be immune privileged, neither susceptible nor contributing to infection/inflammation. It is now evident that CNS infection and neurological diseases trigger local inflammation and consequently activation of the immune response. In particular, the response to aggression is driven by the resident immune cells, the microglia distributed throughout the normal adult brain (Perry and Andersson, 1992; Nimmerjahn et al., 2005). Specifically, Alzheimer's disease (AD) is characterized by an inflammatory response to Amyloid-β (Aβ), inducing the activation of microglia and the recruitment of astrocytes to the sites where Aβ deposits occur (Sastre et al., 2006a).

It is nowadays accepted that there is a dynamic microglia turnover in the brain and that microglia phenotype may change depending on aging, stage of the disease, and/or the presence of peripheral inflammation. In the brain there are also infiltrated macrophages, which play an essential role in the immune response.

The purpose of this review is first, to describe microglia as a cell of the immune system and the effects of peripheral inflammation on their activation. Secondly, our aim is to describe how this system is altered in a neurodegenerative disease, such as AD. Therefore, targeting microglia could serve as a potential therapy to treat AD patients.

MICROGLIA THE INNATE IMMUNITY CELL COMPONENT

ROLE OF MICROGLIA AND MACROPHAGES IN THE BRAIN

– Microglia represent around 10% of the cells in the nervous system. Although there are many theories concerning the origin of microglia, the general consensus today is its hematopoietic origin, derived from myeloid precursor cells, which enter the developing CNS during embryogenesis. Many questions remain about the recruitment and the life of the resident microglial in adult and aging brain (Chan et al., 2007).

Microglia constitute the first line of defense against invading pathogens or other types of brain tissue injury. The general agreement is that microglia are the "sentinels" of the CNS. Their fundamental role is sensing both pathogen- and host-derived ligands within the CNS. By detecting the type of insult and consequently directing the innate to the adaptive immune response (e.g., removal of pathogen) they are fundamental to the resolution of inflammation. Under pathological situations, such as neurodegenerative disease, stroke, and tumor invasion, microglia become activated, surround damaged and dead cells, and clear cellular debris from the area, in analogy to phagocytic macrophages of the immune system (Fetler and Amigorena, 2005). This process plays a fundamental part in the reorganization of neural circuitry and repair mechanisms that arise following injury (Neumann et al., 2009; Neher et al., 2011). As part of a beneficial role microglial phagocytosis is a highly regulated process, with activated microglia expressing a wide, and redundant, variety of distinct receptors for the removal of pathogenic organisms, e.g., Toll-like receptors (TLRs; Neumann et al., 2009), or of apoptotic cell debris, e.g., CD36 and integrins (Napoli and Neumann, 2009; Lue et al., 2010). The microglial phagocytic response is thus a central part of the brain's defense mechanisms, and is a powerful contributor to the systems in place that ensure healthy neural function.

Activated microglia also up-regulate other cell-surface receptors, including the major histocompatibility complex and complement receptors (Liu and Hong, 2003). Microglia experience dramatic morphological changes from ramified cells to activated amoeboid microglia (Kreutzberg, 1996). In addition, when microglia become activated they generate inflammatory mediators like cytokines, chemokines, prostaglandins, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), free radicals, and stimulating an adaptive immune response (Nimmerjahn et al., 2005; Ransohoff and Perry, 2009). The principal goals of such reactions include the repair and the restoration of the homeostasis, but complications often arise, resulting in detrimental effects and actual exacerbation of the occurring damage (Lehnardt, 2010).

– Apart from resident microglia, in the brain there are monocyte-derived macrophages (Schwartz and Shechter, 2010). Perivascular macrophages have a phagocytic role and are also implicated in the presentation of antigens to T cells that have been activated in the periphery, thereby facilitating the recognition of CNS antigens (Perry et al., 2010). The macrophage and microglia phenotype has been defined as M1 (classically activated via TLRs or interferon γ) and M2 (alternatively activated by interleukin 4 or interleukin 13), although it is assumed that a mixed population of both phenotypes exists (Cameron and Landreth, 2010; Perry et al., 2010). Because most techniques are unable to differentiate between both populations of microglia and macrophages in the brain, they are collectively referred as microglia. However, both microglia and infiltrated monocytes are not functionally redundant and have different properties, so they are both necessary to display functions such as brain repair.

IMPACT OF PERIPHERAL INFLAMMATION IN THE BRAIN

Inflammation is a response mounted by the innate immune system in response to injury and infections in order to promote recovery. It was long thought that the brain was protected from systemic inflammation. However, growing evidence shows that systemic inflammatory stimuli, such as infection, also trigger a central response through microglia with consequent release of pro-inflammatory mediators (cytokines, lipid metabolites, free radicals). As part of the host-defense process, this stimulates autonomic, neuroendocrine, and behavioral responses that promote recovery. Under normal conditions the neuroinflammatory response resolves and microglia resume their "resting" state and role of monitoring the microenvironment. However, increasing experimental and clinical evidence indicates that systemic inflammation can worsen, or possibly trigger, neurological diseases (Weller et al., 2005; Ransohoff and Perry, 2009). These include stroke (McColl et al., 2007), Alzheimer's, prion, and motoneuron diseases (Nguyen et al., 2004; Perry, 2004; Rogers et al., 2007). Although the underlying mechanisms are not clear, mounting evidence suggests that microglia enter a primed/hyper-sensitive state in neurodegenerative conditions. This pre-disposes to an exaggerated production of toxic pro-inflammatory mediators in response to systemic inflammogens, thereby exacerbating neuronal loss (Streit et al., 2004; Godbout et al., 2005; Perry et al., 2007).

Understanding the routes of communication between peripheral immune responses and the brain has not been an easy challenge (Perry et al., 2007). The general belief is that immune messages are passed to the brain mainly through three different pathways: first, peripherally derived signals (mainly pro-inflammatory cytokines like IL-1β, TNF-α, and IL-6) and even pathogen-associated molecular patterns (PAMPs; for example the lipopolysaccharide (LPS), the main cell-wall component of Gram-negative bacteria) can access the nervous system through brain sites that lack a proper blood brain barrier (BBB), or through fenestrated capillaries (Rivest, 2003); secondly, on-going peripheral reactions can be sensed and transmitted to the brain via neural afferent pathways, mainly through the vagus nerve (Gao et al., 2008); lastly, the BBB itself, through the role of its numerous cellular components like endothelial cells and perivascular macrophages can sense circulating signals and respond to them, affecting behavior of neurons, astrocytes, and especially resident microglia population (Rivest, 2009; **Figure 1**).

MICROGLIA IN AGING

Despite the similarities with the innate immune response in the peripheral system, there are important differences with brain environment, where the microglia activity could be sometimes deleterious for the brain. This in-balance phenotype is determined by the fact that neuroinflammation may be chronic or acute (Colton, 2009). Both chronic low-grade peripheral inflammation (Ouchi et al., 2005) and microglial priming/hypersensitivity are associated with aging.

It is well known that microglia exhibit significant phenotypic changes during normal aging. Microglia cells from both aged humans and rodents show profoundly altered morphology, characterized by dystrophic processes, and abnormal clustering (Perry et al., 1996; Streit et al., 2008). These changes in morphology are accompanied by increased expression of activation markers such as MHCII and RAGE (Perry et al., 1993), raised basal production of the pro-inflammatory cytokines TNFα, IL-6, and IL-1β (Ye and Johnson, 1999; Xie et al., 2003; Sierra et al., 2007), and hyper-responsiveness to inflammatory stimulation (Njie et al., 2012), together suggesting that microglia become progressively dysfunctional with age. A likely explanation for this fact lies in the loss of endogenous factors which would normally control/drive prevent excessive microglial activation, and promote the beneficial, anti-inflammatory, phenotype. We could further hypothesize that microglia turnover with aging is reduced and that newly attracted

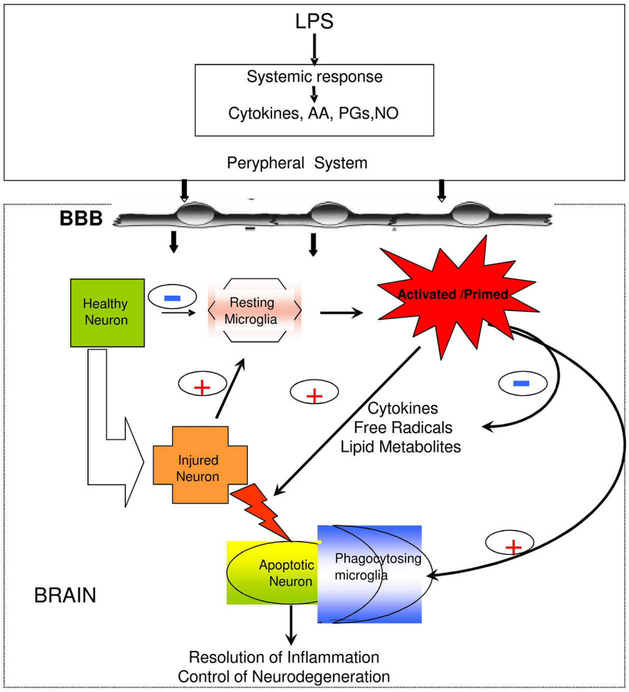

FIGURE 1 | Peripheral infection/inflammation causes the release of pro-inflammatory mediators, including cytokines (TNF-α, IL-1β, IL-6), arachidonic acid (AA), prostaglandins (PGs), and nitric oxide (NO) synthesis. The brain also mounts an inflammatory response to systemic inflammation, as well as to local injury (neurodegeneration, trauma, stroke), with the microglial cells responding soonest and with production of the greatest amounts of pro-inflammatory mediators. However, the central response appears to be under tighter control than the peripheral response in that it is delayed and more modest, probably in order to avoid the dire consequences of a full-blown inflammatory response within the confines of the skull. Modified after Solito et al. (2008).

monistic cells may acquire a wrong phenotype once enriched the brain environment (Kofler and Wiley, 2011).

In summary, microglial activation presents a double-edged sword: from one side we can define it as neuroprotective, forming the first line of defense in the CNS; from the other, it can become a neurodegenerative force, when its power is excessive and to represent risk factors for developing age-associated neurodegenerative disease (AD), contributing to the worsening of symptoms that occur after systemic infection (Perry et al., 2007). Understanding the shift between these two opposite and the changes that occurring with aging will allow us to minimize the harmful and capitalize the beneficial effects and consequently the treatment of neurodegenerative diseases (Yong and Rivest, 2009).

MICROGLIA IN ALZHEIMER'S DISEASE

IMPLICATIONS OF AD PATHOGENESIS IN MICROGLIA ACTIVATION

Inflammation has been implicated in neuronal damage, increased Aβ generation, increased phosphorylation of tau, and cognitive impairment in AD. Cause or consequence of disease progression is still not clear. Clinical work and studies in animal models suggest that microglial activation precede amyloid plaques and tangles formation (Griffin et al., 1989; Heneka et al., 2005a) while PET studies have reported inflammatory changes in one-third of amnestic mild cognitive impairment (MCI; Cagnin et al., 2001; Okello et al., 2009).

Many of the cytokines and chemokines secreted by microglia such as IL-1β, IL-6, TNF-α, IL-8, TGF-β, and macrophage inflammatory protein-1α (MIP-1α) have been found to have altered expression in AD patients compared to control individuals (Sastre et al., 2006a). Animal models of AD, including the APP transgenic line Tg2567 carrying the Swedish mutation, also show elevated levels for TNF-α, IL-1β, IL-1α, chemoattractant protein-1, COX-2, and complement component 1q (Benzing et al., 2000; Matsuoka et al., 2001). In addition, an increased risk of AD has been associated with several polymorphisms of pro-inflammatory genes, including IL-1 (Nicoll et al., 2000), IL-6 (Capurso et al., 2004), TNF-α (McCusker et al., 2001; Perry et al., 2001), and α1-antichymotrypsin (Kamboh et al., 1995).

Amyloid peptides and their precursor protein (APP) are strong glial activators (Barger and Harmon, 1997) and knockdown of APP gene and its proteolytic products delay and decrease microglial activation (DeGiorgio et al., 2002). The extent of astrocytosis and microglial activation is directly dependent on the amyloid load, and treatment with β-sheet breaker peptides leads to reduced brain inflammation (Permanne et al., 2002). Aβ is able to activate a NFκB-dependent pathway that is required for cytokine production (Combs et al., 2001). In addition, the C-terminal (CT) 100 amino acids of βAPP, which is also present in senile plaques, can induce gliosis and neuronal death. CT100 exposure results in activation of mitogen-activated protein kinase (MAPK) pathways as well as NFκB (Bach et al., 2001). At the same time, inflammation may increase Aβ generation by affecting the transcription of the β-secretase (BACE1), the main enzyme responsible for Aβ generation (Sastre et al., 2006b, 2008), therefore creating a feed-forward cycle.

In addition, neuroinflammation participates in tau-mediated neurodegeneration (Jaworski et al., 2011). Animal models of tauopathy such as the P301S tau transgenic mice exhibit accumulation of activated microglial cells around tau-positive nerve cells (Yoshiyama et al., 2007). Eventually, pro-inflammatory cytokines are also able to modify the activity of kinases involved in Tau phosphorylation (Arnaud et al., 2006). Products of inflammation might change the substrate specificity of kinases/phosphatases leading to tau phosphorylation at pathological sites. It was shown recently that inflammation induced by infection increased GSK3 activity in the triple-transgenic mouse model of AD, associated with a shift of tau from the detergent-soluble to the detergent-insoluble fraction (Sy et al., 2011).

On the other hand, other proteins involved in AD, such as presenilin, have been implicated in inflammation. Presenilin conditional knockout mice present differential up-regulation of inflammatory markers in the cerebral cortex, such as strong microglial activation, and elevated levels of glial fibrillary acidic protein (GFAP), complement component C1q, and cathepsin S (Beglopoulos et al., 2004). In fact, γ-secretase inhibitors have been reported to impair microglial activity as measured in gene expression, protein levels, and migration ability, which resulted in a reduction of soluble β-amyloid phagocytosis. Moreover, microglia deficient in presenilin 1 and 2 showed impairment in phagocytosis of soluble β-amyloid (Farfara et al., 2011).

MECHANISMS OF Aβ-INDUCED MICROGLIAL ACTIVATION

As indicated above, Aβ is able to bind and activate microglia. The mechanism of action for this is through interaction with pattern recognition receptors (PRRS). Microglia express many PRRs (Farina et al., 2007; Falsig et al., 2008), which recognize and bind to both PAMPs, or danger-associated molecular patterns (DAMPs), such as Aβ (Salminen et al., 2009). Interaction of microglia with Aβ, via PRRs provokes their inflammatory actions.

Toll-like receptors

Toll-like receptors are a type-1 integral glycoproteins (Pancer and Cooper, 2006; Miyake, 2007). Among the cell-surface TLRs, TLR2 and 4 can recognize Aβ (Carty and Bowie, 2011). Several studies have confirmed that TLR4 mediates microglial-induced neurotoxicity both *in vivo* and *in vitro* (Lehnardt et al., 2003; Walter et al., 2007).

Toll-like receptor activation is regulated by co-receptors, including MD-2, CD14, and CD36 (Akashi-Takamura and Miyake, 2006). Research using knockout mice for TLR4 or TLR2 demonstrated an increase in Aβ deposition and acceleration in cognitive decline (Tahara et al., 2006; Richard et al., 2008). These results suggest that TLR2 and TLR4 may be involved in Aβ clearance *in vivo* and hence provide neuroprotection in AD. In fact, it was shown that response of microglial cells to fibrillar forms of Aβ requires the participation of TLRs and the co-receptor CD14 (Reed-Geaghan et al., 2009). However, microglia internalize soluble Aβ through a non-saturable, fluid phase macropinocytic mechanism that is distinct from phagocytosis and receptor-mediated endocytosis (Mandrekar et al., 2009).

Receptor for advanced end glycation products (RAGE)

RAGE is a member of the immunoglobulin superfamily of cell-surface proteins (Schmidt et al., 2001; Chavakis et al., 2003; Bierhaus et al., 2005). It is a multiligand receptor, which recognizes Aβ peptides and fibrils (Knapp and Prince, 2007). Interestingly, RAGE-expressing microglia are upregulated in AD, and microglial RAGE is reported to mediate the pro-inflammatory effects of Aβ (Yan et al., 1996; Lue et al., 2001; Arancio et al., 2004). This is supported by recent work whereby it was demonstrated in transgenic AD models that the interaction of microglial RAGE with Aβ activates signal transduction cascades (MAP kinase, p38, and ERK1/2), enhances cytokines production (IL-β and TNF-α), and accelerates or amplifies the inflammatory response, leading to recruitment or activation of microglia and astrocytes (Fang et al., 2010).

Scavenger receptors

Scavenger receptor (SR) type-A (SR-A), type B1 (SR-B1), CD36, and CD40 are established receptors for insoluble fibrillar Aβ aggregates, and are expressed by activated microglia, mediating the endocytosis of oligomeric and fibrillar Aβ (El Khoury et al., 1996; Paresce et al., 1996; Coraci et al., 2002; Husemann et al., 2002). Microglial adherence via SR-A binding to fibrillar Aβ leads to microglial immobilization, production of ROS, secretion of cytokines such as TNF-α and complement proteins (El Khoury et al., 1996).

Formyl peptide receptors

Aβ can also bind to members of the seven-transmembrane G protein coupled receptors known as formyl peptide receptors (FPRs; Le et al., 2002). FPR, FPR-like 1 (FPRL-1), and FPR-like 2 (FPRL2) have been characterized as series of receptors, for which the main endogenous ligand is Annexin A1 (ANXA1; Solito et al., 2008). These receptors bind with high affinity to N-formylated bacterial peptides. FPRs are expressed on several immune cells including leukocytes, monocytes, and microglia. Among them the FPRL-1 mediates the chemotactic activity of Aβ42 for mononuclear phagocytes and therefore appear to be pathophysiologically relevant in the AD (Iribarren et al., 2005). In addition, Aβ bound to FPRL-1 is rapidly internalized into the cytoplasmic region as ligand/receptor complexes in mononuclear phagocytes. This process may represent responses of host-defense aiming at the clearance of abnormally elevated, pathogenic Aβ. However, the Aβ interaction with FPRL-1 is clearly associated with cell activation (Cui et al., 2002) and the release of pro-inflammatory and neurotoxic mediators (Pan et al., 2011). Interestingly FPRL-1 is highly express in mononuclear phagocytes surrounding and infiltrating Congo red-positive plaques in AD patients' brain tissue (Le et al., 2001).

Complement receptors

Complement receptors are one of the categories of cell-surface molecules on microglia that are upregulated in response to the activation of these cells (Liu and Hong, 2003). Aβ-induced complement activation leads to generation of C1q, C4, and C3 activation fragments around the plaques. Here microglia express complement proteins C1q, C3, and receptors C1qR, CR3, CR4, and C5aR, which support phagocytic uptake (Keene et al., 2011). Inhibition of the complement system results in an increase of Aβ plaque formation and neurodegeneration in AD transgenic mice (Shen and Meri, 2003).

In contrast, lack of C1q in mice models of AD results in decrease pathology (Hafer-Macko et al., 2000). This indicates that one mechanism by which microglia could recruit further reactive cells to the site of a plaque and cause neurotoxic damage is by activating the classic complement pathway and the inflammatory machinery associated with it (pro-inflammatory cytokines, oxidative products) through production of C1q (McGeer and McGeer, 1998; Bonifati and Kishore, 2007).

The demonstration that the peripheral benzodiazepine receptor is upregulated in activated microglia led to the development of a ligand, $[^{11}C](R)$-PK11195, which binds to this receptor also known as the 18-kDa translocator protein (TSPO). Extensive amyloid deposition and microglial activation can be demonstrated in the same group of AD patients *in vivo* by PET using $[^{11}C]$PK11195 and a negative correlation between microglial activation and levels of cognition has been reported. Both amyloid deposition and microglial activation can be detected *in vivo* with PET in around 50% of patients with MCI. However, amyloid deposition and microglial activation are not necessarily correlated in MCI suggesting both can occur in the absence of the other (Okello et al., 2009; Sastre et al., 2011). On the other hand, a significant age-dependent increase in specific $[^{3}H](R)$-PK11195 binding was also demonstrated in a transgenic mouse model of AD (TASTPM:

APPswxPS1M146V; Roberts et al., 2009). This was consistent with immunohistochemical data showing age-dependent increases in CD68 immunoreactivity co-localized with Aβ deposits. CD68 is a 110-kDa transmembrane glycoprotein, expressed by monocyte/macrophage lineages and serves as a marker for microglia. Interestingly, an antibody to human TSPO revealed induction of TSPO-positive microgliosis by tau fibrils in tauopathy brains. In addition, in transgenic PS19 mice, carrying the P301S Tau mutation, radiolabeling of TSPO with $[^{11}C]$AC-5216 was linearly proportional to the amount of phospho-tau immunolabeling (Maeda et al., 2011). The results of that study indicated that TSPO immunoreactivities are associated with NFTs, neuropil threads, and plaque neuritis rather than Aβ deposits. All together, the analysis of microglia by PET in AD and MCI patients plus the studies of microglial activation over time in animal models suggest that microglia activation occurs before Aβ deposition and correlates better with cognitive deficits and tau phosphorylation.

MICROGLIAL ACTIVATION IN DIFFERENT STAGES OF AD

It has been hypothesized that early microglial activation in AD delays disease progression by promoting clearance of Aβ before formation of senile plaques. It is conceivable that glial activation is protective early in the disease (Wyss-Coray et al., 2003; Maragakis and Rothstein, 2006; Wyss-Coray, 2006). In fact, studies have shown that blood derived macrophages (BMDM) are able to efficiently eliminate amyloid and confer neuroprotection by secretion of growth factors such as the glia-derived neurotrophic factor (GDNF), which are potentially beneficial to the survival of neurons (Liu and Hong, 2003). Activated microglia in early stages of AD can reduce Aβ accumulation by increasing its phagocytosis, clearance, and degradation (Frautschy et al., 1998; Qiu et al., 1998). The mechanism by which Aβ is phagocytosed depends on the physical properties of Aβ and whether it is soluble or fibrillar. Secreted Aβ1-40 and Aβ1-42 peptides are constitutively degraded by neprilysin and the insulin degrading enzyme (IDE), a metalloprotease released by microglia and other neural cells, whose enzymatic activity is enhanced by inflammatory events, such as LPS stimulation (Qiu et al., 1997).

In later stages, with persistent production of pro-inflammatory cytokines, microglia lose their protective effect (Hickman et al., 2008; Jimenez et al., 2008) and may become detrimental through the release of cytokines and chemokines (Hickman et al., 2008). These inflammatory mediators modulate immune and inflammatory function and may also alter neuronal function. In addition, microglia from old transgenic mice have a decrease in the expression of the Aβ-binding SR-A, CD36 and RAGE, and the Aβ degrading enzymes IDE, neprilysin, and matrix metalloprotease 9 (MMP9), compared with wild-type controls (Hickman et al., 2008). Therefore, all the evidences support the idea that over-activated microglia could cause uncontrolled inflammation that may drive the chronic progression of AD by exacerbating Aβ deposition and stimulating neuronal death (Mrak and Griffin, 2005; Gao and Hong, 2008). This concept constitutes the "Neuroinflammatory hypothesis."

By comparison, the "Microglial dysfunction hypothesis" stipulates that rather than an increase of inflammatory function there is a loss of the microglial neuroprotective function in AD (Polazzi and Monti, 2010). Research has shown that the phagocytic abilities of microglia are altered in aging and impaired in neurodegenerative diseases. Therefore this "senescent" or dystrophic microglia can also contribute to the onset of sporadic AD (Streit et al., 2004, 2009).

In addition, other studies have shown that inadequate recruitment of blood monocytes with aging might be a critical event that leads to disease onset. Because the dynamics of the local and systemic inflammatory response may vary with aging and stage of the disease, this would important for the outcome of immunosuppressive treatments (Schwartz and Shechter, 2010).

Studies with animal models have provided controversial data regarding the role of microglia in AD. Experiments crossing APP animal models with Iba1-TK mice, leading to nearly complete ablation of microglia, did not display differences in plaque formation (Grathwohl et al., 2009). These results suggest that microglia may not have a direct role on Aβ deposition, but affect neuronal function. These results also reinforce the role of blood monocytes, which may support the phagocytic function of microglia. However, another study, using two-photon microscopy, performed in triple-transgenic mice crossed with the microglial chemokine receptor CX3CR1 knockout mouse, revealed that microglia is involved in neuron elimination, indicated by locally increased number and migration velocity of microglia around lost neurons (Fuhrmann et al., 2010). Microglia were recruited to the neuron before and not after the elimination of the neuron. Furthermore, CX3CR1 knockout prevented neuronal loss, indicating that neuronal loss depends on the communication between neurons and microglia (Fuhrmann et al., 2010).

MICROGLIA AS TARGET FOR AD THERAPY

Anti-inflammatory drugs

Microglia associated with the senile plaques is thought to be a potential target of non-steroidal anti-inflammatory drugs (NSAIDs). A study by Mackenzie and Muñoz (1998) carried out in non-demented patients showed that those treated with NSAIDs had three times less activated microglia as non-treated controls. These data have been confirmed by *in vivo* treatment with NSAIDs such as ibuprofen in mouse models of AD, which have shown decreases in microglial activation and in inflammatory mediators such as iNOS, cyclooxygenase (COX), and cytokines (Lim et al., 2000; Heneka et al., 2005b). Experiments performed using cultured microglia have revealed that incubation with NSAIDs decreased the secretion of pro-inflammatory cytokines and may increase Aβ phagocytosis (Lleo et al., 2007). However, the reduction of activated microglia and astroglia by NSAIDs was not significant in AD patients, indicating an age or stage dependent difference in the glial response, i.e., in their activation rate (Alafuzoff et al., 2000). Microglia in aged or diseased brains are primed and usually behave differently to those in younger individuals (Gao and Hong, 2008). Thus, it is likely that microglia do not respond equally to anti-inflammatory therapy in old age and therefore, treatment of patients with NSAIDs in advanced stages of the disease may not produce any benefit. In this regard, NSAIDs have been shown to have beneficial effects in young individuals with robust immune systems. In aged patients, these drugs may affect the weak systemic immune response of the patients, exacerbating the local damage by eliminating the capacity of the immune system to introduce disease-modifying factors to the inflamed area (Sastre and Gentleman, 2010) (**Figure 2**).

A potential downstream target of some NSAIDs such as ibuprofen, indomethacin, and naproxen is the peroxisome proliferator-activated receptor-γ (PPAR-γ; Lehmann et al., 1997; Willson et al., 2000). Several PPAR-γ activators including NSAIDs, drugs of the thiazolidinedione class, and the natural ligand prostaglandin J2 (15d-PGJ2) have been shown to be able to inhibit the β-amyloid-stimulated secretion of pro-inflammatory products by microglia and monocytes responsible for neurotoxicity and astrocyte activation (Combs et al., 2000). Furthermore 15d-PGJ(2) caused microglial death, which terminates brain inflammation (Yang et al., 2006).

Interestingly, anti-TNFα treatment reduced Aβ and Tau phosphorylation in transgenic mice. Treatment with the antibody against TNF-α Infliximab increased the number of CD11c-positive dendritic-like cells and the expression of CD11c. These data suggested that the CD11c-positive dendritic-like cells might contribute to the Infliximab-induced reduction of AD-like pathology (Shi et al., 2011).

Therapeutic vaccination with Aβ antibodies in mice evidenced the Fc-mediated uptake and clearance of Aβ antibody complexes by local activated microglia (Bard et al., 2000; Weiner and Selkoe, 2002). Therefore, it was proposed that microglial activation by active immunization might be a valid mechanism for clearance of senile plaques (Gelinas et al., 2004).

Endogenous molecule for the control of microglia detrimental action

The major breakthrough in the therapy of neurodegenerative disease would be controlling the switch between the beneficial versus the detrimental microglia phenotype in order to control inflammation.

Because Aβ stimulates microglia phagocytosis with consequent release of toxic factors, many studies have reported possible mechanism of action implicating receptors on microglia surface. In this regard, therapeutic agents that are able to disrupt the interaction between Aβ42 and FPR may prove beneficial in the treatment of AD.

We have recently published data providing strong indication for a protective role of a protein called annexin A1 (ANXA1), a glucocorticoid anti-inflammatory mediator in the peripheral system (Perretti and D'Acquisto, 2009). ANXA1 plays a key role in ensuring the effective and selective removal of apoptotic neuron-like cells under inflammatory and non-inflammatory conditions which is a ligand for FPRL-1 receptor (McArthur et al., 2010).

Our studies have shown that ANXA1 is upregulated in human microglia in AD, supporting a possible role for the protein in regulating the microglial response to amyloid plaques and inflammatory response in neurodegeneration. This view is strongly supported by further findings in which recombinant ANXA1 administration *in vitro* suppress microglial activation following an inflammatory challenge (McArthur et al., 2010).

The identification of microglial FPRL-1 as a receptor for ANXA1, together with our identification of strong expression of ANXA1 in neuritic plaque-associated microglia in AD, suggests a fascinating connection with published data indicating a link between Aβ and FPRL-1 (Heurtaux et al., 2010). Microglia have clearly been shown to phagocytose Aβ through this receptor, but they appear unable to digest this protein, leading to persistent internalization of Aβ/FPRL-1 complexes and culminating in intracellular fibril formation and apoptosis (Pan et al., 2011). The binding of ANXA1 to FPRL-1 in microglia may thus be able to disrupt the interaction of the receptor with Aβ, potentially being of significant benefit in the treatment of AD (**Figure 3**).

CONCLUSION

Microglia effects on AD seem to have double component. On one hand their activation seems to be neuroprotective at early stages of the disease but at older ages and in severely ill patients the effects could be counterproductive. There is therefore the need

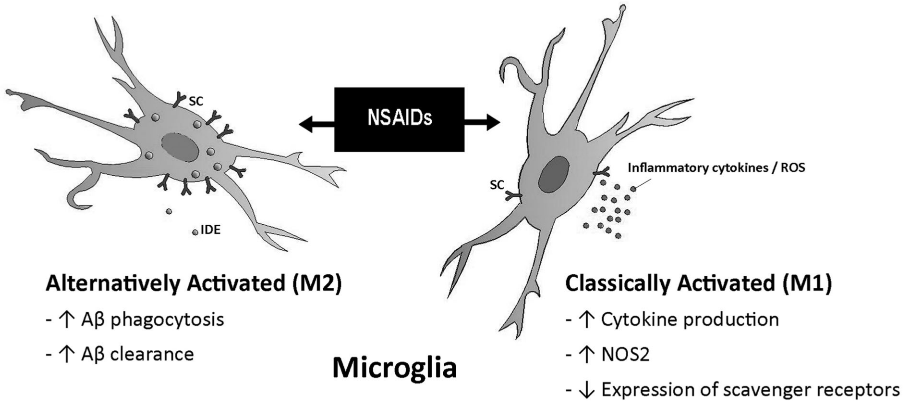

FIGURE 2 | Different effects of NSAIDs on microglia. The response to NSAIDs may differ depending on whether they are used in early stages of disease, in which microglia present an alternatively activated phenotype compared with late stages which is associated with a classical microglia phenotype. (Adapted from Sastre and Gentleman, 2010). Abbreviations: ROS, reactive oxygen species; NOS2, same as iNOS; IDE, insulin degrading enzyme; SC, scavenger receptors.

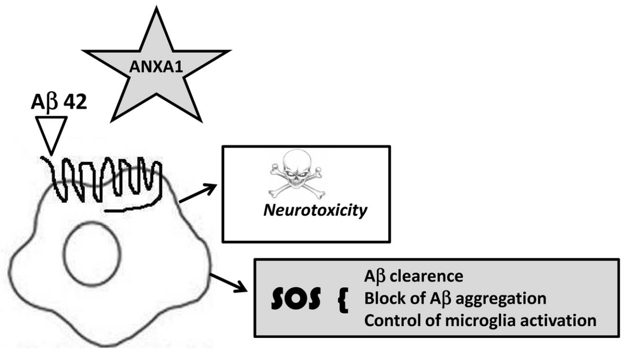

FIGURE 3 | Hypothetical annexin A1- FPRLI mechanism of action in microglia. Annexin A1 binds to FPRL-1 triggering an SOS signaling limiting microglia response to Aβ and preventing a constant uptake and apoptosis, altering microglia pro-inflammatory phenotype.

to investigate the changes in phenotype of resident microglia and how they react to anti-inflammatory therapy over age. In addition, it would be relevant to determine the role of monocyte-derived macrophages along different stages of the disease. One could speculate that the use of BMDM, which are more effective at degrading Aβ could compensate the defective functions of senescent resident microglia (Davoust et al., 2008).

In the future, the combined use of amyloid and microglial imaging should allow one to determine how different anti-amyloid strategies exert their therapeutic effects. Follow-up and further imaging of patients at risk of developing AD with radiotracers that bind to the TSPO/peripheral benzodiazepine receptor will enable a better understanding of both the pattern and the time course of microglial activation and will also provide the opportunity to monitor the pathophysiological effects of anti-inflammatory agents in these patients *in vivo*.

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Aspects of innate immunity and Parkinson's disease

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Genetic studies on PARK genes have identified dysfunction in proteasomal, lysosomal, and mitochondrial enzymes as pathogenic for Parkinson's disease (PD). We review the role of these and similar enzymes in mediating innate immune signaling. In particular, we have identified that a number of PARK gene products as well as other enzymes have roles in innate immune signaling as well as DNA repair and regulation, ubiquitination, mitochondrial functioning, and synaptic trafficking. PD enzymatic dysfunction is likely to contribute to inadequate innate immune responses to a variety of extra- and intra-cellular stimuli, with a number of the innate immunity related enzymes found in the characteristic Lewy body pathology of PD. The decrease in innate immunity in PD is associated with an increase in markers of adaptive immunity, and recent GWAS studies have identified variants in human leukocyte antigen region as associated with late-onset sporadic PD (Hamza et al., 2010; Hill-Burns et al., 2011). Intriguing new data also suggest that peripheral immune responses may be involved, giving some potential to alleviate such peripheral dysfunction more directly in patients with PD. It is now important to identify the cell type specific immune responses contributing to the initial changes that occur in PD, as well as to the propagating immune responses important for the progression of PD pathology between cells and within the brain. Overall, a complex interplay between different types of immunity appear to be involved in the underlying pathology of PD.

Keywords: immunity, mitochondrial dysfunction, Parkinson's disease, synapse dysfunction, ubiquitination

INTRODUCTION

Although Parkinson's disease (PD) is the most common progressive movement disorder in the elderly, there is still considerable uncertainty about its etiology. Recent advances in genetics have identified a number of PARK genes in families with PD, which have pointed to common underlying intrinsically driven intracellular pathways (Corti et al., 2011). These include dysfunction of the proteosome (McNaught et al., 2001), lysosome (Shin et al., 2005), and mitochondria (Muqit et al., 2006). In particular, leucine repeat rich kinase 2 (LRRK2, PARK8) and PTEN-induced kinase 1 (PINK1, PARK6) are linked to familial dominant and recessive PD respectively. PINK1 deletion causes aberrant expression of genes that regulate innate immune responses (Akundi et al., 2011). LRRK2 expression is enriched in human immune cells and is a target gene of IFN-γ (Gardet et al., 2010). Increased LRRK2 expression occurs when an immune response is required (see below). As mutations in these proteins cause PD, this suggests that innate immunity may play a more fundamental role in PD. How these systems interact to cause the fundamental pathology of PD (intracellular Lewy body inclusions made from fibrilized α-synuclein protein) to occur within affected neurons needs to be addressed.

Despite considerable organelle dysfunction, neuronal death occurs slowly, initially, and selectively targeting certain brainstem regions with a predisposition for the substantia nigra (Fearnley and Lees, 1991). Neuronal death does not occur in isolation, but is accompanied by considerable neuroinflammation (Orr et al., 2002; Wilms et al., 2003) and intrinsically driven glial cell changes (Halliday and Stevens, 2011). These changes include the accumulation of α-synuclein in glia, with many of the PARK gene proteins also concentrating in glia in the human brain (LRRK2, PINK1, DJ-1, parkin; Gandhi et al., 2006; Huang et al., 2008; Halliday and Stevens, 2011; Song et al., 2011). As glia interact most closely with the immune system, we will review how innate immunity is involved in these processes.

The innate immune system is an immediate, non-specific, first line of defense against pathogen invasion, contrasting with the delayed and targeted adaptive immune response (Stone et al., 2009). The specific molecular structure of pathogens (pathogen-associated molecular patterns or PAMPs), like lipopolysaccharides (LPS) or the bacterial DNA motif polycytosine guanine (CpG), are directly recognized by phagocytes, granulocytes, and natural killer cells of the immune system (West et al., 2006). These PAMPs

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Abbreviations: CARD, caspase recruitment domain; CpG, polycytosine guanine; ER, endoplasmic reticulum; HDAC, histone deacetylase; IKK, IκB kinase; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharides; LRRK2, leucine-rich repeat kinase 2; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAVS, mitochondrial antiviral signaling; Mda5, melanoma differentiation-associated gene 5; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PAMPs, pathogen-associated molecular pattern; PARP, poly (ADP-ribose) polymerase; PD, Parkinson's disease; PINK1, PTEN-induced putative kinase 1; PKR, Protein kinase RNA-activated; RIG-I, RNA helicases protein retinoic acid-inducible gene I protein; TAK1, Transforming growth factor-beta-activated kinase 1; TBK-1, TANK-binding kinase 1; TLR, Toll-like receptor; TRAF6, tumor necrosis factor receptor-associated factor 6.

bind to Toll-like receptors (TLR) on cells, triggering a cascade of signaling that results in pro-inflammatory cytokine release and complement activation to clear the pathogen or the infected/dead cell through macroautophagy (West et al., 2006).

Pathogens activate cells by binding to specific TLRs through characteristic extracellular multiple leucine-rich repeats domains of TLR (Nguyen et al., 2002). The ultimate induction of interferons and inflammatory cytokines by different pathogens is led by several phosphorylation and subsequent ubiquitination events, with activation of TBK-1 and IKKε, JNK and p38 kinase, IKK, MAPK and MAPKK, IRAK, and TAK1 [detailed in previous reviews (Kawai and Akira, 2006; Seth et al., 2006)]. A major pathway is NF-κB activation by its release from IκB allowing its nuclear translocation and the subsequent activation of target genes to occur. IκB release occurs following IκB phosphorylation and consequent degradation through the lysine 48 linked ubiquitin–proteasome system (Silverman and Maniatis, 2001; Wu et al., 2006).

INNATE IMMUNITY AND PD

Classic PD is not considered an immune disease like multiple sclerosis, and can not be associated with common infectious agents. However, Parkinsonian symptoms can occur after Epstein–Barr virus (EBV) encephalitic infection in patients, with EBV DNA detected in the brain (Espay and Henderson, 2011). This shows that an immune activation in the brain can produce PD-like symptoms, and a number of genetic studies suggest the immune system is commonly involved. Genome-wide association studies (GWAS) show that common variants in human leukocyte antigen (HLA) region are associated with late-onset sporadic PD (Hamza et al., 2010; Hill-Burns et al., 2011). The brains of individuals with PD show upregulation of HLA-DR antigens and the presence of HLA-DR-immunopositive and highly reactive microglia (McGeer et al., 1988). Microglia are the only cells in the substantia nigra that express the initial recognition component of the complement cascade, C1q (Carlsson et al., 2011). Finally, nonsteroidal anti-inflammatory drugs reduce PD risk (Wahner et al., 2007; Steurer, 2011), further supporting some involvement of the immune system in PD.

From a PARK gene perspective, it is of interest that both parkin (PARK2) and LRRK2 (PARK8) are genes associated with leprosy (Cardoso et al., 2011), which is a chronic infectious disease of peripheral nerves. LRRK2 is also a risk gene for inflammatory bowel disease (Van Limbergen et al., 2009; Torkvist et al., 2010; Umeno et al., 2011), and there is much discussion about PD being initiated from peripheral neurons within the gut (enteric nervous system; Braak et al., 2003). In an animal model of ulcerative colitis, there is an alteration of the blood–brain-barrier (BBB) permeability that leads to more substantive cell loss to pathogen-induced cell loss of the substantia nigra (Villaran et al., 2010). Interestingly, treatment of the ulcerative colitis ameliorated cell loss in the brain in this model (Villaran et al., 2010). This could suggest a more direct link between immune defense mechanisms in peripheral neurons and the later onset of PD. Deletions within PINK1 (PARK6) or DJ-1 (PARK7) genes cause aberrant expression of genes involved in the p38 MAP kinase/NF-κB signaling pathway causing changes in the regulation of the innate immune response (Cornejo Castro et al., 2010; Akundi et al., 2011). This suggests that many PARK genes also have significant influence on the immune system which may be important for the onset and/or progression of PD.

A number of animal models of the neuronal loss in PD use direct initiation of the innate immune system to model this aspect of PD. Administration of LPS induces dramatic cell loss in primary neuronal cultures and direct injection of LPS into the substantia nigra produces progressive nigrostriatal degeneration and movement abnormalities in animal models of PD (Liu, 2006; Dutta et al., 2008). These LPS PD models trigger TLR4 mediated signaling pathways (Carvey et al., 2003; Visintin et al., 2003). Even intraperitoneal and *in utero* injections of LPS cause degeneration of the nigrostriatal system (Ling et al., 2002; Perry, 2004), consistent with peripheral effects causing PD-like neurodegeneration. These innate immunity models of PD cause neurodegeneration and microglial activation in a time and LPS dose dependent manner (Liu, 2006; Dutta et al., 2008). Similar direct injections of TLR3 into the substantia nigra produce nigrostriatal degeneration (Deleidi et al., 2010). These studies show that strong stimulators of the innate immune system and increased numbers of innate immune receptors can produce significant site-specific neurodegeneration, perhaps suggesting that the nigrostriatal system is particularly vulnerable to immune activation.

Innate immune signaling also plays a role in the abnormal deposition of α-synuclein. In α-synuclein overexpressing models, ablation of TLR4 augments the deposition of α-synculein due to disruption of the ability of microglia to adequately phagocytose α-synuclein (Stefanova et al., 2011). This suggests that certain aspects of innate immunity are required for initial protection from excessive extracellular α-synuclein. Whether these mechanisms play a role in patients is more difficult to determine. Overall, these studies suggest that the innate immune system can play both neuroprotective and neurotoxic roles depending on the circumstances.

ADAPTIVE IMMUNITY AND PD

In contrast to the "non-specific" innate immune system, the adaptive immune response is cell mediated and highly specialized to remove a specific antigen. It is mainly composed of two parts: humoral immunity, which is mediated by B-lymphocytes, and cell mediated immunity, which is mediated by T-lymphocytes. The CNS was traditionally considered as an "immune privileged" site due to its protection by the BBB, which prevents toxins and infections from reaching the CNS. However, in PD the BBB is disrupted due to activated microglia and monocytes in PD brain (Stone et al., 2009). IgG, but not IgM, has been shown bound to dopamine neurons in the substantia nigra of idiopathic and familial PD patients, but not in age-matched controls (Orr et al., 2005). In addition, LRRK2, a causative gene for PD (see above), regulates B2-lymphocyte function (Kubo et al., 2010). This suggests that adaptive immunity may also be involved in the progression of PD (**Figure 1**).

Apart from B-lymphocyte involvement in PD, both CD4$^+$and CD8$^+$ T-lymphocytes have been found in the SN of PD patients, and CD4$^+$ T-lymphocytes are responsible for the T-cell-mediated immunopathology (Brochard et al., 2009). The expression of CD95 ligand (Fas) has been shown to be important for the capacity

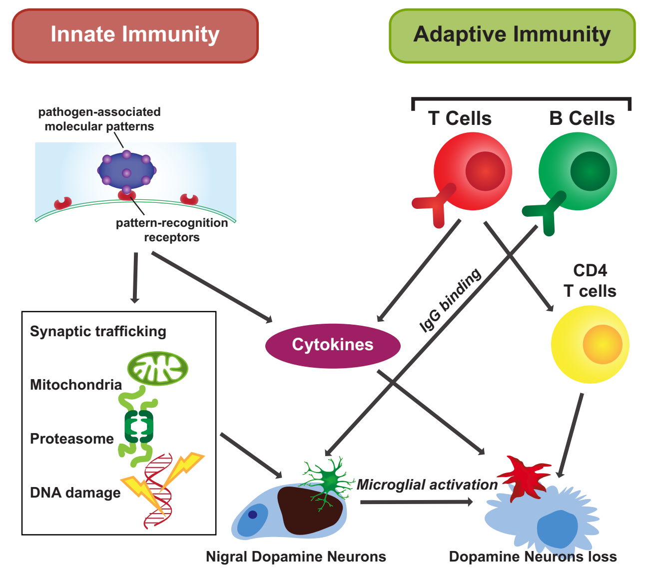

FIGURE 1 | Innate and adaptive immunity in PD. Innate and adaptive immunity are both thought to affect the cellular response to PD. Both produce cytokines facilitating microglial activation. Innate immune signaling is initiated by PAMP binding. Innate immunity influences cell functions through different organelles, such as the nucleus to affect DNA, the mitochondria and proteasome, and through impacting on synaptic trafficking. Adaptive immunity is a subsequent response. Antigen specific adaptive immune processes occur as immune cells survey the brain. In response to stimuli, B cells secrete IgG and CD4 T cells are activated impacting on selective neuronal degeneration.

of T-lymphocytes to induce neuronal death (Brochard et al., 2009). T-lymphocyte cells and T cell receptor (TCR) associated CD3, a polypeptide complex comprised of four distinct chains (CD3γ, CD3δ, CD3ε, and CD3ζ), are found in the PD characteristic Lewy body lesions (Castellani et al., 2011), providing another direct link between cell mediated immunity and PD pathology (**Figure 1**).

Through activation of both the adaptive and innate immune systems, cytokines are induced and secreted. Circulating IL-12 and IL-10 are significantly elevated in PD patients compared with controls (Rentzos et al., 2009) and our data show that the protein levels of IL-10 and GM-CSF are elevated in the cortex of patients with PD (unpublished data). Intercellular adhesion molecule-1 (ICAM-1)-positive glia are increased in the substantia nigra of PD brains (Miklossy et al., 2006). Further analysis of these immune responses in the brains of patients with PD may provide more specific cytokine targets for modifying detrimental immune responses in PD.

INNATE IMMUNITY AND DNA REPAIR AND REGULATION IN PD

The presence of genomic DNA single and double-strand breaks is very common in PD affected brain regions (Hegde et al., 2006). At early stages of DNA damage, DNA sensing molecules (such as PARP and ATM) activate and cause a signaling cascade for repair (Herceg and Wang, 2001). Histone deacetylases (HDAC) are important suppressors of gene transcription, but also deacetylates the p62 subunit of NF-κB increasing its binding to IκB and suppressing innate immunity and interferon-stimulated transcription (Babic et al., 2004; Into et al., 2010). HDACs have been localized to Lewy bodies in patients with PD (Takahashi-Fujigasaki and Fujigasaki, 2006), indicating that they are involved in PD pathogenesis. Inhibiting HDAC alleviates dopamine depletion in models of PD (Outeiro et al., 2007) and is protective by enhancing α-synuclein expression in neurons (Nusinzon and Horvath, 2003; Leng and Chuang, 2006), possibly in concert with an increased innate immune response.

Poly (ADP-ribose) polymerase catalyzes the attachment of ADP ribose units from NAD to nuclear proteins after DNA damage, resulting in three major outcomes, DNA repair, activation of transcription factors (notably NF-κB), and/or cell death due to NAD depletion and release of apoptosis releasing factor from mitochondria (Kauppinen and Swanson, 2007). PARP negatively regulates α-synuclein expression by binding to the α-synuclein promoter Rep1 region (Chiba-Falek et al., 2005) and α-synuclein protein suppresses PARP activity (Adamczyk and Kazmierczak, 2009). Inhibition of PARP activation protects mice from MPTP-induced parkinsonism (Mandir et al., 1999; Leng and Chuang, 2006). PARP may protect against PD through increasing innate immune signaling.

ATM is a kinase that is recruited to phosphorylate histones for DNA repair, signaling molecules for cell cycle arrest or apoptosis, and nuclear IKKγ (NF-κB essential modulator; Habraken and Piette, 2006; Hinz et al., 2010; Hadian and Krappmann, 2011). Phosphorylation of IKKγ leads to its ubiquitination and cytoplasmic translocation where it associates with the rest of the IKK complex, resulting in IKK activation followed by subsequent NF-κB activation and inflammatory cytokines production (Chen, 2005). ATM has also recently been shown to regulate proteasome-mediated protein turnover through suppression of the ISG15 pathway (Wood et al., 2011). ISG15 is an interferon-stimulated gene activated by viral infection and important in antiviral defense and innate immunity (Skaug and Chen, 2010). It is a ubiquitin-like protein that can be attached to both host and viral proteins (Skaug and Chen, 2010). ATM-deficient mice exhibit a selective loss of dopamine nigrostriatal neurons (Eilam et al., 1998), implicating dysfunction of ubiquitination as a factor in PD neurodegeneration, and specifically of ubiquitination in the innate immune system.

INNATE IMMUNITY AND UBIQUITINATION IN PD

The expression of ISG15 is increased in Crohn's disease (Labbe et al., 2011), an inflammatory bowel disorder sharing common genetic and environmental risk factors with PD (Bihari and Lees, 1987; Bialecka et al., 2007). Both ISG15 and LRRK2 (PARK8) are targets of interferon regulation (**Figure 2**). Interferon also regulates proteasome processing of ubiquitinated protein degradation into small peptides (Rivett et al., 2001; Piccinini et al., 2003; **Figure 2**). Ubiquitin is a small molecule and through lysine 48 and lysine 63 forms polyubiquitin chains for protein degradation and cellular localization (Ikeda and Dikic, 2008).

Among PARK genes, parkin has a variety of functions. It not only promotes lysine 48 linked ubiquitination for substrate degradation via the proteasome, but also functions in lysine 63 ubiquitin chain assembly to regulate diverse cellular processes, such as ribosome control, protein sorting and trafficking, and endocytosis of

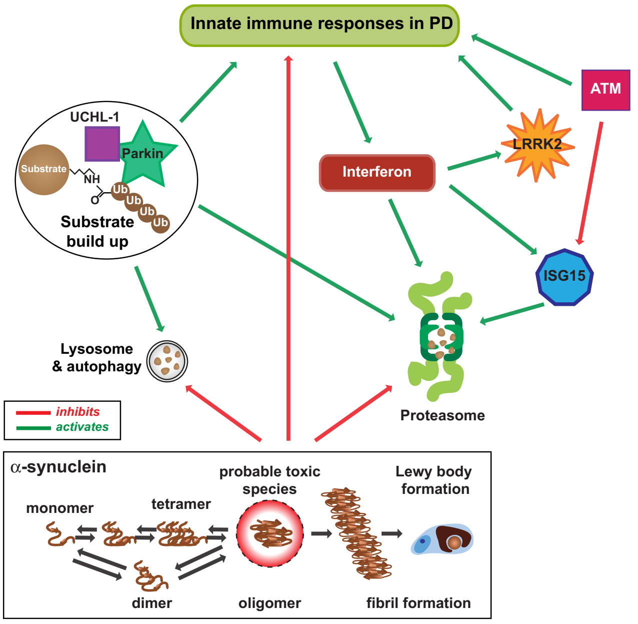

FIGURE 2 | Innate immunity and the ubiquitin–proteasome system in PD. Innate immunity is used by the cell to increase proteasome degradation of unwanted proteins by direct and indirect stimulation of the protesome through interferon-γ and ISG15, a ubiquitin-like protein that can be attached to both host and viral proteins. ATM (and appropriately functioning LRRK2) usually stimulates innate immune signaling when necessary. However, ATM also regulates proteasome-mediated protein turnover through suppression of the ISG15 pathway, and together with dysfunctional LRRK2 could decrease proteasome activity necessary for an adequate immune response and the degradation of normal cellular protein substrates, like dimer or tetramer forms of α-synuclein. As these forms are normally degraded through the ubiquitin/proteasome and lysosome/autophagy pathways, along with other cellular substrates, dysfunction in these pathways would increase α-synuclein to form toxic oligomeric species that also inhibit the proteasome and autophagy pathways, as well as innate immunity. This scenario would lead to the increase in toxic oligomers of α-synuclein that aggregate into fibrils to form Lewy bodies over time.

membrane proteins (Doss-Pepe et al., 2005). Lysine 63 linked ubiquitin chains promote the degradation of membrane proteins by the lysosome (Doss-Pepe et al., 2005). It is notable that α-synuclein is degraded via both the proteasome and lysosome pathways (Shin et al., 2005), suggestive that lysine 48 and lysine 63 linked ubiquitin chains are both important in the pathogenesis of PD (**Figure 2**). UCH-L1 has two opposing enzymatic activities, deubiquitination and ubiquityl ligase. The UCH-L1 ligase activity forms lysine 63 linked polyubiquitin chains (Liu et al., 2002). Lysine 63 multiubiquitin chains are also stimulated by α-synuclein (Doss-Pepe et al., 2005), and α-synuclein filaments and oligomers or its mutants inhibit proteasome function (Tanaka et al., 2001; Lindersson et al., 2004). These three genetically linked PD proteins all contribute to lysine 63 multiubiquitin chain formation. Dysfunction of this system is likely to decrease innate immune responses in PD.

Both lysine 48 and lysine 63 linked ubiquitin chains are known to be involved in anti-pathogen signaling cascades. In particular, lysine 63 linked ubiquitination is essential for NF-κB activation (detailed in review of Wu et al., 2006). Phosphorylation of IκB that releases NF-κB only occurs when the kinase IKKβ is released from its complex following IKKγ phosphorylation and lysine 63 linked degradation. Some other key immune response signaling molecules, such as TRAF6, also function as the lysine 63 linked ubiquitin E3 ligase to activate kinase activity (Kawai and Akira, 2006), and TRAF5 has been identified as a downstream target of VISA (see below) that mediates both IRF-3 and NF-κB activation (Tang and Wang, 2010).

INNATE IMMUNITY AND MITOCHONDRIAL FUNCTION IN PD

The proper functioning of mitochondria is crucial for adequate innate immune defense (Qi et al., 2007; Tang and Wang, 2010; West et al., 2011). Intracellular double-stranded RNA originating from viruses or virus replication is recognized by RIG-I and Mda5, which contain a caspase recruitment domain (CARD; **Figure 3**). Activated RIG-I/Mda5 passes the signal to mitochondrial antiviral signaling (MAVS/VISA) proteins via a CARD–CARD domain interaction (**Figure 3**). The C-terminal of MAVS/VISA bind to mitochondria to activate NF-κB and start interferon α and β production (Qi et al., 2007; **Figure 3**). Upon viral infection, MAVS/VISA immigrate from the outer membrane into the mitochondrial detergent-resistant membrane fraction, suggesting that mitochondria react and contribute to antiviral signaling (Qi et al., 2007; **Figure 3**). In addition to regulating antiviral signaling, there is mounting evidence that mitochondria facilitate antibacterial immunity by generating reactive oxygen species and contributing to innate immune activation following cellular damage and stress (Nakahira et al., 2011).

Most nuclear encoded PARK gene products are located within mitochondria (**Table 1**), such as PINK1, DJ-1, Omi/HtrA2, and LRRK2, suggesting mitochondrial dysfunction is common in PD. The mitochondrial complex I has been shown to be selectively reduced in PD vulnerable regions, such as the substantia nigra (Schapira et al., 1989). The mitochondrial complex I inhibitor rotenone and mitochondrial toxin MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) induce selective dopamine neuron loss and a parkinsonian syndrome and have been used as a major model for studying the tissue aspects of PD (Langston and Ballard, 1984; Betarbet et al., 2000). These changes may decrease the cellular responses of the innate immune system in PD.

INNATE IMMUNITY AND SYNAPTIC TRAFFICKING IN PD

Most enveloped animal RNA and DNA viruses enter host cells via receptor-medicated endocytosis or viral membrane fusion with the plasma membrane in order to replicate. Endocytosis impinges on viral infection at a number of different cellular levels, from the plasma membrane to endosome to lysosome or alternatively to Golgi, and to ER (**Figure 4**; Hacker et al., 1998; Leadbetter et al., 2002; Diebold et al., 2004). Viral proteins accumulate in the ER during viral replication and elicit ER stress. Viral replication induced ER stress disturbs vesicle trafficking within the intracellular membranous web (Goodridge et al., 2011).

Many PARK genes are associated with endocytosis and synaptic vesicle trafficking. α-Synuclein, parkin and UCH-L1 concentrate in the presynapse and associate with synaptic vesicles, anchoring and regulating the undocked pool of presynaptic vesicles (Di Rosa et al., 2003; Ferrer, 2009). α-Synuclein acts as a molecular

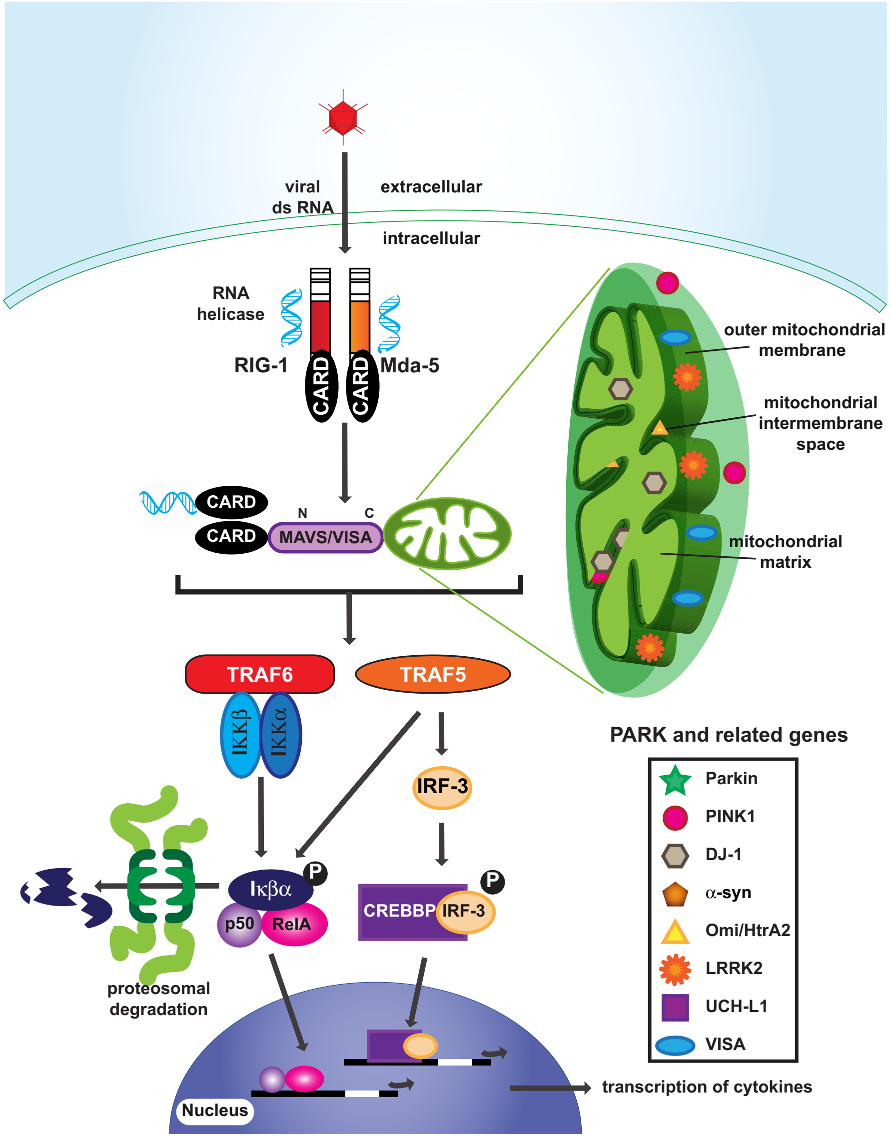

FIGURE 3 | Innate immunity and mitochondria in PD. Many PARK related gene products are located in mitochondria, implicating mitochondrial dysfunction in PD. Interestingly, MAVS/VISA that are attached to the outer membrane of mitochondria are involved in viral infection signaling by activating TRAF6 and TRAF5. Phosphorylation of Iκβα and IRF-3 leads to nuclear translocation of transcription factors and the production of cytokines.

Table 1 | Cellular localization and functions PARK and related genes

	Cellular location indicative functions in mitochondria and synaptic trafficking	Ubiquitination	Reference
Parkin	Outer mitochondrial membrane Synaptic vesicles and the synaptic membrane	Lysine 48-, 63-linked ubiquitin E3 ligase	Kubo et al. (2001); Darios et al. (2003)
DJ-1	Mitochondrial matrix and inter-membrane space	NA	Zhang et al., 2005)
PINK1	Mitochondria	Upstream of parkin	Valente et al. (2004)
Omi/HtrA2	Mitochondrial inter-membrane space	NA	Strauss et al. (2005)
LRRK2	Outer mitochondrial membrane	NA	West et al. (2005)
α-synuclein	Presynaptic terminal	Promote 63 linked ubiquitin assemble	Iwai et al. (1995)
UCH-L1	Synaptic vesicle	Lysine 63 linked ubiquitin E3 ligase	Liu et al. (2002)
VISA	Outer mitochondrial membrane	NA	Seth et al. (2005)

NA, not available.

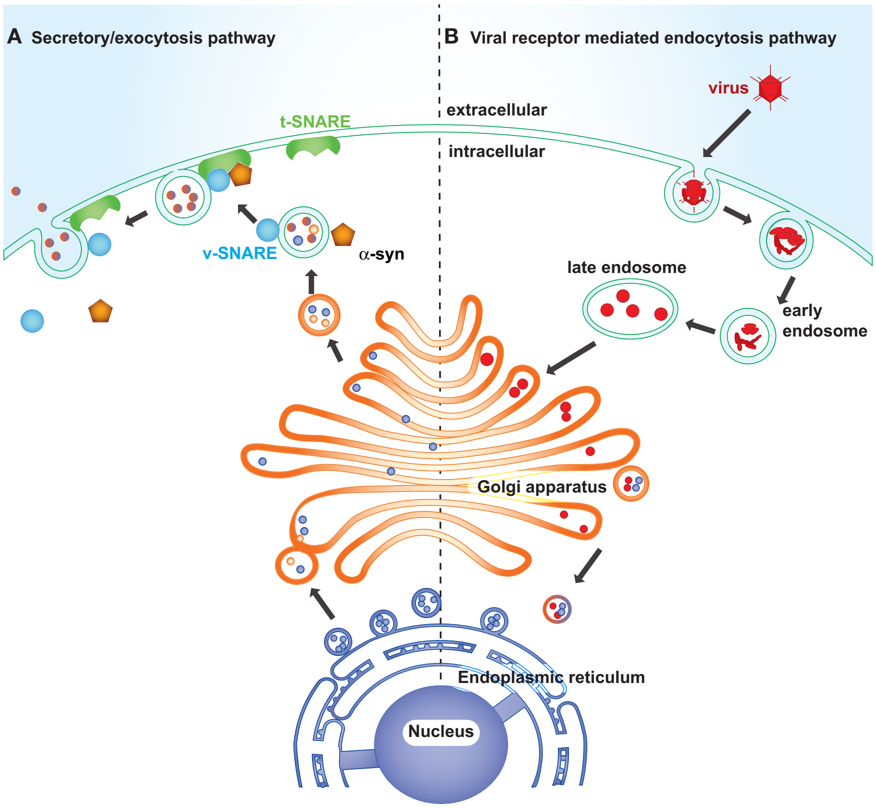

FIGURE 4 | Synaptic trafficking failure and viral intake leading to synaptic dysfunction in PD. Left: The endoplasmic reticulum is composed of a membranous network of tubes and cisternae enclosed in a membrane. It is continuous with the membrane surrounding the nuclear membrane and connects with the Golgi network via the vesicles carrying newly synthesized proteins. Certain vesicle-trafficking steps are required for the translocation of a vesicle from the Golgi apparatus to the plasma membrane. A transport vesicle is tethered and docked at the target membrane to form a tight SNARE complex, thus to merge the vesicle membrane with the target membrane and evacuate neurotransmitters into the extracellular space. The vesicle releasing process is under the assistance of α-synuclein, CDCrel-1, and synaptotagmin. Right: Viruses enter host cells through endosomes. Late endosomes fuse with or convert to lysosomes to degrade its contents, or alternatively it forms budding vesicles that pass to the trans-Golgi region and translocate to the Golgi and endoplasmic reticulum. The endoplasmic reticulum is coated with ribosomes, which assemble the viral amino acids into viral proteins.

chaperone, assisting in the folding and refolding of synaptic proteins called SNAREs, which are crucial for the release of neurotransmitters at the neuronal synapse, vesicle recycling, and synaptic integrity (Chandra et al., 2005). Upon the arrival of action potentials at the synaptic terminal, SNARE complexes, assembly of vesicle membrane (v-SNARE) and the presynaptic plasma membrane (t-SNARE), fuse the vesicle at the plasma membrane to release neurotransmitter into the synaptic cleft (Chandra et al., 2005; Fortin et al., 2005). Parkin ubiquitinates and regulates the turnover of the synaptic vesicle-associated protein, CDCrel-1, a synaptic vesicle-enriched septin GTPase implicated in the inhibition of exocytosis through its interactions with the SNARE complex component, syntaxin (Zhang et al., 2000). Another substrate of parkin is synaptotagmin XI, a member of the synaptotagmin family that is well characterized in vesicle formation and docking by interaction with SNAREs (Huynh et al., 2003). Loss of function of α-synuclein, parkin, or DJ-1 reduces neurotransmission and synaptic vesicle trafficking (Abeliovich et al., 2000; Goldberg et al., 2005). UCH-L1 deficient mice also show altered vesicle transport gene expression (Bonin et al., 2004). Reduced innate immunity allowing for pathogen replication would significantly exacerbate any loss of synaptic function caused by PARK gene products (**Figure 4**) precipitating frank degeneration.

CONCLUSION

A number of intrinsically driven cellular pathways shown to be dysfunctional in PD are important in mediating innate immune signaling. These important intracellular pathways have been identified through genetic studies in patients with PD. More recent genetic studies have also implicated immune pathways in the pathogenesis of PD. In particular, we have identified a number of PARK gene products as well as other enzymes that have dual roles in innate immune signaling as well as proteasome, lysosome, and mitochondrial functioning. The additional enzymes identified are important for DNA repair and regulation, ubiquitination, mitochondrial function, and synaptic trafficking. These pathways appear to lose their ability to mount an adequate innate immune response to PD and experimental manipulation of many of these intracellular molecules has been associated with characteristic PD pathologies in animal models. Intriguing new data also suggest that peripheral immune responses may be involved, with the potential to alleviate such peripheral dysfunction more directly in patients with PD.

Most important, the molecular pathways involved are likely to function in a cell type specific manner (Ledesma et al., 2002), a concept that has not yet been evaluated substantially in patients with PD. The cross-talk between neurons and glia is crucial for an adequate immune response, with astrocytes considered particularly important (Halliday and Stevens, 2011; Schmidt et al., 2011). Astrocytic endfeet form part of the BBB and may serve as ideal sentinel cells able to sense the appearance of toxins and viral pathogens (Scumpia et al., 2005), similar to their cousins found in the gut (Savidge et al., 2007; Daneman and Rescigno, 2009). Astroglia in both the brain and gut regulate barrier function and membrane permeability (Savidge et al., 2007; Daneman and Rescigno, 2009), functions which may be important for the cell to cell transfer of α-synuclein pathology as well as more traditional infectious agents. The increased expression of PARK gene proteins in astrocytes (see above) and their lack of activation to PD pathology in patients (Mirza et al., 2000; Halliday and Stevens, 2011) lends weight to a loss of innate immunity and BBB function in PD that potentially increases membrane and cellular permeability leading to increased toxin and pathogen infiltration in the brain. In addition to the direct cellular damage to vulnerable neurons this would initiate, pathogen exposure may also activate the alternate adaptive immune responses that are thought to participate in propagating the pathology of PD.

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The glial response to intracerebrally delivered therapies for neurodegenerative disorders: is this a critical issue?

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The role of glial cells in the pathogenesis of many neurodegenerative conditions of the central nervous system (CNS) is now well established (as is discussed in other reviews in this special issue of Frontiers in Neuropharmacology). What is less clear is whether there are changes in these same cells in terms of their behavior and function in response to invasive experimental therapeutic interventions for these diseases. This has, and will continue to become more of an issue as we enter a new era of novel treatments which require the agent to be directly placed/infused into the CNS such as deep brain stimulation (DBS), cell transplants, gene therapies and growth factor infusions. To date, all of these treatments have produced variable outcomes and the reasons for this have been widely debated but the host astrocytic and/or microglial response induced by such invasively delivered agents has not been discussed in any detail. In this review, we have attempted to summarize the limited published data on this, in particular we discuss the small number of human post-mortem studies reported in this field. By so doing, we hope to provide a better description and understanding of the extent and nature of both the astrocytic and microglial response, which in turn could lead to modifications in the way these therapeutic interventions are delivered.

Keywords: gene therapy for neurodegenerative diseases, cell transplantation, deep brain stimulation, Alzheimer's disease, Huntington's disease, Parkinson's disease, astrocytes, microglia

INTRODUCTION

Over the last 30 years, there has been a burgeoning of new therapeutic approaches to treat chronic neurodegenerative conditions of the central nervous system (CNS). These approaches have essentially been of two main types:

(i) Better symptomatic agents most notably deep brain stimulation (DBS) for Parkinson's Disease (PD) as well as gene therapy approaches (e.g., AAV2-GAD; ProSavin®; hAADC gene therapy).
(ii) Restorative agents such as growth factor administration or cell transplants. This has involved either directly infusing growth factors into the CNS (e.g., GDNF in the case of PD) or the use of viral delivery systems (e.g., AAV2-Neurturin). In the case of cell therapies a number of different cell types have been grafted into the diseased brain especially in patients with PD (e.g., adrenal medulla; carotid body; ventral mesencephalon, amongst others) and Huntington's Disease (HD) (fetal striatal cells).

Whilst the efficacy of these approaches has been the subject of many reviews, one area that has received rather less attention is the host response to these therapeutic agents. This involves not only an anticipated and beneficial response (e.g., cell integration, fiber sprouting, synapse formation, and so on) but also a glial reaction to it, involving both astrocytes and microglia. In the adult CNS, astrocytes constitute the predominant glial cell type that function to control the CNS environment by removing excess ions and recycling neurotransmitters, supporting endothelial cells that form the blood-brain-barrier, as well as playing a key role in tissue scarring and repair following injury. In recent years, the importance of cross-communication between astrocytes, as well as between astrocytes and neuronal cells has started to be better understood. Microglial cells, in contrast, have more of a phagocytic function but like astrocytes, are known to release cytokines in inflammatory/infectious conditions. Whilst both astrocytes and microglia react to, and convey messages within their immediate environment, they also can communicate over extended distances through syncytial networks.

In this review, we will discuss the extent to which the glial response may limit, or possibly augment, the potential effects of intracerebrally delivered therapeutic approaches for neurodegenerative disorders. The focus of this mini review will be largely restricted to the astrocytic and microglial responses in human post-mortem studies, as there is no published data on the oligodendroglial response to these types of treatments. However, because of the importance of pre-clinical data in this field, we have also summarized studies that have reported on the nature of the glial response in the context of DBS (**Table 1**), neurotrophic therapies (**Table 2**), and neural grafting (**Table 3**) *in vivo*.

DEEP BRAIN STIMULATION AND THE GLIAL RESPONSE

In the last 20 years, DBS has been used to treat a range of neurological disorders, most notably in patients with advancing PD and those with essential tremor refractory to medical therapy (Lyons, 2011). DBS involves applying high frequency stimulation (HFS) via implanted electrodes into strategic CNS nuclei which serves to modulate the output of these nuclei with therapeutic benefits in the majority of patients. This benefit persists for many years and has encouraged the adoption of this approach for a whole range of new indications including neuropsychiatric conditions and pain, as well as in earlier stage PD. However, the host CNS glial response to the implantation of this foreign body and the HFS that it delivers has been the subject of only a few reports, especially in the clinical field.

In contrast, there has been a significant amount of experimental work over the years to look at how the CNS reacts to chronically implanted electrodes, although more often in the context of micro-recording than DBS. This is because micro-recording is a well-established method for studying CNS function and DBS has been developed for use primarily in non-human primates and man rather than rodents. Nevertheless, as one would expect, the insertion of a metal electrode into the parenchyma of the brain will induce an astrocytic response (both in terms of the number and degree of astrocytes activated), the magnitude of which may be dependent, in part, on the electrode architecture (Szarowski et al., 2003; Groothuis et al., 2014), and the biomaterial being used (Ereifej et al., 2011). Studies of this type have led some to look at non-metallic electrodes with the hope that this will induce less of a glial response (Ereifej et al., 2011). For example, stainless-steel, as oppose to platinum-iridium or tungsten electrodes, are more prone to electrolytic dissolution which may increase the likelihood of local cell death and a secondary astroglial response (Harnack et al., 2004).

In all cases, the astrocytic and microglial responses—which typically include a change in cell morphology and density of activated cells as well as the release of various pro-inflammatory molecules—are evident soon after implantation (**Figure 1.1**), persist for years (Jarraya et al., 2003) and seem to occur independently of where the electrode is placed in the brain and the clinical condition for which it is being used (Moss et al., 2004). The extent to which this reactive astroglial and microglial response changes over time is unknown (although see Griffith and Humphrey, 2006), as are the effects that such changes have on the efficacy of current delivered by the stimulating electrode. Nevertheless, a number of experimental studies have shown that these glial reactions could adversely affect electrode impedance and thus efficacy (Spataro et al., 2005; Polikov et al., 2009; Frampton et al., 2010). Indeed, there is even some evidence that chronically implanted electrodes cause sustained local inflammation and neurodegeneration (McConnell et al., 2009). For example, in a study performed in rodents, McConnell et al. demonstrated that heightened levels of chronic inflammation correlated with increased neuronal and dendritic loss, a phenomenon which was more pronounced at 16 weeks than at 8 weeks post-implantation. Remarkably, this local but progressive neurodegeneration was accompanied by axonal pathology, as evidenced by tau phosphorylation, a prominent feature of neurodegenerative conditions. Exactly how this tau pathology arises is unclear but it does suggest that inflammation may be able to induce some forms of proteinopathy.

Additionally, in a recent paper by Vedam-Mai et al. they have interestingly shown in patients that there are variable responses to chronic DBS electrodes, ranging from minimal astrocytosis and microglia activation in the majority of cases to the formation of a dense collagenous band at the electrode tip in at least one case (Vedam-Mai et al., 2012b). In this paper, the authors comment on the possibility of gliotic encapsulation altering electrical thresholds of the stimulating electrode and with this its clinical efficacy—although this study would suggest that this is a rare event. Understanding and predicting such tissue responses to the stimulating and recording electrodes, or even just cannula guidewires, is critical as the magnitude of the foreign body response can lead to electrode failure (Groothuis et al., 2014) and suboptimal clinical outcomes. However, in this study, they did not look to see whether the inflammation induced altered the pathology within the brain, which is of course not straightforward in patients with pre-existing neurodegenerative disorders.

Based on data of this type, some experimental studies have sought to minimize this microglial and inflammatory response, or so-called foreign body response, by using agents such as minocycline and steroids in combination with the stimulating electrode (Rennaker et al., 2007; Zhong and Bellamkonda, 2007) as well as nanoparticle delivery systems (Kim and Martin, 2006; Mercanzini et al., 2010). These different strategies are all predicated on the grounds that suppressing the microglial/inflammatory response to DBS will improve the brain-electrode interface in these chronically implanted micro-electrodes and thus maintain efficacy. Whether this is really a problem in patients with DBS is unknown (see above and Vedam-Mai et al., 2012a,b), but one interesting study by Hirshler et al. (2010) (see also **Table 1**) in rats showed that merely inserting an electrode into the brain could induce widespread and chronic (i.e., over weeks) neuroinflammation which was correlated with deficits in cognitive function—deficits which are also seen in patients who have had DBS (Witt et al., 2008). No such studies using microglia markers and positron emission tomography (PET) have been performed clinically, although a recent study found that in patients with DBS of the pedunculopontine nucleus, there was an improvement in cognition in association with improvements in cortical activity as measured by fluorodeoxyglucose (FDG)-PET (Stefani et al., 2010). This aspect of DBS, namely its ability to induce widespread inflammation (i.e., not just at the site of implantation of the electrode) across whole neural networks may prove to be much more important in understanding the positive and negative effects on cognition in the large number of conditions for which it is now being used. However, this is clearly complicated by the fact that the stimulation itself will alter the function of neural networks directly, as has been seen in studies looking at cognitive function in patients in receipt of subthalamic DBS (Funkiewiez et al., 2006).

However, more recently, others have argued that the induction of a significant astrocytic response actually enhances any DBS-mediated effects (Fenoy et al., 2014). Fenoy et al. suggest that the inevitable stimulation of astrocytes by DBS can set in motion the release of gliotransmitters (e.g., glutamate, adenosine,

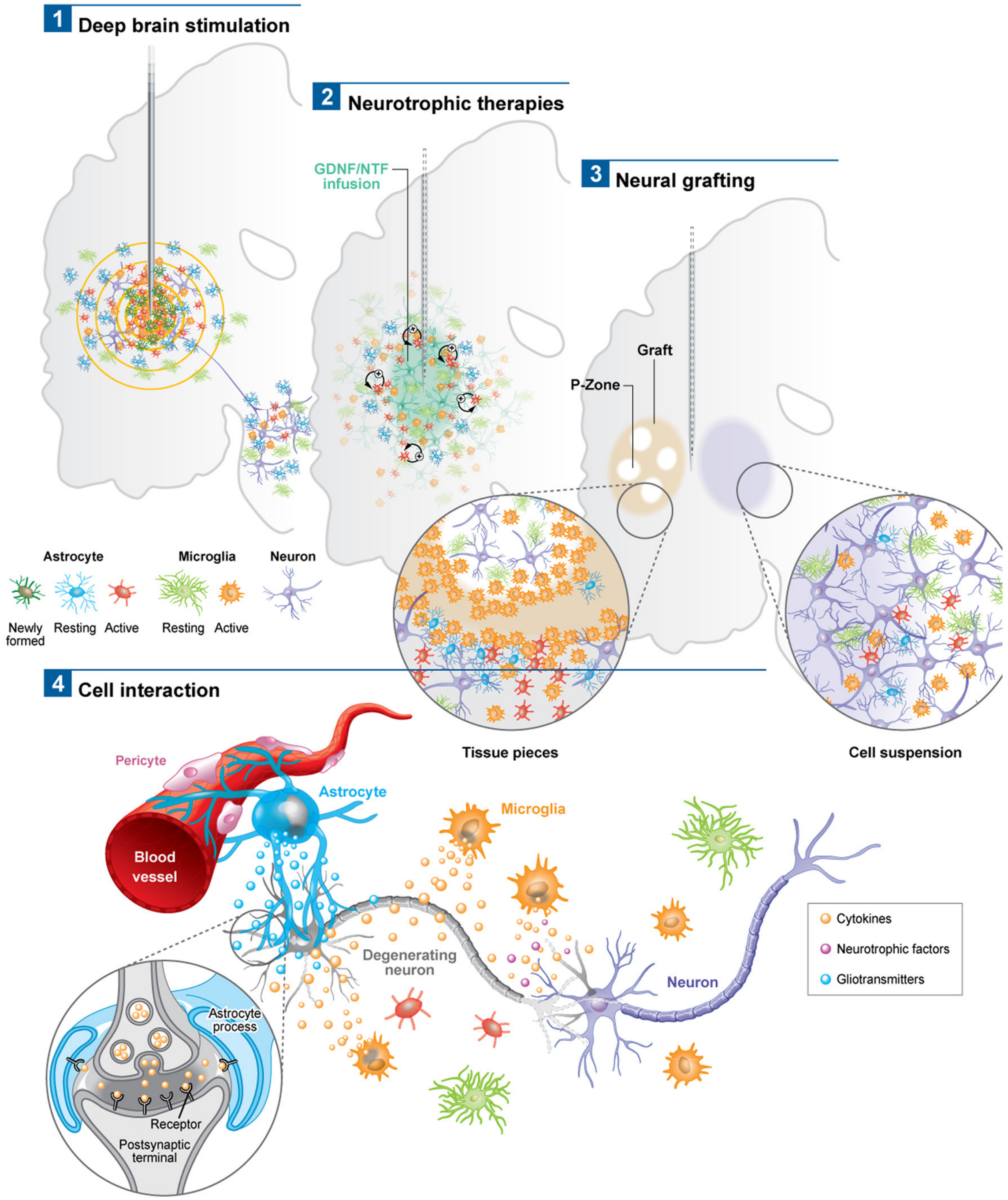

FIGURE 1 | Schematic depicting the astroglial and microglial responses to a variety of invasive experimental therapeutic approaches being trialed for neurodegenerative disorders (1–3). (4) Potential cell interactions that may further influence the outcome of the therapeutic intervention, or, that could be used to potentiate the effects of these therapies. For example the astrocytic response may lead to the release of trophic factors, the modulation of local blow flow as well as the activation of axonal circuits via tripartite synapses. The microglial response may produce both neuroprotective and neurotoxic products, all of which may impact not only on local neurons but also on local vasculature to influence the efficacy of the delivered therapeutic agent. Note: *p*-zone refers to areas of the graft expressing markers for striatal cells.

and D-serine) that can in turn trigger axonal activation which mediates part of the therapeutic benefits of the stimulation. For example, application of adenosine A1-receptor antagonists abolishes HFS-induced suppression of thalamic neuronal activity. More specifically, adenosine, which results from the conversion of ATP by astrocytes, significantly increases in the electrodes' surroundings, endorsing its role in stimulation-mediated effects on tremor (Bekar et al., 2008). In support of this, the release of glutamate and adenosine both terminate oscillations generated by HFS in thalamic sites (Tawfik et al., 2010).

Table 1 | Glial response to DBS and electrode implantation in *in vivo* models.

References	Species	Condition	Target site	Type of stimulation	Period of experimentation/ observation	Nature of the glial response
Han et al., 2012	Cat	Normal	Inferior colliculus	Chronic	3–18 months	Observable astrogliosis near the probe at 2 months, but reduced at 5 months
Morimoto et al., 2011	Male Wistar rat	Stroke	Striatum	Chronic	1 week	Diminished microglial activation with stimulation
Hirshler et al., 2010	Male Sprague-Dawley rat	Normal	Subthalamic nucleus	Electrode implantation only	1–8 weeks	Astrocytic and microglial activation; more significant in cortex, striatum, and thalamus
Baba et al., 2009	Wistar rat	Stroke	Cortex (ischemic boundary)	Chronic	1 week	Diminished microglial and astrocytic proliferation
Harnack et al., 2008	Male Wistar rat	Normal	Subthalamic nucleus	Chronic	3 weeks	Increased number of GFAP$^+$ astrocytes at all anatomical sites as well as thickening of processes
Leung et al., 2008	Rat	Normal	Cortex	Electrode implantation only	12 weeks	Observable activated microglia attached to the electrodes' external coatings
Biran et al., 2007	Male Fischer-344 rat	Normal	Cortex	Electrode implantation only	2–4 weeks	Microglia and astrocyte activation around the electrode
Lenarz et al., 2007	Cat	Normal	Inferior colliculus	Semi-chronic (4 h/day)	Implantation: 3 months Stimulation: 60 days beginning 4 weeks post-implantation	Increased GFAP$^+$ cell density around the electrode (greater with the stimulated than the non-stimulated electrode)
Stice et al., 2007	Female Sprague-Dawley rat	Normal	Cortex	Electrode implantation only	2 and 4 weeks	Astrocytic scar around the electrodes
Griffith and Humphrey, 2006	Rhesus macaque	Normal	Cortex	Electrode implantation only	3 months and 3 years	Persistent reactive astrogliosis around the electrodes (3 months to 3 years). Transient microglial reaction (present at 3 months but not at 3 years)
Biran et al., 2005	Male Fischer-344 rat	Normal	Cortex	Electrode implantation only	4 weeks	Persistent activated microglia around the electrode
Kim et al., 2004	Male Fischer-344 rat	Normal	Striatum	Electrode implantation only	4 weeks	Significant increase in activated microglia in all brain regions

Astrocytes, which have the capacity to interact with up to 2 million synapses in the human brain, can have a profound effect on synaptic activity as well as other astrocytes across large neural networks (Oberheim et al., 2006). The contact that astrocytes have with blood vessels also uniquely enables them to regulate local blood flow in response to increased neuronal activity, which again may help explain the beneficial effects of DBS (Takano et al., 2006). Using *in vivo* two-photon imaging and photolysis, the authors elegantly demonstrated the association between increases in Ca^{2+} levels in astrocytic end feet with vasodilation, a process which more specifically involves COX-1 metabolites. Finally, DBS can also promote the proliferation of "neurogenic astrocytes" which can differentiate into functional neurons (Vedam-Mai et al., 2012a) (**Figure 1**). Taken together, this data suggests a prominent role for astrocytes in DBS-mediated effects implying that there may be an optimal balance in the astrocytic response, in terms of positive and negative effects on the stimulated neural pathways, which may in part be responsible for determining the clinical success of this treatment.

In summary, DBS electrodes will induce an astrocytic and microglial response (**Figure 1.1**) although the extent to which this adversely affects their efficacy in patients is unproven. Indeed, recent data may even suggest the opposite (Moro et al., 2010). However, the data is limited and there is therefore a need to collect systemically more information in this area and moves are afoot to do this (Vedam-Mai et al., 2011), with the expectation that this will enable us to better understand this hitherto poorly described effect of DBS (Vedam-Mai et al., 2011).

NEUROTROPHIC THERAPIES AND THE GLIAL RESPONSE

GENE THERAPY

There are a number of gene therapy trials that have been undertaken in PD which seek to either improve the delivery of dopaminergic stimulation to the striatum; restore to normal the abnormal circuitry of the basal ganglia or promote host repair through rescuing dopaminergic neurons and their striatal innervation (reviewed in Berry and Foltynie, 2011). Other gene therapy trials have targeted HD (Bloch et al., 2004) and Alzheimer's disease (AD) (Mandel, 2010). In this last respect, Nerve growth Factor (NGF) transfected fibroblasts were used and one patient died shortly after receiving the therapy for unrelated reasons. At post-mortem, the brain showed surviving cells and NGF expression, and although the glial response to the therapy was not explicitly studied, they did comment there was a minimal inflammatory and glial response, as evidenced by the fact that they found a few granulomatous cells only (Tuszynski et al., 2005).

In the PD studies, again only a few patients have come to post-mortem, so any data as to the host response to these virally delivered agents is very limited and most have concentrated on the extent of tyrosine hydroxylase (TH) fiber sprouting and the volume of distribution of the therapeutic agent, as well as systemic toxicity and local inflammation (e.g., Herzog et al., 2008, 2009; Bartus et al., 2011). However, there are studies showing that these agents may have a primary effect on the astrocytic compartment and that this is the route by which they actually rescue neurons. For example, Hauck et al. (2006) showed that GDNF works indirectly on photoreceptors to rescue them via Mueller glial cells, and so unlike other invasive therapies, part of their efficacy may actually be enhanced through the host glial compartment.

Overall, evidence to date would suggest that the viral delivery of neuroactive substances to the CNS induces very little glial and inflammatory cell responses, including AAV2-Neurturin (Jeff Kordower; Personal Commun.) (**Figure 1.2**) as well as pre-clinical reports pertaining to this field (**Table 2**).

DIRECT NEUROTROPHIC FACTOR INFUSION

The direct infusion of GDNF into the brain of patients with PD has been the subject of a number of trials. This was initially undertaken using an intraventricular delivery approach that proved ineffective (Nutt et al., 2003), almost certainly because the agent failed to reach the dopaminergic neurons and their projections. This would be consistent with the single post-mortem case from this study showing no evidence of regeneration within the affected nigrostriatal pathway (Kordower et al., 1999). This was then followed by a number of studies in which GDNF was directly infused into the brain parenchyma with variable efficacy—the open label studies showed benefits whilst a double blind placebo controlled study showed no such effects (Gill et al., 2003; Patel et al., 2005; Slevin et al., 2005, 2007; Lang et al., 2006). Although there are various reasons as to why this may have occurred (Barker, 2006), it is of interest to know whether the infusion provoked a glial reaction. This is important not only to see the extent to which chronically implanted catheters delivering neuroactive agents induce a glial response but, given that astrocytes themselves can actually release neurotrophic factors (e.g., Iravani et al., 2012), it is also critical to know whether they may also be capable of enhancing any regenerative response.

In the single post-mortem case looking at intra-parenchymal GDNF delivery in a patient with PD, there was evidence of host TH fiber sprouting around the catheter tip with a low grade astrocytic, microglial and T-cell response (Love et al., 2005). This patient showed a 38% improvement in their contralateral UPDRS (Unified Parkinson's disease rating scale) "off" motor score with a 91% increase in Fluoro-dopa signal in the infused posterior putamen. He died of a myocardial infarction, 3 months after stopping the GDNF therapy that he had been receiving for 43 months. Neuropathologically, there was a slight astrogliosis around the catheter track and tip with a few MHC class II expressing microglia and where this was most intense, there was a reduction in the synaptic protein, synaptophysin. Overall, the glial response to the chronic infusion of GDNF was localized.

NEURAL GRAFTING AND THE GLIAL RESPONSE

The experimental approach of cell transplantation is one in which the glial response has been more specifically addressed. Two distinct types of cell transplantation have been tested in the clinic—cell suspensions, prepared by mechanically dissociating the cells prior to implantation (Lindvall et al., 1990; Mendez et al., 2002; Freeman and Brundin, 2006) and solid grafts, where donor tissue is transplanted as small pieces (Freeman et al., 1995, 2000; Kordower et al., 1997; Olanow et al., 2003; Freeman and Brundin, 2006; Cicchetti et al., 2009). Each of these strategies is associated with a different pattern and intensity of gliosis. In addition, different cell types may induce different host responses, depending

Table 2 | Glial response to neurotrophic therapies in *in vivo* models.

References	Species	Condition	Vector	Delivery site	Nature of the glial response
Rahim et al., 2012	MF1 mouse (fetal)	Normal	Ad5 and AAV pseudotypes 2/5, 2/8, 2/9	Lateral ventricle (trans-uterine injection)	No significant microglia-mediated immune response (with any of the vectors)
Louboutin et al., 2011	Rhesus macaque	Normal	Recombinant SV40-derived vector	Caudate nucleus	No microglia or astrocyte reactions
Rahim et al., 2011	MF1 mouse (fetal and neonatal)	Normal	AAV pseudotype 2/9	Intravenous	No microglia-mediated immune response
Hadaczek et al., 2010	Male Rhesus macaque	PD (MPTP lesion)	AAV	Striatum	No signs of neuroinflammation or reactive gliosis up to 8 years
Lattanzi et al., 2010	Mouse	Globoid cell or metachromatic leukodystrophy	Lentivirus (coding for beta-galactocerebrosidase or arylsulfatase A)	External capsule	Decrease in activated astrocytes and microglia
Snyder-Keller et al., 2010	B6.HDR6/1 mouse	HD	AAV2/1 (delivering anti-htt scFv-C4)	Striatum	Modest glial reaction (activated microglia) at the injection site
Toupet et al., 2008	C57Bl/6J mouse	Prion disease	Lentivirus	Hippocampus	Remarkable decrease in astrogliosis
Louboutin et al., 2007	Female Sprague-Dawley rat	Normal	Recombinant SV40-derived	Caudate-putamen or lateral ventricle	Increased number of astrocytes along the needle track (suggested to be reparative gliosis in response to the minor lesion provoked by the needle)
Zou et al., 2001	Male Fischer-344 rat	Aged brain	hdAdv and fgAdv	Intraventricular or hippocampus	Activation of microglia and astrocytes at injection sites: lower with hdAdv than with fgAdv
Driesse et al., 1998	Rhesus macaque	Normal	Adenovirus	Frontal lobe white matter (unilateral)	Astrocyte activation

Abbreviations: AAV, Adeno-associated virus; Ad5, Adenovirus serotype 5; fgAdv, first-generation adenoviral vectors; HD, Huntington's disease; hdAdv, Helper-dependent adenovirus; htt, huntingtin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease.

on their source of origin, preparation, and mode of implantation (see **Table 3**).

In PD, various post-mortem analyses conducted in transplanted patients have shown that solid grafts evoke a more robust and durable immune response in comparison to cell suspensions (Kordower et al., 1997; Olanow et al., 2003; Mendez et al., 2005, 2008; Freeman and Brundin, 2006; Cooper et al., 2009), a finding which is further supported by observations collected in grafted animal models of the disease (Leigh et al., 1994; see also **Table 3**). Few microglial cells are observed around the needle track with only a mild response around the graft deposits themselves. Similarly, the astrocytic response, which accompanies cell suspension grafts, is predominantly confined to the borders of the graft, with the core of the transplant largely devoid of this cell type (**Figure 1.3**).

Histological analyses in HD patients transplanted with solid embryonic neural grafts show microglial activation, particularly around the transplant and within the grafted areas expressing striatal markers (referred to as p-zones). Importantly, this significant microglial cuffing is still present 12 years following

Table 3 | Glial response to neural grafting in *in vivo* models.

References	Species	Condition	Cell type	Implantation site	Nature of the glial response
De Vocht et al., 2013	Male FVB/NCrl mouse	Normal	Autologous mesenchymal stromal cells	Right hemisphere	M1-microglia and severe astrogliosis surrounding the graft (2 weeks post-tp)
Ma et al., 2013	APP/PS1 double transgenic mouse	AD	Adipose-derived mesenchymal stem cells	Hippocampus and cortex	Increased number of activated microglia in transplanted regions
Osman et al., 2013	Mouse	Irradiated	Syngenic enteric neural stem/progenitor cells	Hippocampus	Microgliosis and astrogliosis associated with grafted cell clusters
Tripathy et al., 2013	Male Sprague-Dawley rat	PD	Differentiated neurons from murine embryonic stem cells	Striatum	Increased expression of microglia-derived factors (CD11b and Iba1). Astrocytosis in the grafted region. Increase in GDNF
Mosher et al., 2012	C57Bl/6J mouse	Normal	Mouse neural progenitor cells	Striatum	Increased number of Iba1$^+$ microglia in transplanted regions
Praet et al., 2012	C56Bl/6 mouse	Normal and cuprizone-treated	Neural stem cells	Below the capsula externa	Extensive invasion of GFAP$^+$ astrocytes and Iba1$^+$ microglia (few CD11b$^+$) within graft sites. Astrocytic scar surrounding graft
Khoo et al., 2011	Wistar Ob rat	PD (6-OHDA lesion)	Bone marrow-derived human mesenchymal stem cells (undifferentiated and neuronal-primed)	Striatum and substantia nigra	Iba-1$^+$ microglia and GFAP$^+$ astrocytes surrounding the grafts (7 days post-tp)
Coyne et al., 2006	Female Sprague-Dawley rat	Normal	Allogeneic marrow stromal cells	Hippocampus or striatum	Massive infiltration of ED1$^+$ microglia leading to graft rejection. Marked astrogliosis surrounding grafts
Muraoka et al., 2006	Male Fischer 344 rat	Normal	Autologous vs. allogeneic neural stem cells	Hippocampus	Astrocyte and microglia reactivity in the host tissue (lower in autologous than in allogeneic tp)
Jiang et al., 1999	Monkey	PD	Microencapsulated rat myoblasts transfected with the tyrosine hydroxylase gene	Striatum	No obvious gliosis around microcapsules
Dunnett et al., 1998	R6/2 mouse	HD	Syngenic striatal cell suspension	Striatum	Modest astroglial reaction at the graft-host border
Pennell and Streit, 1997	Rat	Normal	Embryonic neural cell suspension (whole, or microglial and endothelial cell-depleted)	Striatum	Ameboid microglial cells within grafts early post-tp. By 30 days post-tp, microglia display a resting phenotype within grafts

(Continued)

Table 3 | Continued

References	Species	Condition	Cell type	Implantation site	Nature of the glial response
Barker et al., 1996	Female Sprague-Dawley rat	PD (6-OHDA lesion)	Embryonic ventral mesencephalic tissue	Striatum	Transient astrogliosis and microglial reaction surrounding grafts
Kosno-Kruszewska et al., 1996	Rat	Normal	Cryopreserved ventral mesencephalic tissue	Striatum	Similar glial scar in both grafted and sham-lesion conditions
Duan et al., 1995	Female Sprague-Dawley rat	Normal	Dissociated embryonic ventral mesencephalic: (murine syngeneic, allogeneic, or xenogeneic)	Striatum	Similar reactions in syngeneic and allogeneic: activated microglia infiltration on day 4, decreasing at 6 weeks. More intense reaction in xenografts leading to rejection
Helm et al., 1993	Female Rhesus macaque	HD (ibotenic acid lesion)	Monkey fetal neostriatal neurons	Striatum	Dense gliosis of degenerating grafts at 8 months post-tp

Abbreviations: AD, Alzheimer's disease; GDNF, Glial cell line derived neurotrophic factor; HD, Huntington's disease; PD, Parkinson's disease; tp, transplantation; 6-OHDA, 6-hydroxydopamine.

transplantation (Cicchetti et al., 2009). As observed in cell suspension grafts, solid piece transplants induce little astrogliosis although a number of cells of a reactive phenotype are found around the grafted tissue (Cisbani et al., 2013) (**Figure 1.3**). This pattern of astroglial activation and distribution may have important implications for the survival of the grafts long-term, as astrocytes play not only a critical role in growth factor release but also in glutamate buffering, and thus excitotoxicity (Cicchetti et al., 2011).

Comparing the post-mortem analysis of HD and PD allografted tissue, irrespective of the type of graft implanted, there appears to be a distinctly different glial reaction. In HD, there seems to be a much more aggressive glial (both in terms of the number of activated cells and their phenotype) response whilst in PD, the evidence would suggest that the microglial response is much less obvious. Therefore, transplantation in different diseases may produce different glial responses which in turn may influence the long term survival, integration and outcome of the grafted issue (Cicchetti et al., 2011, 2014).

Furthermore, we have recently shown the presence of mutant huntingtin protein aggregates within genetically unrelated grafted tissue in HD patients. This observation, which is similar to that reported earlier with the discovery of lewy bodies in grafted fetal ventral mesencephalon tissue in PD patients, could also result, at least in part, from oxidative stress and the host inflammatory response induced by the graft (Li et al., 2008; Cicchetti et al., 2014).

Taken together, it is possible that the host response to solid grafts in the absence of immunosuppression (which was restricted to the first 6 months following transplantation in those HD post-mortem cases described above) generates enhanced antigenic stimulation and a stronger immunological/inflammatory response than with suspension grafts (Kordower et al., 1997; Olanow et al., 2003; Mendez et al., 2005, 2008; Freeman and Brundin, 2006; Redmond et al., 2008). Indeed, the type and duration of immunosuppressive treatment is likely to contribute to some of the discrepancies in the immune/glial responses observed using these different transplants. Nevertheless, the accumulating evidence of an attenuated glial response following transplantation of cell suspension grafts favors their use in future clinical cell transplant programs in patients with neurodegenerative disorders (Freeman and Brundin, 2006).

CONCLUSION

There are now a number of new therapies emerging for the treatment of chronic neurodegenerative disorders of the CNS which are invasive but seek to restore to normal the dysfunctional circuits that underlie these conditions. These approaches have generally used a targeted intracerebral delivery of a neuroactive substance or therapeutic device. To date, these therapies have produced mixed results and many explanations to account for this have been put forward including patient selection, disease stage and subtype, mode of delivery, dose given, trial design, and the nature and timing for the primary end-points. However, another area that requires further investigation is the glial and inflammatory response to the novel therapeutic agent as this may impact on its efficacy. To date there are very few studies looking at this and in this review we have sought to highlight this with reference to the limited literature on the microglial and astrocytic reactions to these therapies. At the present time, such responses seem likely to be less of an issue with growth factor infusions and gene therapies—although this may simply reflect the limited post-mortem analyses on this to date—but may be highly relevant for the short and long term viability of neural transplants as well as DBS efficacy, and to a greater magnitude than originally thought. The only way such information can meaningfully be obtained is through the detailed analyses of the post-mortem cases that can be made available by such trials and which will allow us to understand which cells are affected and to what extent. Indeed, one of the critical questions will be the extent to which each cell type influences the behavior of another, as it is now clear that there is a dialog between the glial cells, neurons, and vasculature (**Figure 1.4**). An improved understanding of these interactions may ultimately impact on our ability to better treat patients using these novel approaches as well as modifications as to how they are can be optimally delivered.

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Current understanding of the glial response to disorders of the aging CNS

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In this special issue of Frontiers in Pharmacology, we have asked leading experts to comment and review the evidence that inflammatory cells play a leading role in the pathological processes underlying neurodegenerative disorders. We now seek to draw these various observations together into a conclusion, with the hope that this will inform further work in this area and result in the identification of new therapeutic targets that will have a disease modifying effect.

Keywords: inflammation, microglia, Alzheimer's disease, Parkinson's disease, Huntington's disease, motorneuron disease

INTRODUCTION

For many years, chronic neurodegenerative disorders of the central nervous system (CNS) were thought of in terms of primary neuronal dysfunction and loss with secondary glial and inflammatory responses. These responses were defined by an astrogliosis and the presence of a few activated microglia, but of late this theory has required revision.

Microglia, which account for approximately 10% of the adult brain cell population, were first described by Pio Del Rio Hortega in 1919 (McGeer and McGeer, 2012) and their identity as a discrete cell population was met with great skepticism. Nevertheless, the recognition of microglia as a distinct cell type was taken on by Ralph van Furth who proposed that they may have some function akin to phagocytes found in other parts of the body. However, it was not until the late 1980s that this field came of age when, using the new technique of immunohistochemistry, the McGeers showed that within the Alzheimer's disease (AD) brain there were large numbers of activated HLA-DR II positive microglia (McGeer et al., 1987). This paper, which struggled to find its way into publication, has now been cited many hundreds of times and paved the way for a whole new area of work on the glial response in neurodegenerative disorders. This first demonstration of class II positive microglial cells in AD led to further discoveries of such cells in a whole range of different neurodegenerative disorders and with it the concept of neuroinflammation. The pioneering work of the McGeers was to radically change how these diseases were seen as they went on to show that microglia were not only intimately bound to central inflammatory responses and antigen presentation, but in fact the whole innate immune system itself had a role to play in these CNS disorders.

We now know that microglia continuously and actively survey the CNS microenvironment (Nimmerjahn et al., 2005), although their distribution is not uniform across the brain which may impact on their pathogenic capacity in different disease states (Lawson et al., 1990). They arise from the primitive myeloid progenitors and enter the developing CNS during embryogenesis and continue to be produced throughout life, sharing many similarities to peripheral macrophages. The original description of the phagocytic properties of microglia was taken to show that their primary function was to remove dying and dead cells and by so doing prevent the release of pro-inflammatory cytokines, chemoattractants, and infiltrating T-cells. However of late, Guy Brown and colleagues have shown that under certain conditions, activated microglia can actually phagocytose viable neurons and synapses and that this may be especially prominent in areas of CNS neuroinflammation as is seen in many, if not all, neurodegenerative disorders (Neher et al., 2012). This would fit well with their role in synaptic pruning in normal development as well as their emerging role in plasticity in the normal brain (Tremblay et al., 2011). In addition, microglia have an important role in adult neurogenesis through their ability to remove apoptotic cells and by so doing allow the integration of those cells that survive and mature into neurons [reviewed in Ekdahl (2012)]. Thus microglia have a repertoire of roles throughout life in the CNS.

MICROGLIA AND ALZHEIMER'S DISEASE

An appreciable number of brain diseases seem to share the common feature of a marked glial response and in this respect the role of microglia in AD has been particularly well studied following on from the original observations of the McGeers (McGeer et al., 1987). This disorder, the most common of the chronic neurodegenerative diseases, typically presents with an evolving amnesic syndrome and is characterized pathologically by the deposition of extracellular beta-amyloid plaques and intracellularly by tau tangles. The way in which these two pathologies interact with each other to cause disease is unclear, as is how they relate to the inflammation seen within the brains of patients dying with AD. Nevertheless, it is known that inflammation starts early in the disease process, probably ahead of any obvious amyloid deposition [reviewed in Politis et al. (2012)] and at post-mortem there is a substantial inflammatory response especially around the Aβ plaques. Indeed, Aβ itself can activate microglia which in turn can phagocytose neurons, suggesting that microglia are a critical determinant of neuronal cell loss in this condition (Fuhrmann et al., 2010). Neuroinflammation has also been linked to tau-mediated neurodegeneration as well.

For many years, though, the role of inflammation in AD, whilst being recognized as being present, was always relegated to be a secondary or downstream event. However, the recent GWAS in AD have shown that a number of genetic loci linked to the disease possibly code for inflammatory factors (Harold et al., 2009), which implies that they may be much more intimately involved in the actual disease process. In this respect, it is of interest to note that systemic inflammation can also influence what happens within the CNS, which has led some investigators to propose that it may be a critical initiating factor in the genesis of disorders such as AD, and it is through this non-CNS route that the GWAS loci mediate their susceptibility effects (Ransohoff and Perry, 2009).

Whilst microglia responses can be thought to be detrimental to neuronal viability and survival, it is possible that some of the microglia responses may actually be neuroprotective by virtue of the fact that they could help clear Aβ, remove damaged cells and secrete a range of growth factors and anti-inflammatory agents [reviewed in Solito and Sastre (2012)]. Whilst all this may be the case in the young CNS, there is now an emerging concept that AD results primarily from microglial senescence and a progressive breakdown of innate CNS immunity. This is because the major function of microglia is a neuroprotective one given their ability to phagocytose pathogens, secrete neurotrophic factors as well as dampen down free radical production and sequester glutamate in conjunction with astrocytes. Indeed, the astrocytic response has an equally important role in AD pathogenesis with evidence that astrocytes and neurons can interact in a vicious cycle of chronic, sustained, progressive neuroinflammation and cell death. For example, in advanced stages of AD, it has been shown that TRAIL secreted from astrocytes binds to death receptors on neurons to trigger apoptosis (Li et al., 2011).

All of this complexity of action of inflammatory cells in an aging CNS presents a challenge in knowing how best to target them therapeutically in AD.

MICROGLIA AND PARKINSON'S DISEASE

Parkinson's disease (PD) has long been described clinically through its motor manifestations of a resting tremor, rigidity, and bradykinesia and, pathologically, by the formation of alpha synuclein positive Lewy bodies in the substantia nigra with the loss of the nigrostriatal dopaminergic projection. However, it is now clear that the pathology, and the clinical features of PD, are much more extensive and that the disease may even start outside the CNS (Ferrer et al., 2011). This change in our understanding of the extent and pattern of evolution of PD pathology has led to a re-examination of the pathogenic process and with this the potential role for inflammation and microglia, as first suggested by McGeer et al. (1988a) as early as 1988.

Several studies have now shown that microglial activation and elevated levels of inflammatory cytokines accompany neurodegeneration in PD patients (McGeer et al., 1988b; Mogi et al., 1994, 1995a,b; Banati et al., 1998). For example, Langston et al. (1999) in the post-mortem analysis of MPTP intoxicated patients and Brownell et al. (1999) in primates exposed to the same toxin, pointed to the possibility of an inflammatory and glial response in MPTP induced nigral cell death [also see review by McGeer et al. (2001)]. Subsequent studies have confirmed that activated microglia can be seen in the brains of patients with idiopathic PD both using PET imaging [reviewed in Politis et al. (2012)] and pathologically (reviewed in Huang and Halliday (2012)). These changes occur early in the disease course and thereafter remain stable, which suggests that they may be an important initiating event (Ouchi et al., 2005). In this respect, it has now been shown that in animal models of PD, dopaminergic cell loss can be induced solely by inflammatory insults (e.g., Lipopolysaccharide, LPS; Cicchetti et al., 2009a) and that in more traditional neurotoxin induced animal models of PD, inflammation may be necessary for the full expression of the lesion (e.g., Drouin-Ouellet et al., 2011). Furthermore, it has been shown that α- synuclein itself can directly activate microglia (Zhang et al., 2005), and that many of the gene products from the mendelian forms of PD, not only have a role in the intracellular handling of PD related proteins, but also in modulating innate immune signaling (Huang and Halliday, 2012). This sits well with the recent genetic studies linking PD to HLA (Hamza et al., 2010; Saiki et al., 2010). Finally it is worth noting that activated microglial cells in PD are predominantly found in proximity to a key sign of aging namely free neuromelanin (McGeer et al., 1988b) – again highlighting the complex interplay between microglia, disease processes, and an aging CNS.

Other inflammatory cells, such as astrocytes, may also be important in PD not only by virtue of their known ability to support normal neuronal function but also through a role in maintaining the integrity of the blood brain barrier (BBB; Halliday and Stevens, 2011). This may be especially important as abnormalities in this structure (namely the BBB) could allow other parts of the immune system to gain access to the CNS and by so doing contribute to the pathology seen in PD (Kortekaas et al., 2005).

MICROGLIA AND HUNTINGTON'S DISEASE

Huntington's disease (HD) is a rare autosomal dominant disorder characterized by motor, cognitive, and psychiatric problems with extensive pathology across the CNS. Driven by this dominant genetic mutation in the *huntingtin* gene, the neuropathological signs of HD are visible in many structures of the CNS, but predominantly within the striatum and cortex from an early stage of disease. This prominent neuronal loss is accompanied by protein aggregates composed of the mutated form of the huntingtin protein and with this there is significant activation of microglia (Sapp et al., 2001). This activation has been shown in both premanifest (Tai et al., 2007) and manifest HD patients (Sapp et al., 2001) and there appears to be a correlation between the level of microglial activation and disease severity (Pavese et al., 2006), all of which suggests that the microglia are intimately involved in the disease process in HD.

There is now emerging evidence that the ubiquitous expression of the mutant huntingtin protein affects the function of cells outside the CNS. In particular, the mutant huntingtin protein interacts with the immune system with accumulating evidence that changes in this system may critically contribute to the pathology of HD. However, the nature of this contribution remains unclear, to the extent that it is not even known whether the immune system plays a beneficial or detrimental role in HD patients (Soulet and Cicchetti, 2011). What is clear is that analysis of blood samples from HD patients shows abnormal release of pro-inflammatory cytokines in early stages of the disease (Bjorkqvist et al., 2008).

The astrocytic response in HD is substantial and in fact defined early descriptions of the pathology. Indeed the original grading system of striatal atrophy and disease severity by Vonsattel et al. (1985) used this response. This work also highlighted how the astrocytic response follows neuronal loss such that the striking striatal atrophy following a dorso-ventral pattern is mirrored by the intensity of the astrocytosis.

As in the other neurodegenerative conditions we have discussed, the glia are not passive bystanders to the disease process, but seem to be an integral part of the pathological process from the time of disease onset.

MICROGLIA AND MOTORNEURON DISEASE

Motorneuron disease covers a range of disorders all of which are characterized by the loss of motorneurons (MNs), and the extent to which this selectively targets the upper or lower MN defines the disease type, e.g., primary lateral sclerosis (PLS) for a pure upper motor neuron (UMN) disorder whilst spinal muscular atrophy (SMA) singles out the lower motorneurons (LMNs). However, the commonest type involves both UMN and LMNs and is termed amyotrophic lateral sclerosis (ALS), and whilst there are known genetic forms of this disorder, the vast majority are sporadic in nature (Talbot, 2011). Nevertheless, the rare genetic forms of ALS, especially those with mutations in SOD1, have been used to model the disease in the laboratory and of late this has led to studies where the mutant gene has been differentially expressed in various cellular compartments within the CNS. These studies have clearly shown that the effects of the mutant gene in non-neuronal cells is not insignificant, and coupled to the pathological findings in patients dying with ALS, has suggested that the disorder is one that is critically dependent on events in neurons, astrocytes, and microglia (Phani et al, submitted).

Within the MN itself, many pathogenic pathways, which compromise that cell – subsequently leading to dysfunction and death – have been postulated (Ince et al., 2011). These pathways involve the production of abnormal reactive oxidative species (ROS) which in turn compromises mitochondrial function, energy production, and cell integrity. This abnormal production of ROS is enhanced in SOD mutant cells and can further be exacerbated by excessive glutamate stimulation of the MNs and calcium influx. These latter processes may be in part mediated by abnormal glutamate handling by astrocytes, as further supported by studies demonstrating the beneficial effects of glial cell grafts in transgenic models of ALS (Lepore et al., 2008).

In addition, whilst the astrocyte-neuron interactions may be a critical component in the disease process, it is also known that in the brains of patients dying with ALS there is a marked microglial response. As is common to other neurodegenerative disorders, the question arises as to where this reaction lies in the causal cascade of pathogenic events and whether this changes over time. In this respect, there is evidence in animal models that minocycline can have deleterious effects on microglia and astrocytes once the disease has begun (Keller et al., 2011), which is in line with a clinical study of this drug in ALS showing that it was ineffective and even harmful (Gordon et al., 2007). As such, it is likely that the microglia, as with the astrocytes, do play a role in the loss of MNs in ALS, although the extent to which selectively targeting them therapeutically will truly change the disease course is less clear. What is clear however, is that in ALS, the disease is not localized to the motorneuronal compartment and as such, strategies designed around studying patient specific induced pluripotent stem cell derived MNs may only give partial answers. This is especially true if such assays are being used for patient selective drug screens (Ebert et al., 2009).

INFLAMMATORY CELL TARGETING FOR FUTURE THERAPEUTIC APPROACHES TO NEURODEGENERATIVE DISORDERS

Initial views on the role of microglia suggested that these cells were simply there to scavenge up debris and dead cells, while astrocytes fulfilled some supportive role in the CNS. However, microglia are now recognized to have a complex array of supportive and destructive roles in the CNS and that the balance between the two may be critical in driving some aspects of disease processes. Astrocytes are now seen as being fundamental in shaping and maintaining the developing and mature CNS, including a role in adult neurogenesis, axonal regeneration, and the BBB. The dynamic interplay between all of these different CNS compartments is becoming more evident, such that some neurodegenerative disorders of the CNS may have a pathology as much in the glial cells as in the neurons themselves. This all means that understanding what happens in disease states is far more complex than originally conceived and that targeting each element of the interaction may be the route by which true disease modification can be achieved.

In this special issue of Frontiers in Pharmacology we have repeatedly seen how the glia, immune response, and neurons interact to drive disease, and that our abilities to more accurately define and follow this *in vivo* has enabled us to better understand the temporal relationships that exist between these cellular players and when and how they can best be modulated for therapeutic benefit. Indeed, our capacity to better visualize the glial cells in patients with neurodegenerative disorders especially with respect to microglia is well covered by Politis et al. (2012). This is particularly important given the limitations of animal models of neurodegeneration which includes the fact that they often model disease using a transgenic approach, even though the commonest neurodegenerative disorders (AD and PD) are largely sporadic in nature. In addition, these animals typically develop disease over weeks and months when in patients they evolve over months or years, and thus short acute therapies in the laboratory may not be relevant to the clinic. Finally, our capacity to better define the heterogeneity of patient populations has meant that the therapeutic idea that "one size fits all" is no longer tenable, and that disease processes may follow very different trajectories in different patients and as such require completely different therapeutic approaches (see, e.g., Williams-Gray et al., 2009). A point reinforced by the heterogeneity of glial responses as a function of age and disease state.

As we move toward an era of ever more sophisticated therapeutic agents, our ability to better understand networks of disease pathogenesis will become increasingly important as our capacity to dissect the role of each component will be critical to the success of any such therapy. This is perhaps best shown in the world of cellular transplants for HD, where simply delivering a cell replacement therapy to a diseased brain may not be useful in itself, not because there is anything intrinsically wrong with replacing dysfunctional and lost neurons, but because the necessary support for those cells is no longer there and may even be replaced by a hostile environment (Cicchetti et al., 2009b, 2011). It has even been proposed and demonstrated that in some animal models of motorneuron disease, transplanting glial cells is better than trying to replace the MNs *per se*, as the former have a more critical role in disease pathogenesis through their handling of glutamate (Lepore et al., 2008).

In conclusion, there is now a growing body of evidence from many different sources demonstrating that glial and inflammatory responses are central to these diseases – this includes findings from epidemiological studies looking at anti-inflammatory drugs and the risk of PD and AD; the GWAS in AD and PD; pathological and imaging studies in patients as well as the study of peripheral markers of inflammation [e.g., in HD – reviewed in Wild et al. (2008)]. All of this makes for a very persuasive role for the glia and inflammation in chronic neurodegenerative disorders of the CNS, which will set the scene for a whole new approach to disease modifying therapeutics for a group of disorders that will become increasingly more common as the population ages. Exactly what form these agents will take is unresolved but may involve using drugs that are already in clinical use such as minocycline and non-steroidal anti-inflammatories (NSAIs). Indeed, the use of anti-inflammatory agents for treating AD has long been considered, outside of the amyloid immunization approach (Menendez-Gonzalez et al., 2011). Their efficacy has yet to be proven especially with respect to the commonly available NSAIs which appear to be much less effective in controlling the activation of aged, as compared to young microglia and are likely to be too wide-spectrum thereby suppressing both the detrimental (e.g., pro-inflammatory cytokines, oxidative stress, etc.) and beneficial roles (e.g., pro-repair processes, phagocytosis, and neuroprotection) of the immune cells (Soulet and Cicchetti, 2011). Thus selecting the right agent in the right aged patient group at the right stage of their disease will be critical, and as our understanding of the role of microglia, astrocytes and other related cells evolves, and how they relate to each other, so will our capacity to target them in disease settings.

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