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REVIEW ARTICLE

Phenotypic heterogeneity of astrocytes in motor neuron disease

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Abstract

Accumulating evidence has shown that astrocytes do not just support the function of neurons, but play key roles in maintaining the brain environment in health and disease. Contrary to the traditional understanding of astrocytes as static cells, reactive astrocytes possess more diverse functions and phenotypes than previously predicted. In the present focused review, we summarize the evidence showing that astrocytes are playing profound roles in the disease process of amyotrophic lateral sclerosis. Aberrantly activated astrocytes in amyotrophic lateral sclerosis rodents express microglial molecular markers and provoke toxicities to accelerate disease progression. In addition, TIR domain–containing adapter protein–inducing interferon-β-dependent innate immune pathway in astrocytes also has a novel function in terminating glial activation and neuroinflammation. Furthermore, heterogeneity in phenotypes and functions of astrocytes are also observed in various disease conditions, such as other neurodegenerative diseases, ischemia, aging and acute lesions in the central nervous system. Through accumulating knowledge of the phenotypic and functional diversity of astrocytes, these cells will become more attractive therapeutic targets for neurological diseases.

Introduction

Neurodegenerative diseases are characterized by the selective death of certain types of neurons. In addition, activation of glial cells surrounding the degenerating neurons is also a common pathological finding in almost all neurodegenerative diseases. For a long time, glial activation has been regarded just as a consequence of neurodegeneration; however, accumulating evidence has shown the active roles of glial cells in neurodegenerative diseases, and the term "neuroinflammation" has been used to describe the key phenomenon involving the glia-mediated pathology of these diseases.[1]

Among the glial cell types, such as astrocytes, oligodendrocytes, microglia and NG2 cells, astrocytes are a key component in maintaining the brain environment. Astrocytes used to be regarded as static cells, simply supporting neurons, and participating in wound healing by forming glial scars. However, recent research results have shown that astrocytes actively control synaptic functions and formation, regulate the concentration of neurotransmitters at synapses, control the vasculature to increase the blood flow, and are involved in a wide range of homeostatic functions, including sleep.[2]

In the lesions of neurodegenerative diseases, astrocytes robustly change their morphology and the expression of molecules, and are referred to as reactive or activated astrocytes.[3–6] Furthermore, several lines of evidence show that the activation phenotypes of astrocytes are more complex and heterogenous than previously predicted. The present focused review summarizes the accumulating evidence showing that astrocytes are playing critical roles in the disease process of amyotrophic lateral sclerosis

(ALS). Furthermore, we discuss the phenotypic heterogeneity of activated astrocytes mainly in ALS, and also in other neurological diseases based on studies using rodent models.

Non-cell autonomous neurodegeneration in ALS

Patients with ALS develop progressive paralysis of skeletal muscles and respiratory failure within 2–5 years of disease onset as a result of selective degeneration of both the upper and lower motor neurons. Most ALS cases develop disease sporadically; however, approximately 10% of them are familial cases, and >20 causative genes have been identified to date. Dominant mutations in the gene for copper/zinc superoxide dismutase 1 (SOD1) are the second most frequent cause of inherited ALS after the *C9orf72* gene.[7] Ubiquitous overexpression of the mutant human *SOD1* gene in mice leads to progressive motor neuron degeneration accompanied by extensive gliosis. It is now generally recognized that all SOD1 mutant proteins uniformly provoke unidentified toxicities in degenerating neurons, and that toxicities are not mediated by changes in the enzymatic activity.[7,8] To date, many hypotheses have been proposed to explain the mutant SOD1-mediated toxicity in SOD1-linked ALS, including damage to mitochondria, endoplasmic reticulum stress, defects in protein degradation machinery, axonal transport dysfunction, excitotoxicity from excess glutamate at synapse and overproduction of neurotoxic molecules through neuroinflammation.[9–11] It is likely that the combination of the aforementioned mechanisms, rather than a single one, contributes to neurodegeneration in ALS.

Although pathologies within motor neurons are a key determinant of triggering disease, several studies including ours showed that a non-cell autonomous mechanism also plays an important role in motor neuron degeneration.[12,13] Studies using chimeric mice derived from wild-type and mutant SOD1 mice,[12] as well as those derived from mutant SOD1 and Olig$^{-/-}$ mice,[13] showed that wild-type non-neuronal cells are capable of protecting mutant SOD1-expressing motor neurons, supporting the concept of non-cell autonomous neurodegeneration in ALS.

Astrocytes in ALS

To identify the non-neuronal cell types crucial for non-cell autonomous neurodegeneration in ALS, we and others have created mouse models of ALS in which the mutant SOD1 transgene can be eliminated in a cell type-specific manner using the Cre-loxP system.[14,15] Ablation of the mutant SOD1 transgene in either astrocytes, microglia, or oligodendrocytes from floxed SOD1^{G37R} or SOD1^{G85R} mice using Cre recombinase significantly slowed the disease progression and extended survival times of mice.[14–18] Mutant SOD1-ablated astrocytes delayed the degree of microglial activation and conferred neuroprotection, suggesting that an interaction between astrocytes and microglia modifies neuroinflammation and disease progression in ALS. An interplay between astrocytes and motor neurons has also been examined using *in vitro* co-culture experiments. Co-culture studies using embryonic stem cell- or induced pluripotent stem cells-derived motor neurons and mutant SOD1-expressing astrocytes have shown that mutant SOD1 astrocytes selectively provoke toxicity to motor neurons, providing additional support for the role of astrocytes in non-cell autonomous neurodegeneration in ALS.[19–22] The adverse role of ALS astrocytes has also been shown in sporadic and non-SOD1 inherited ALS. Astrocytes derived from post-mortem ALS spinal cord or differentiated directly from the fibroblasts of sporadic and C9orf72-linked ALS patients appear to be harmful to motor neurons *in vitro*.[23–25]

Astrocyte-mediated toxicity to motor neurons is associated with profound changes of astrocytic phenotype

A noteworthy question in ALS pathogenesis is, why the degenerating spinal cord in both sporadic and familial ALS cases does generate glial cells capable of killing motor neurons. Astrocytes and microglia in ALS do not seem to be constitutively toxic for motor neurons, as the entire motor system develops normally in ALS rodents and patients carrying ALS genes until adulthood. However, it appears that glial cells in ALS show a predisposition to become neurotoxic when subjected to cellular stress, such as the expression of mutant ALS-linked genes or the cell culture condition. Such glial vulnerability might be associated with permanent epigenetic changes, prompting an activated glial phenotype. After activation, the neurotoxic astrocyte phenotype seems to be maintained by mitochondria dysfunction, oxidative stress, disrupted inflammatory signaling, endoplasmic reticulum stress and so on.[26–29] In addition, activation of astrocytes in ALS is associated with increased proliferation and their inability to reach final differentiation,[30,31] a condition involving decrease in the expression of glutamate transporters,[32–34] elevated levels of nicotinamide adenine

dinucleotide phosphate oxidase, reactive oxygen species and inducible nitric oxide synthase,[22,26] and increased productions of pro-inflammatory cytokines/mediators, such as interferon-γ,[35] prostaglandin D_2,[21] and transforming growth factor-β.[36] Even wild-type cultured rat neonatal astrocytes can be induced to develop a permanent neurotoxic phenotype when subjected to different acute and sublethal stressful conditions, such as exposure to lipopolysaccharide or peroxynitrite.[37–39] A strikingly similar switch to a neurotoxic phenotype has been reported in cultured microglia obtained from murine models of ALS or when activated by means of inflammatory or toxic stimuli.[40–43] This evidence further shows that glial cells are prone to switch to a neurotoxic phenotype in response to sublethal cytotoxic damage, and that this phenotype can be perpetuated by autocrine or epigenetic mechanisms.

Long-lasting activation of glial cells in the ventral horn is likely triggered by factors released by damaged motor neurons. After peripheral nerve lesions or spinal cord injury, as well as ALS, motor neurons upregulate several inflammatory mediators and growth factors that induce microglia activation including CSF1,[44,45] CX3CL1 (fractalkine),[46] fibroblast growth factors,[47,48] HBMG1[49] and major histocompatibility complex encoded antigens.[50,51]

From activated glial cells to the emergence of aberrant phenotypes

Aberrant glial cells drive neurodegeneration in ALS

Motor neuron death in the spinal cord of symptomatic ALS rodents is closely associated with local microglia activation, immune cell infiltration and astrocytosis, the latter involving major changes in cell morphology and proliferation rate. This observation led to the prediction that motor neuron pathology in ALS could be initiated by the emergence of phenotypically "aberrant" astrocytes playing an active pathogenic role during disease progression.[47,48] Subsequent reports have established that astrocytes and microglia cells expressing mutant SOD1 are directly toxic to motor neurons in rodent models, as well as in ALS patients.[19,20,23,26,52,53] Furthermore, the discovery by Diaz-Amarilla et al. of a cell type different from reactive astrocytes or microglia and directly associated with rapid disease progression in $SOD1^{G93A}$ rats provided a new avenue to study and understand ALS pathogenesis.[54]

In the degenerating spinal cord of $SOD1^{G93A}$ rats, aberrant glial cells are characterized by the simultaneous expression of microglia and astrocytic markers.[54,55] These cells can typically be localized in areas surrounding the dying motor neurons in the ventral horn of the spinal cord, and can be identified by immunostaining for astrocytic markers, such as GFAP, S100β and Cx43, as well as microglia markers, such as Iba1 and CD163.[55] These aberrant features have not been previously described in other neurodegenerative diseases, but are commonly observed in glioblastoma multiforme, an aggressive type of human astroglial tumor undergoing intense inflammation.[56,57] Notably, the emergence of aberrant glia directly correlates with disease onset and progression, suggesting that they might mediate the rapid course of disease characteristics of the $SOD1^{G93A}$ rat model.[54] Aberrant glia seem to actively proliferate, as estimated by the high proportion of cells labeled with BrdU or the proliferation marker Ki67.[40] Thus, the potential pathogenic role of aberrant glia in mediating motor neuron damage and neuroinflammation is evidenced not only by observational analysis in ALS rats, but also by cell transplant[23,58,59] and pharmacological experiments.[60]

As described below, aberrant glia likely originate from overactivated and inflammatory microglia undergoing a phenotypic transition to astrocyte-like cells. Microglia in ALS rats show exceptional overactivation and atypical behaviors, such as microglia clusters[32,61] and multinucleated giant cells,[62] further indicating major phenotypic instability. Aberrant features would denote chronic inflammatory overactivation, dedifferentiation and epigenetic changes, resulting in a loss or gain of function, finally leading to neuronal toxicity.

One feature of aberrant glia cells is that they can be easily cultured and expanded from the spinal cord of symptomatic adult transgenic $SOD1^{G93A}$ rats, as compared with cultures from non-transgenic rat cords yielding only a few or no cells.[54,55] When first established, cell morphology is that of hypertrophic and rapidly dividing phagocytic microglia. After a few days in culture, the cells transition to clusters of proliferating flat cells resembling astrocytic monolayers, which can be further propagated for months. Such cells were named AbAs (aberrant astrocytes), and are characterized by the simultaneous expression of astrocyte and microglial markers (GFAP, S100β, vimentin, connexin 43, Glutamine synthase, Iba1, CD11b, CD206; Fig. 1a).[54,55] These atypical features define the "aberrant" immunophenotype. Aberrant glia show a robust proliferating capacity and a lack of replicative senescence after several passages in cell culture.

Cultured aberrant glia appear to be the most toxic cells yet identified for embryonic motor neurons, as

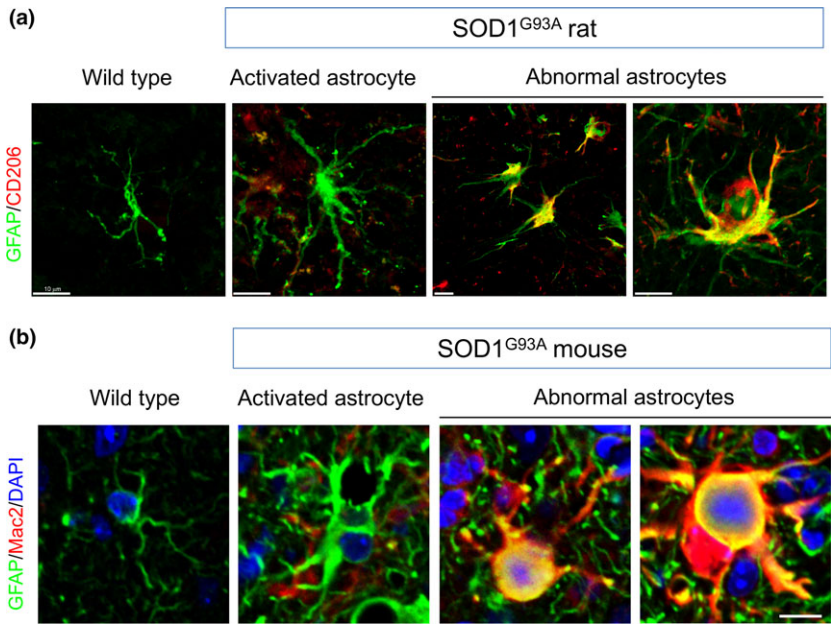

Figure 1 Features of aberrantly activated astrocytes. (a) Representative confocal images of spinal cord astrocytes from wild-type and symptomatic superoxide dismutase 1 $(SOD1)^{G93A}$ rats stained for GFAP (green) and CD206 (red). (b) Representative confocal images of spinal cord astrocytes from wild-type and end-stage $SOD1^{G93A}$ mice stained for GFAP (green), Mac-2 (red) and DAPI (blue). Note that aberrantly activated astrocytes show a large round cell body with shorter processes. Scale bars, 10 μm.

compared with mutant SOD1-bearing astrocytes or microglia.[54] When seeded on confluent monolayers of aberrant glia, motor neuron survival was <10%, suggesting a non-permissive environment for motor neuron growth and differentiation. The conditioned medium from aberrant glia also showed a potent toxicity, producing significant motor neuron loss at 1:1000-fold dilutions, more than 10-fold higher than that of $SOD1^{G93A}$-expressing neonatal astrocytes.[54] Thus, AbAs potentially play an important role in mediating motor neuron damage through various complex molecular mechanisms.

Although it remains unknown whether aberrant glial cells emerge in patients with sporadic or familial ALS, few studies have reported increased levels of aberrant cell markers. A subset of hypertrophic astrocytes expressing S100β were identified close or in indirect contact to motor neurons in the spinal cord of ALS patients,[63] such proximity being strongly evocative of aberrant glial cells found in the ALS rat model.[54] Also, the motor cortex and spinal cord from ALS patients showed increased levels of connexin 43, a protein highly expressed in aberrant glial cells.[64] Connexin 43 was also increased in astrocytes obtained from human-induced pluripotent stem cells, further suggesting an association of this protein with ALS pathology.[64]

Pro-inflammatory effects of aberrant glia after transplantation into the spinal cord

While aberrant glia appear as a distinct but relevant glial cell type associated with rapid disease progression in ALS rats, Ibarburu et al. analyzed the neurotoxic and inflammatory potential of aberrant glia isolated from $SOD1^{G93A}$ rats at 7 days after the focal transplantation into the spinal cord of wild-type syngeneic rats.[59] Although transplanted glia survived and proliferated within the site of injection, they strongly activated endogenous astrocytes and microglia that appeared to isolate the exogenous cells, restricting the migration and neurotoxicity on host motor neurons. Neuroinflammation induced by transplanted aberrant glia propagated well beyond the lumbar injection site, extending to the cervical spinal cord, and was associated with incipient motor neuron damage assessed by ubiquitin aggregation. These results suggest that the emergence of aberrant glial cells could be sufficient to initiate ALS-like pathology, even in wild-type rats. Results are also in agreement with a previous study showing neuroinflammation and motor neuron death induced by transplantation of glial-restricted precursors bearing $SOD1^{G93A}$ into the wild-type rat spinal cord.[58] Aberrant glia could release colony-stimulating factor 1 or interleukin-34 to potently induce microgliosis and inflammation in the neuroaxis. Astrocytes are a major source of colony-stimulating factor 1 and interleukin-34, both factors being potent agonists of the colony-stimulating factor 1 receptor promoting proliferation and activation of microglia and aberrant glia.[45,60] Interestingly, transplanted aberrant cells expressed misfolded $SOD1^{G93A}$ species, which might have a relevant pathogenic role in ALS pathology, both in familial and sporadic cases.[65,66]

Ultrastructural features of aberrant glial cells

Further evidence for the aberrant nature of cultured aberrant glial cells isolated from $SOD1^{G93A}$ symptomatic rats has been obtained from ultrastructural analysis.[67] Cells show an absence of intermediate filaments, an abundance of microtubules together with an important production of extracellular matrix components, suggesting a pro-fibrotic activity. In addition, cells showed exacerbated endoplasmic reticulum stress together with a significant abundance of lipid droplets, autophagy images and many heterogeneous formations including vesicles, suggesting a role in secretion. Cells express markers of secretory granules, such as chromogranin A and secretogranin II (chromogranin C),[68,69] which might interact with mutant SOD1 to promote inflammation and neuronal death.[70] Thus, considering that aberrant glia proliferate and migrate actively, the ultrastructural features are indicative of a profound cellular pathology only comparable with tumor cells.

Phenotypic changes and elimination of activated astrocytes

A previous study reported that astrocytes of symptomatic $SOD1^{G85R}$ mice were immunopositive for ubiquitinated-SOD1 aggregates, suggesting that they are defective in proteostasis.[71] A subsequent study showed that activated astrocytes in SOD1 mice had an atypical shape, and were co-labeled with ubiquitin and cleaved caspase-3, concluding that they were degenerating astrocytes.[72] This phenotype is similar to that of aberrant astrocytes isolated from $SOD1^{G93A}$ rats, discussed previously.[54] A recent study also showed that aberrantly activated astrocytes are accumulated in the spinal cord of several lines of SOD1-ALS mice, and are immunopositive for GFAP, ALDH1L1 and S100β, and surprisingly expressing Mac-2 (galectin-3), an activation marker for microglia. However, they are negative for CD68 and Iba-1, typical microglial markers, concluding that these cells are aberrantly activated astrocytes (Fig. 1b).[73] As these cells do not express typical microglial markers, CD68 and Iba-1, the origin and identity of these cells might be different from aberrant glia discussed in the prior section.

Although the phenotypic changes of reactive astrocytes and their characteristics were described, the fate of those activated astrocytes has not been shown. The authors recently uncovered the mechanism for eliminating overactivated astrocytes in SOD1-ALS models.[73] When TIR domain–containing adapter protein–inducing interferon-β (TRIF), an innate immune adaptor protein essential for the Toll-like receptor (TLR) 3/4 was deleted, disease progression was substantially accelerated, thereby shortening the survival time of SOD1 mice. In contrast, gene ablation of MyD88, which is crucial for all TLR signaling except TLR3, had a marginal impact on the survival time of SOD1 mice. Aberrantly activated Mac-2^+ astrocytes often express cleaved caspase-3, showing that they undergo apoptosis. In TRIF-deficient ALS mice, the number of Mac-2^+ astrocytes increased through insufficient apoptosis of those cells. The TRIF-dependent TLR pathway is known to induce apoptosis in multiple cell types, such as microglia and macrophages, for eliminating those cells after infection by pathogens. The cited study uncovers the novel role of TRIF signaling in eliminating aberrantly activated astrocytes.

In Mac-2^+ astrocytes, accumulation of p62 and ubiquitin, as well as elevated expression of nicotinamide adenine dinucleotide phosphate oxidase, suggests that they are neurotoxic by overproducing reactive oxygen species. Correlation analysis in SOD1-ALS mice showed that greater numbers of Mac-2^+ astrocytes predicted shorter survival times of ALS mice, suggesting that they are harmful to motor neurons.[73] It is possible that the pathways other than TRIF signaling might participate in eliminating abnormal reactive astrocytes. Therefore, further studies are required to provide a complete picture of the mechanisms for eliminating activated glial cells and terminating neuroinflammation.

Phenotypic heterogeneity of astrocytes in ALS and other neurological diseases

Phenotypic heterogeneity of astrocytes is not restricted to the context of ALS. A study showed that toxic reactive astrocytes, referred to as A1 astrocytes, were induced by three cytokines released from activated microglia *in vitro*. These astrocytes were also observed in the lesions of neurodegenerative diseases, including sporadic ALS, Alzheimer's disease, Parkinson's disease and Huntington's disease.[74] A1 astrocytes lose their ability to support neuronal survival and phagocytosis, and induce cell death in cultured neurons. Questions remain about the detailed molecular basis of astrocyte-mediated toxicities of A1 astrocytes, and whether the mechanism of A1 astrocytes-mediated toxicities is common to the above-mentioned neurodegenerative diseases.

Table 1 shows some features of aberrant glial cells, including astrocytes or microglia, abnormally-

Table 1 Characteristics of aberrant glial cells reported in neurodegenerative diseases, central nervous system acute lesion and aging

Disease	Cell type	Model/human	Markers	Features	Function	References
Amyotrophic lateral sclerosis	Aberrant glial cells	$SOD1^{G93A}$ rats and mice.	S100β/GFAP/Cx43 coexpressing Iba1 and CD163. GFAP/S100β co-expression with Mac2. Elevated levels of p62 and ubiquitin.	Abnormally activated astrocytes. High proliferation rate, no replicative senescence when isolated. Defects in autophagy-lysosome and ubiquitin-proteasomal degradation Pathways. SOD1 inclusions.	Toxic to motor neurons. Secrete neurotoxic factors. Induce oxidative stress.	54,55,72,73
Huntington's disease	Aberrant astrocytes	R6/2 HD mice models and human patients	Increased VEGF-A levels.	Through VEGF-A release, mediate neurovascular abnormalities	Reduced pericyte survival.	76
Alzheimer's disease	Aberrant astrocytes	Alzheimer's disease patients – iPSC-derived astrocytes	Nuclear S100β, lower nuclear EAAT1 and GS levels.	Reduced morphological heterogeneity, atrophy	Altered release of soluble inflammatory mediators	75
Alexander disease	Neurotoxic reactive astrocytes	AxD mice model carrying hGFAP (R239H mutation)	Increase GFAP expression, vimentin, lipocalin 2, SerpinA3N	Downregulation of Ca^{2+} homeostasis molecules	Produce aberrant extra-large Ca^{2+} signals	88
Neuroinflammation/aging	A1 astrocytes	Microglia activation induce A1 astrocytes	Complement component 3 (C3). Co-expression of C3 with GFAP and S100β	Do not promote synapse formation or function. Reduced phagocytic capacity. Could constitute part of toxic astrocytes present in neurodegenerative conditions	Highly neurotoxic, Impair oligodendrocytes differentiation and division. Release neurotoxic factors.	74,80
CNS acute lesion	Cells expressing astrocyte/ microglia markers	Cortex and spinal cord injury/ Chronic neurodegeneration	Co-expression of GFAP/ Tmem119/Aldh1. GFAP/Cx3Cr1/Iba1/CD68 co-expression	A subpopulation of cells expressing both markers might be a fusion of astrocytes with monocytes.	-	77
	Neurotoxic Microglia expressing astrocyte markers	Mouse spinal cord injury	A subpopulation of Iba1+ microglia expressing GFAP, vimentin, serpina3n and Aldh1 l1. Up-regulation of Brca1	Proliferation and DNA damage. Dual phenotype with an acute increase in anti-inflammatory factors followed by later upregulation of pro- and anti-inflammatory factors.	Upregulation of anti- and pro-inflammatory transcripts being neuroprotective but also neurotoxic. Activation of DNA damage pathway	78
	IDAs: ischemia-derived astrocytes	Rat brain focal ischemic lesion	Nestin. GFAP overexpression. Isolated cells express Iba1 and S100β	Show reduced replicative senescence, increased cell division and spontaneous migration. Contribute to glial scar formation.	Potentiate death of oxygen-glucose deprived cortical neurons. Propagate reactive gliosis on quiescent astrocytes *in vitro* and *in vivo*.	79
CNS tumor	Human gliomas	Astrocytoma/GBM	GFAP/CD68/HLA-class II/MAC 387	Potential fusion of both linages in the tumor microenvironment.	Functional behavior as mesenchymal cells with phagocytic activities. Astrocytes with phagocytic-like properties.	56,57,81

The lists of diseases, glial cell types and names, model animals/human, molecular markers, features, and functions are summarized with references. CNS, central nervous system; iPSC, induced pluripotent stems cells; SOD1, superoxide dismutase 1.

expressing markers from different cell lineages have been also reported in Alzheimer's disease,[75] Huntington's disease,[76] central nervous system acute lesions[77–79] and aging,[74,80] as well as glioma,[56,57,81] brain ischemia and trauma,[78,79,82] further suggesting the phenotypic switch is strongly associated with inflammation and tissue remodeling after damage.

Compared with microglia/macrophages, astrocytes have been regarded to retain fewer phagocytic abilities. However, recent studies uncovered the phagocytic function of astrocytes under the various settings; synaptic elimination,[83] clearance of dead cells,[84] brain ischemia[82] and glaucoma.[85] For example, $Mac2^+$ astrocytes are also observed in the specific subpopulation in the myelination transition zone of the optic nerve head, indicating that those astrocytes are phagocytic and contribute to neurodegeneration in glaucoma.[85] In a brain ischemia lesion, $Mac2^+$ reactive astrocytes can function as phagocytes through inducing ABCA1, a molecule required for the engulfment and phagocytosis of debris and dead cells.[82] An apolipoprotein E4 variant is known as the most prominent genetic risk factor in Alzheimer's disease. In apolipoprotein E4 knock-in mice, apolipoprotein E-4-producing astrocytes are defective in phagocytic activity and failed to eliminate synapses in a complement-dependent manner.[86] Phagocytosis of astrocytes is also promoted by sleep deprivation.[87] In this context, enhanced phagocytic activity of astrocytes seems to be protective to the brain by cleaning worn components of heavily used synapses on prolonged wakefulness.

Conclusion

In the present review, we provided evidence supporting that the activation phenotypes of astrocytes are more heterogeneous mainly from the research for ALS. Contrary to the traditional understanding of astrocytes as static cells, reactive astrocytes possess more diverse functions than previously thought. Through achieving more knowledge of the phenotypic and functional diversity of astrocytes, astrocytes will become more attractive therapeutic targets for neurodegenerative diseases.

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Conflicts of interest

None declared.

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