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Reactive astrocyte nomenclature, definitions, and future directions

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Abstract

Reactive astrocytes are astrocytes undergoing morphological, molecular, and functional remodeling in response to injury, disease, or infection of the CNS. Although this remodeling was first described over a century ago, uncertainties and controversies remain regarding the contribution of reactive astrocytes to CNS diseases, repair, and aging. It is also unclear whether fixed categories of reactive astrocytes exist and, if so, how to identify them. We point out the shortcomings of binary divisions of reactive astrocytes into good-vs-bad, neurotoxic-vs-neuroprotective or A1-vs-A2. We advocate, instead, that research on reactive astrocytes include assessment of multiple molecular and functional parameters—preferably in vivo—plus multivariate statistics and determination of impact on pathological hallmarks in relevant models. These guidelines may spur the discovery of astrocyte-based biomarkers as well as astrocyte-targeting therapies that abrogate detrimental actions of reactive astrocytes, potentiate their neuro- and glioprotective actions, and restore or augment their homeostatic, modulatory, and defensive functions.

‘Neuroglia’ or ‘glia’ are collective terms describing cells of neuroepithelial (oligodendrocytes, astrocytes, oligodendrocyte progenitor cells, ependymal cells), neural crest (peripheral glia), and myeloid (microglia) origin. Changes in neuroglia associated with diseases of the CNS have been noted, characterized, and conceptualized from the very dawn of neuroglial research. Rudolf Virchow, in a lecture to students and medical doctors in 1858, stressed that ‘this very interstitial tissue [that is, neuroglia] of the brain and spinal marrow is one of the most frequent seats of morbid change...’¹. Changes in the shape, size, or number of glial cells in various pathological contexts have been frequently described by prominent neuroanatomists². In particular, hypertrophy of astrocytes was recognized very early as an almost universal sign of CNS pathology: ‘the protoplasmic glia elements [that is, astrocytes]

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are really the elements which exhibit a morbid hypertrophy in pathological conditions'³. Neuroglial proliferation was thought to accompany CNS lesions, leading to early suggestions that proliferating glia fully replaced damaged neuronal elements⁴. Thus, a historical consensus was formed that a change in 'the appearance of neuroglia serves as a delicate indicator of the action of noxious influences upon the central nervous system,' and the concept of 'reactionary change or gliosis' was accepted⁵. While the origin of 'gliosis' is unclear (*glia* + *osis* in Greek means 'glial condition or process'; in Latin the suffix *-osis* acquired the additional meaning of 'disease'; hence 'astrogliosis' may also carry a connotation of 'glial disorder'), the term became universally adopted to denote astrocytic remodeling in response to pathologic conditions. The role of reactive astrocytes in forming a scar-border to seal the nervous tissue against penetrating lesions was recognized, with distinct stages being visualised⁵. In the 21st century, astrocytes are increasingly viewed as having a critical contribution to neurological disorders. Research into the roles of astrocytes in neurology and psychiatry is accelerating and drawing in increasing numbers of researchers. This rapid expansion has exposed a pressing need for unifying nomenclature and refining of concepts⁶. Here we start by providing a working consensus on nomenclature and definitions and by critically evaluating widely used markers of reactive astrocytes. Then, we describe the advances and take positions on controversies regarding the impact of astrocytes in CNS diseases and aging. Finally, we discuss the need for new names to grasp astrocyte heterogeneity, and we outline a systematic approach to unraveling the contribution of astrocytes to disorders of the CNS. This article is expected to inform clinical thinking and research on astrocytes and to promote the development of astrocyte-based biomarkers and therapies.

Too many names

'Astrocytosis', 'astrogliosis', 'reactive gliosis', 'astrocyte activation', 'astrocyte reactivity', 'astrocyte re-activation', and 'astrocyte reaction' have been all used to describe astrocyte responses to abnormal events in the CNS, including neurodegenerative and demyelinating diseases, epilepsy, trauma, ischemia, infection, and cancer. We suggest 'reactive astrogliosis' to define the process whereby, in response to pathology, astrocytes engage in molecularly defined programs involving changes in transcriptional regulation, as well as biochemical, morphological, metabolic, and physiological remodeling, which ultimately result in gain of new function(s) or loss or upregulation of homeostatic ones. Although for some researchers, particularly neuropathologists, reactive astrogliosis is invariably associated with irreversible changes such as astrocyte proliferation, scar-border formation, and immune-cell recruitment⁶, these phenomena mainly occur when there is disruption of the blood-brain barrier (Fig. 1a)⁷. We also support the term 'astrocyte reactivity' as being broadly equivalent to reactive astrogliosis, but emphasizing the capacity of astrocytes to adopt distinct state(s) in response to diverse pathologies. Therefore, 'reactive astrocytes', referring to the cells undergoing this remodeling, is an umbrella term encompassing multiple potential states. We define 'state' as a transient or long-lasting astrocyte condition characterized by a specific molecular profile, specific functions, and distinct impact on diseases, while 'phenotype' is the measurable outcome of that state. Importantly, the changes in astrocytes in response to pathological stimuli are not to be confused with the plasticity of healthy astrocytes, which

are constantly being activated by physiological signals in the CNS. For this reason, although transitions from physiology to pathology are progressive and sometimes difficult to define, ‘astrocyte activation’ should be reserved for physiological conditions and not used in pathological contexts, which should be referred to as ‘astrocyte reactivity’.

The pathological contexts in which astrocyte reactivity occurs can markedly vary and may be sporadic or genetically mediated; acute or chronic; and due to a systemic pathology (for example, sepsis), specific injury or disease of the CNS, or a deleterious experimental manipulation. By definition, astrocyte reactivity is secondary to an extrinsic signal, may evolve with time, and, in many situations, is reversible. Astrocytes may also exhibit cell-autonomous disturbances⁸, as happens in astrocytopathies resulting from mutated alleles of astrocytic genes (for example, *GFAP* in Alexander disease)⁹, as well as from direct viral infections or exposure to toxic substances that specifically damage astrocytes (for example, ammonium in hepatic encephalopathy)¹⁰. These astrocytes can be considered ‘diseased astrocytes’ that unequivocally initiate the diseases and may secondarily acquire a reactive phenotype with a distinct impact on disease progression. Mutations in ubiquitously expressed genes, as in familial neurodegenerative disorders (for example, Huntington’s disease, HD), or disease-risk polymorphisms in genes highly expressed in astrocytes (for example, *APOE* in Alzheimer’s disease, AD)¹¹, may also lead to dysfunctional astrocytes that, without being the sole or primary initiators of pathology, may adversely affect outcomes. Terminology recommendations and caveats are summarized in Box 1 and in the section ‘Are new names needed?’ below.

GFAP as a marker

Glial fibrillary acidic protein (GFAP)—a major protein constituent of astrocyte intermediate filaments—is the most widely used marker of reactive astrocytes (Table 1)¹². Indeed, upregulation of GFAP mRNA and protein, as shown with multiple techniques including quantitative PCR (qPCR), RNA sequencing (RNAseq), in situ hybridization, electron microscopy, and immunostaining (Fig. 1a,d), is a prominent feature of many, but not necessarily all, reactive astrocytes: (i) increased GFAP content occurs across diverse types of CNS disorders, (ii) is an early response to injury, and, moreover, (iii) is a sensitive indicator, detectable even in the absence of overt neuronal death (for example, when there is synapse loss, minor demyelination, and extracellular amyloid- β oligomers). However, while the degree of GFAP upregulation in reactive astrocytes often parallels the severity of the injury⁶, this correlation is not always proportional, perhaps due to regional differences of astrocytes, including basal GFAP content^{13,14}. In the healthy mouse brain, hippocampal astrocytes have a higher GFAP content than cortical, thalamic, or striatal astrocytes; this, however, does not make hippocampal astrocytes more reactive. GFAP is also expressed by progenitor cells¹⁵ and its expression depends on developmental stages^{16,17}. In addition, GFAP immunoreactivity has been reported to decrease in a subpopulation of astrocytes in mouse cortex following repetitive trauma⁶ and in the spinal cord of a mouse model of amyotrophic lateral sclerosis (ALS), probably due to cleavage of GFAP by caspase-3 (ref. 18). Expression of *GFAP* is also modulated by physiological stimuli such as physical activity¹⁹, exposure to enriched environments¹⁹, and glucocorticoids²⁰, and it fluctuates with circadian rhythms in the suprachiasmatic nucleus²¹. Therefore, changes in *GFAP* expression may also reflect

physiological adaptive plasticity rather than being simply a reactive response to pathological stimuli. A common mistake is to interpret higher numbers of GFAP⁺ cells as local recruitment or proliferation of astrocytes. We recommend using markers of proliferation (Ki67, PCNA, and BrdU incorporation; Table 2) and combining GFAP immunostaining with other ubiquitous astrocyte markers such as aldehyde dehydrogenase-1 L1 (ALDH1L1), glutamine synthetase (GS), and aldolase-C (ALDOC) to correctly estimate astrocyte numbers²², provided that their expression is stable. Finally, there are discrepancies between observed GFAP mRNA and protein levels, perhaps due to differential regulation of translation, post-translational modifications, protein half-life, and antibody epitope accessibility. Overall, although an increase in GFAP content is a strong indication of reactive astrocyte remodeling, it is not an absolute marker of reactivity, nor does it strictly correlate with the extent thereof or indicate altered functions of reactive astrocytes.

Morphology revisited

Increased GFAP immunoreactivity largely reflects changes in the astrocytic cytoskeleton and tends to exaggerate the degree of hypertrophy because, with the exception of scar-border astrocytes, the volume accessed by reactive astrocytes does not change, as they remain in their territorial domains²³. In other words, cytoskeletal reorganization does not necessarily equal astrocyte hypertrophy. Immunohistochemical staining for cytosolic enzymes such as ALDH1L1, ALDOC, GS, and S100B allow the visualization of the somata and proximal processes of astrocytes, although, like GFAP, these markers fail to reveal small processes. Membrane proteins such as the glutamate transporters EAAT1 and EAAT2 are not optimal candidates to assess complex astrocyte morphology, as they tend to produce widespread and diffuse staining²⁴. In addition, the expression of some of these proteins may change in reactive astrocytes²² (Table 1), and some might be expressed by other cell types in specific brain regions¹³. Animal models expressing fluorescent proteins in the astrocyte cytosol or membrane through astrocyte-specific transgenesis or after gene transfer with viral vectors²⁵ circumvent the limitations of immunohistochemical analysis. Further, dye-filling methods can be used to visualize whole astrocytes in mice²³, as well as in human brain samples from surgical resections (Fig. 1b)²⁴. Thorough visualization is necessary because astrocytes undergo distinct morphological changes other than hypertrophy in pathological contexts, including elongation, process extension toward injury site, and some three-dimensional (3D) domain overlap²⁶. In addition, although astrocytes appear to be more resistant than neurons to degeneration and death, loss of primary and secondary astrocyte branches has been reported in mouse models of AD²⁷ and ALS¹⁸, and in patients with multiple sclerosis (MS)²⁸. Detailed analyses of astrocyte arborization in CNS diseases and injuries, however, are pending, given that the fine perisynaptic and perivascular astrocytic processes can only be revealed with super-resolution, expansion, or electron microscopy. Finally, *clasmotodendrosis* (from Greek *klasma*, fragment, + *dendron*, tree, + *osis*, condition or process) is a form of astrodegeneration characterized by an extreme fragmentation or beading and disappearance of distal fine processes, along with swelling and vacuolation of the cell body. It is observed in neuropathological specimens after severe trauma and ischemia, as well as in the aged brain²⁹. However, although astrocytes may suffer plasma membrane disruption due to mechanical damage and cleavage of membrane proteins and

cytoskeletal proteins, including GFAP, by proteases in acute brain trauma^{30,31}, the phenomenon of clasmatodendrosis should be approached with caution, because it may be an artifact derived from postmortem autolysis with no pathophysiological bearing, as suggested by Cajal³². In summary, GFAP upregulation and hypertrophy are useful but insufficient markers of astrocyte reactivity that need to be complemented by additional markers (Table 1 and Box 1).

Impact in CNS diseases

Research on astrocytes in CNS diseases has advanced in the last century in line with conceptual and technological progress in astrocyte biology. New approaches have been progressively integrated with existing ones, and these continue to evolve. At present, research in reactive astrocytes is an interdisciplinary endeavor combining -omics approaches with physiology and genetic manipulation. Below, we summarize advances and controversies with regards to the impact of astrocytes in CNS diseases from a historical perspective, punctuated by technical advances.

From morphology to functional studies.

From the early 20th century up to the 1980s, the morphological appearance of astrocytes was the only readout of their role in neuropathology. Hypertrophy and increased GFAP content were generally regarded as reflections of a detrimental astrocyte phenotype. The advent of genetic engineering in the early 1990s opened a new phase of research based on astrocyte-targeted manipulation of gene expression. For example, depletion or overexpression of receptors, membrane proteins^{33,34}, cytoskeleton proteins³⁵, acute-phase proteins³⁶, heat-shock proteins³⁷, and transcription factors^{38–40} in astrocytes or ablation of proliferative scar-border forming astrocytes⁴¹ was reported to modify (protect or exacerbate) the course of neurological diseases in mouse models. An important conclusion drawn from these studies is that the morphological appearance of astrocytes does not correlate with functional phenotypes or with their impact on other cell types. Moreover, the overall impact of reactive astrocytes on each disease is complex. For example, the manipulation of reactive astrocytes has resulted in improved outcomes^{38,42,43}, worse³⁵ outcomes, and no change⁴⁴ in mouse models of AD and MS^{40,45,46}. Plausibly, such differences arise from several scenarios: (i) pathways that ultimately exacerbate, attenuate, or have no impact on ongoing pathology occur in the same astrocyte, such that the selective manipulation of one pathway may mask, or secondarily impact, the manifestation of others; (ii) coexisting astrocyte subpopulations may have opposing effects on pathology⁴⁵; (iii) in neurodegenerative diseases, a spectrum of reactive-astrocyte phenotypes conceivably coexist in the same brain at a given time point because of the asynchronous progression of neuropathology in different brain regions; or (iv) the pathological impact of astrocytes is stage-dependent, as shown in mouse models of MS^{40,45,46}. Finally, pathways inducing astrocyte reactivity may be beneficial in one disease and detrimental in another. For example, activation of STAT3-dependent transcription is beneficial in neonatal white matter injury⁴⁷, traumatic brain injury³⁰, spinal cord injury^{48,49}, and motor neuron injury⁵⁰, but detrimental in AD models^{42,43}. That is, STAT3-mediated transcriptional programs may contribute to malfunctioning astrocyte states in AD models and to resilient states in other conditions. We broadly define ‘astrocyte resilience’ as the set of

successful astroprotective responses that maintain cell-intrinsic homeostatic functions in neural circuits (Table 2) while promoting both neuronal and astrocyte survival. Lastly, responses of reactive astrocytes may be maladaptive and result in dysfunctional astrocytes, which, in addition to losing homeostatic functions, may also gain detrimental functions, thus exacerbating ongoing pathology⁶. Numerous mixed scenarios of dysfunctional and resilient astrocytes plausibly exist, with multidirectional transitions among them.

Research in the last decade has begun to unravel specific functional alterations in reactive astrocytes underlying complex phenotypic changes. In normal conditions, astrocyte Ca^{2+} -based responses, and downstream signaling via neuroactive mediators, exert multifarious effects on synaptic function and plasticity, neural-network oscillations, and, ultimately, on behavior^{51,52}. In pathology, various functional changes emerge. Astrocyte Ca^{2+} dynamics and network responses become aberrant in mouse models of HD⁵³, AD⁵⁴, and ALS⁵⁵, possibly contributing to cognitive impairment and neuropathology^{43,53,56}. Reactive microglia may shift astrocyte signaling from physiological to pathological by increasing production of tumor necrosis factor α (TNF α), thus altering synaptic functions and behavior⁵⁷. Functions lost or altered in reactive astrocytes include neurotransmitter and ion buffering in mouse HD models⁵⁸, communication via gap junctions in the sclerotic hippocampus of patients with epilepsy⁵⁹, phagocytic clearance of dystrophic neurites⁶⁰, and metabolic coupling by glycolysis-derived d-serine⁶¹ and lactate⁶² in mouse AD models. The excessive release of GABA by reactive astrocytes in AD⁶³ and Parkinson's disease⁶⁴ may be a case of gain of detrimental function. Another example may be what is sometimes called 'astrocyte neurotoxicity', but we recommend using this term only when increased neuronal death is due to the verified release of an identified toxic factor by reactive astrocytes, and not merely due to loss of trophic or antioxidant support from astrocytes. An example is neuronal damage due to nitrosative stress caused by astrocyte-derived nitric oxide in MS³³. Finally, a classical gain of beneficial function is the restriction of immune cell infiltration in open injuries by scar-border forming reactive astrocytes⁷.

Transcriptomics and A1–A2 classification.

Transcriptomics has contributed to a fundamental discovery: astrocytes in the healthy brain are diverse and specialized to perform specific roles in distinct CNS circuits^{14,65}. Astrocyte diversity in healthy tissue arises from embryonic patterning programs or local neuronal cues¹⁴. Likewise, reactive astrocytes are also diverse, as unequivocally demonstrated by microarray-based^{66–68} and RNAseq-based^{48,69–71} transcriptomic profiling of mouse bulk astrocytes^{48,66–70} or of astrocyte populations preselected according to cell-surface markers⁷¹. Such transcriptomic profiling specifically shows that reactive astrocytes adopt distinct molecular states in different disease models^{48,66–70}, in different CNS regions⁷⁰, and in brain tumors⁷¹. These studies also suggested complex functional changes in reactive astrocytes, including novel regenerative functions⁷⁰, proliferation, and neural stem cell potential⁶⁸, as well as loss of homeostatic functions⁶⁶. They have also identified drug candidates to establish the impact of altered astrocytic pathways in mouse models^{68,70}. Whether baseline astrocyte heterogeneity influences astrocyte reactivity is an outstanding question.

In one early transcriptome study⁶⁶ and its follow-up⁷², it was proposed that mouse astrocytes adopted an ‘A1’ neurotoxic phenotype after exposure to specific cytokines secreted by microglia exposed to lipopolysaccharide (LPS), whereas they acquire an ‘A2’ neuroprotective phenotype after ischemic stroke—two acute pathological conditions. Two correlative signatures of 12 genes with 14 pan-reactive genes were proposed as fingerprints identifying these phenotypes and, for A1 astrocytes, combined with thorough functional analyses *in vitro*⁷². Although the A1 and A2 phenotypes were not proposed to be universal or all-encompassing, they became widely misinterpreted as evidence for a binary polarization of reactive astrocytes in either neurotoxic or neuroprotective states, which could be readily identified in any CNS disease, acute or chronic, by their correlative marker genes in a manner similar to the once-popular, but now discarded, Th1–Th2 lymphocyte and M1–M2 microglia polarization theories⁷³. For multiple reasons, we now collectively recommend moving beyond the A1–A2 labels and the misuse of their marker genes. Importantly, only a subset, often a mix of A1 and A2 or pan-reactive transcripts, are upregulated in astrocytes from brains of study participants with HD⁷⁴ or AD^{75,76}, or from several mouse models of acute injuries and chronic diseases of the CNS^{42,69,76,77}. Moreover, the functions of these genes are not known, because, to date, no experimental evidence has causally linked any of the proposed marker genes of A1 or A2 astrocytes to either toxic or protective functions. Thus, the mere expression of some or even all these marker genes does not prove the presence of functions that these genes have not been demonstrated to exert. Specifically, complement factor 3 (C3) should not be regarded as a single and definitive marker that unequivocally labels astrocytes with a net detrimental effect. In addition, steadily increasing evidence indicates that any binary polarization of reactive astrocytes falls short of capturing their phenotypic diversity across disorders. For example, single-cell and single-nucleus RNAseq (scRNAseq and snRNAseq, respectively) studies in mouse models and human brains of chronic neurodegenerative diseases have unraveled numerous stage-dependent transcriptomic states in HD⁷⁴, AD^{75,78}, and MS⁴⁰ that do not clearly comply with A1–A2 profiles. In addition, advanced statistics using multidimensional data and co-clustering approaches reveals that the A1 and A2 transcriptomes represent only two of many potential astrocyte transcriptomes segregating along several latent variables⁷⁹. The analyses also indicate that multidimensional data are necessary to establish the distinctiveness of astrocyte phenotypes (Fig. 2). Characterization of the potentially extensive and subtle functional diversity of reactive astrocytes suggested by transcriptomic data is an important future goal.

Human stem cells.

Advances in human induced pluripotent stem cell (hiPSC) technology are being adapted to astrocyte research. Interestingly, astrocytes generated from hiPSC derived from fibroblasts obtained from patients with CNS diseases (usually with a genetic mutation causative of disease or a risk polymorphism) show pathological phenotypes, including dysregulation of lipid metabolism¹¹, alteration in the contents of the extracellular vesicles released by astrocytes⁸⁰, reduced autophagy⁸¹, or altered STAT3 signaling⁸². hiPSC-derived astrocytes are also amenable to study responses to viral infection⁸³ and to specific stimuli⁸⁴. Nevertheless, caution is in order, for more research is needed to establish hiPSC-derived astrocytes as bona fide models of human astrocytes and to determine whether they recapitulate the maturity as well as the temporal, regional, and individual heterogeneity of in

vivo astrocytes. Importantly, not only are these cells removed from their original milieu, but the serum pervasively used in culture media may render them reactive⁸⁴. In addition, generation of astrocytes from neural stem cells is inherently difficult, and derivation and culture conditions have not yet been standardized, leading to diversity of clone phenotypes. Finally, aging-related neurodegenerative diseases should be modeled with astrocytes derived from cells from aged individuals, but, in this case, the epigenetic rejuvenation intrinsic to the reprogramming of adult cells arises as a confounding factor to be controlled for.

Are aging astrocytes reactive or senescent?

Healthy brain aging is not pathological and may be defined as an adaptive evolution of global cell physiology over time⁸⁵. Aged human brains display only mild and heterogeneous changes in astrocyte morphology or GFAP levels⁸⁶. Studies in rodents document region-dependent and often contradictory changes in aging astrocytes, such as an increase in cellular volume and overlap of astrocyte processes, but also atrophy, increase in GFAP content, or even a reduction in the number of GFAP⁺ and GS⁺ astrocytes^{87–89}. Notably, aging is also associated with pronounced regional differences in astrocyte gene expression in mouse brains^{90,91}. However, only a few studies have directly assessed astrocyte functions in the aging mouse brain^{85,92}. Thus, although the data suggest complex changes in aging astrocytes, the evidence is not yet sufficient to qualify astrocytes as being bona fide reactive during physiological aging. Nonetheless, with advanced age, cumulative exposure to pathological stimuli may render some astrocytes reactive. To test this hypothesis, a systematic investigation of the molecular properties of aging astrocytes across different CNS regions in humans and comparison of physiologically aged and reactive astrocytes in various pathological conditions are needed, together with functional validations in mouse models. Finally, we suggest caution about extending the concept of senescence to astrocytes based upon the expression of cell senescence marker p16^{INK4A}, increased β -galactosidase activity, and secretion of cytokines⁹³, because the core definition of senescence (that is, irreversible cell-cycle arrest in proliferative cells) may not apply to astrocytes, which are essentially post-mitotic cells that rarely divide in healthy tissue. Molecular and functional profiling of putative senescent astrocytes in different diseases is needed to clarify the meaning of p16^{INK4A} expression in post-mitotic astrocytes, as well as the interplay between senescence-like features, reactivity, and aging in astrocytes.

Are new names needed?

Arguably, new names are needed to capture the variety of reactive astrocytes, but current knowledge does not yet allow the objective categorizing of reactive astrocytes. Indeed, the existence of fixed categories defined by molecular and functional features consistently observed in different disease contexts is not yet certain. Nonetheless, two new names have recently been coined to describe the extremes of six astrocytic transcriptional clusters detected by snRNAseq in the hippocampus of AD transgenic and wild-type mice⁷⁸. In this study, ‘homeostatic astrocytes’ were predominant in healthy mice, whereas ‘disease-associated astrocytes’ were unique to AD mice. We do not support generalization of this ‘disease-associated’ classification to other conditions because only one disease was studied. In addition, the term ‘homeostatic astrocytes’ implies the unproven assumption that other

transcriptional astrocyte clusters are dys-homeostatic, while they may be successful homeostasis-preserving adaptations to disease.

We stress that the expression in full or in part of a predetermined correlative signature of molecular markers is not, on its own, sufficient to define a functional phenotype of reactive astrocyte. In addition, vague and binary terms such as ‘neuroprotective’ or ‘neurotoxic’ are best avoided in describing astrocyte phenotypes as they are too simplistic to be meaningful, unless they are supported by specific molecular mechanisms and direct causative experimental evidence. Future classification of reactive astrocytes should, instead, consider multiple criteria including transcriptome, proteome, morphology, and specific cellular functions (Table 2), together with demonstrated impact on pathological hallmarks (Fig. 2).

For now, we recommend ‘reactive astrocytes’ as the general term for astrocytes observed in pathological conditions (Box 1). The term ‘injured’ or ‘wounded’ astrocytes should be reserved for astrocytes with unequivocal morphological signs of damage (for example, beaded processes), as observed in ischemia and trauma^{30,31}. Descriptions based on misleading generalizations of functional changes and over-interpretation of correlative data should be avoided. We call for a clear operational terminology that includes information about morphology (for example, hypertrophic, atrophic), molecular markers (Table 1), and functional readouts (Table 2), as well as brain region, disease, disease stage, sex, species, and any other relevant source of heterogeneity (Fig. 2). Indeed, the goal is to go beyond the mere categorization of reactive astrocytes and identify the key variables driving specific reactive astrocyte states, phenotypes, and functions in specific contexts. When addressing similar issues for neurons, scientists are not concerned about categorizing disease-associated neurons into simple generalizable subtypes; rather, the emphasis is placed on understanding specific changes of defined neuronal populations in specific diseases. This principle should also apply to astrocytes.

Toward astrocyte-targeting therapies

One goal of research on reactive astrocytes is to develop astrocyte-targeting therapies for CNS diseases. Two challenges preclude translating the wealth of functional and molecular data described in the previous sections into therapies. First, there is a need to unequivocally clarify whether or not reactive astrocytes and their associated signaling pathways significantly contribute to the pathogenesis of specific CNS diseases. The approach should be reciprocal, such that human data inform experimental manipulations in animal models and animal data are validated in human materials. The second challenge is to develop astrocyte therapies tailored to specific disease contexts. Specific research directions include heterogeneity characterization, signaling, humanizing research, and systems biology.

Heterogeneity characterization.

To define astrocyte phenotypes, all sources of heterogeneity should be considered and integrated with multidimensional statistical analyses (Fig. 2). ScRNAseq and snRNAseq are becoming established as valuable tools to gain insight into basal⁹⁴ and reactive-astrocyte heterogeneity (Fig. 1e)^{40,78,95}. Notably, isolation protocols may not always be optimal for astrocytes, resulting in low numbers of cells or nuclei being sequenced, and some highly

relevant but weakly expressed transcripts, such as transcription factors and plasma-membrane receptors, may be overlooked, particularly in snRNAseq. Translation from scRNAseq or snRNAseq data to in situ immunohistochemical detection and functional validations is far from trivial, because the molecular profiles of astrocyte clusters and subpopulations partly overlap. Thus, instead of individual markers, signatures composed of a combination of markers with specified levels of expression or relative fold-changes are required to identify astrocyte phenotypes⁷⁴. Such signatures must be statistically validated to the point of predicting phenotypes. Alternatively, the diversity within astrocyte populations from mouse models may be dissected out by combining fluorescence-activated cell sorting (FACS) and cell-surface markers identified in screens⁷¹. Further, emerging spatial transcriptomics that allow the simultaneous in situ detection of numerous genes will be of value to study the heterogeneity of reactive astrocytes at local and topographical levels (Fig. 1f)⁹⁶. Importantly, molecular signatures based on the expression of genes or proteins need to be validated by assessing specific astrocyte functions (Table 2), since post-transcriptional and post-translational events critically shape functional outcomes. Functional validations should preferably be performed in vivo or using in vitro models closely mimicking human diseases. Classical knockout-, knockdown-, or CRISPR-based approaches to inactivate gene expression are available to gain insight into the impact on disease of a given pathway within previously identified astrocyte subsets⁴⁰.

Signaling.

An important implication of the disease-specific induction of distinct reactive astrocyte states is that the damage- and pathogen-associated stimuli from one disorder cannot be assumed to be active in another. For example, the now widely-used cocktail of factors released by LPS-treated neonatal microglia⁷² cannot be simply assumed to model reactive astrocytes in diseases other than neonatal septic shock due to infection by gram-negative bacteria. Likewise, exposure to Tau, amyloid- β , or α -synuclein needs to be carefully designed in vivo and in vitro to replicate the concentration, protein species, and combinations thereof found in patient brains. Acute metabolic damage with the mitochondrial toxin MPTP does not replicate chronic Parkinson's disease (PD), to cite another example of in vivo inappropriate modelling. To complicate things further, the outcome of activating a signaling pathway may depend on the upstream stimuli⁸² or priming caused by previous exposure to other stimuli⁹⁷, perhaps through epigenetic control⁴⁰. Thus, careful selection of upstream stimuli is essential for appropriate in vivo and in vitro modelling of disease-specific reactive astrocytes. Finally, interventional strategies such as classical pharmacology^{56,98}, genetic manipulation^{42,56}, and biomaterials⁹⁹ are available tools to modify pathological signaling in reactive astrocytes for therapeutic purposes. Optogenetics²⁵ and designer receptors exclusively activated by designer drugs (DREADDs)²⁵ are potential tools to manipulate reactive astrocytes or to restore their aberrant Ca^{2+} signaling observed in mouse models of neurodegenerative diseases⁵³⁻⁵⁵. However, it is unknown whether and how the changes in Na^+ , K^+ , Cl^- , and Ca^{2+} fluxes and second messengers triggered by these approaches²⁵ modulate signaling cascades driving phenotypical changes of reactive astrocytes (for example, JAK-STAT and NF- κ B pathways)⁶.

Humanizing research.

Although some basic functional properties of astrocytes have been shown to be evolutionarily conserved between humans and rodents¹⁰⁰, it is still critical to study patient samples and develop models of human reactive astrocytes, because morphological and transcriptomic comparisons have revealed prominent differences between mice and humans^{101–103}. In addition to astrocytes from postmortem samples and biopsies⁵⁹ (Fig. 1b), hiPSC-derived astrocytes, which can be generated with a fast protocol in 2D layers¹⁰⁴ or integrated in 3D systems such as spheroids and organoids^{105–108}, are rapidly becoming commonplace in basic research^{11,82} and therapy development¹⁰⁹. Researchers need to be aware of the pros and cons of the various protocols available, as discussed in previous sections and elsewhere^{110–112}. Also, hiPSC glial mouse chimeric brains, in which hiPSC differentiate into human astrocytes, oligodendrocytes, and their progenitors, offer the possibility to study human astrocytes from patients in contexts amenable to in vivo experimentation^{113,114}. In addition, proteins released by injured astrocytes are currently being considered as fluid biomarkers of neurological trauma³¹. Biomarkers of reactive astrocytes in human disease will indeed be needed to demonstrate target engagement of future astrocyte-directed therapies in clinical trials. Emerging reactive-astrocyte biomarkers are either measured in blood or cerebrospinal fluid (for example, YKL-40)¹¹⁵ or used for brain imaging, such as MAO-B-based positron emission tomography (PET)¹¹⁶, which provides important topographical information (Table 1)¹¹⁷. Plausibly, disease-specific biomarker signatures rather than single ubiquitous biomarkers will be needed.

Use of systems biology.

Computerized tools, including systems biology and artificial intelligence, are essential to organizing and interpreting the increasing wealth of high-throughput, multidimensional molecular and functional data from reactive astrocytes. Currently, molecular data (for example, -omics) can be transformed into mathematical maps by artificial intelligence¹¹⁸, thereby providing quantitative representations of the otherwise-vague notion of phenotypes. An example of functional data is 2D and 3D Ca^{2+} imaging that generates kinetic profiles and maps for single astrocytes and 2D or 3D networks (Fig. 1c)^{119,120}. Artificial intelligence can identify patterns of Ca^{2+} signaling in astrocytes^{55,120}. Multidimensional molecular and functional data have then two applications. First, multivariate analysis may unravel molecules, pathways, and variables shaping astrocyte phenotypes in acute versus chronic degenerative conditions, different disease stages, sexes, and CNS regions (Fig. 2). Second, these data can be used to predict the net functional outcome of a complex mix of potentially protective or deleterious pathways and identification of hubs, such as master transcription factors or epigenetic regulators, that, when activated, promote globally beneficial transformations. Importantly, the inhibition of detrimental pathways must not secondarily impair protective ones or damage basic astrocyte functions. Finally, no astrocyte-targeting therapy can be successful if it does not consider the complex interactions of reactive astrocytes with other CNS cells.

Concluding remarks

The dawn of neuropathology in the late 19th and early 20th centuries witnessed widespread interest in neuroglia. Today, research on astrocytes and their remodeling in the context of injury, disease, and infection is undergoing a renaissance, with new researchers bringing exciting new techniques, approaches, and hypotheses. Given the scarcity of disease-modifying treatments for chronic diseases and acute injuries of the CNS, this astrocyte revival represents an opportunity to develop largely unexplored therapeutic niches, such as the manipulation of reactive astrocytes. However, despite the substantial body of knowledge accumulated since the discovery of reactive astrocytes a century ago, there are no therapies purposely designed against astrocyte-specific targets in clinical practice. The present working consensus for research guidelines will hopefully boost more coordinated and better focused efforts to improve and therapeutically exploit our knowledge about the role(s) of reactive astrocytes in CNS diseases and injuries.

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Box 1 |**Basic consensus and recommendations for research on reactive astrocytes****Basic consensus**

1. Reactive astrocytes are astrocytes that undergo morphological, molecular, and functional changes in response to pathological situations in surrounding tissue (that is, due to CNS disease, injury, deleterious experimental manipulation).
2. Astrocytes with disease-causing genetic mutations are diseased astrocytes that initiate or contribute to pathology and later become reactive in ways that may differ from the astrocyte reactivity normally triggered by external stimuli. Genetic polymorphisms linked to CNS diseases may also influence astrocytic functions and prime astrocytes to acquire distinct reactive states.
3. There is no prototypical reactive astrocyte, nor do reactive astrocytes polarize into simple binary phenotypes, such as good–bad, neurotoxic–neuroprotective, A1–A2, etc. Rather, reactive astrocytes may adopt multiple states depending on context, with only a fraction of common changes between different states.
4. Loss of some homeostatic functions, and gain of some protective or detrimental functions, may happen simultaneously. Whether the overall impact on disease is beneficial or detrimental will be determined by the balance and nature of lost and gained functions and the relative abundance of different astrocyte subpopulations.

Recommendations

5. Astrocyte phenotypes should be defined by a combination of molecular markers (Table 1) and functional readouts (Table 2), preferably in vivo. GFAP and morphology alone are not sufficient criteria to qualify astrocytes as reactive.
6. The specifics of the astrocytes under study should be spelled out in titles, abstracts, and results of articles (for example, *X*-positive astrocytes in *Y* region showed *Z* phenomenon).
7. Multivariate and clustering analysis of molecular and functional data will facilitate the identification of distinct phenotypes of reactive astrocytes (Fig. 2).
8. Local, regional, temporal, subject or patient, and sexual heterogeneity of reactive astrocytes should be studied (Fig. 2).
9. The discovery and validation of plasma or serum and cerebrospinal fluid biomarkers, as well as of PET radiotracers of astrocyte reactivity, is a research priority, as it will facilitate astrocyte-directed drug development.

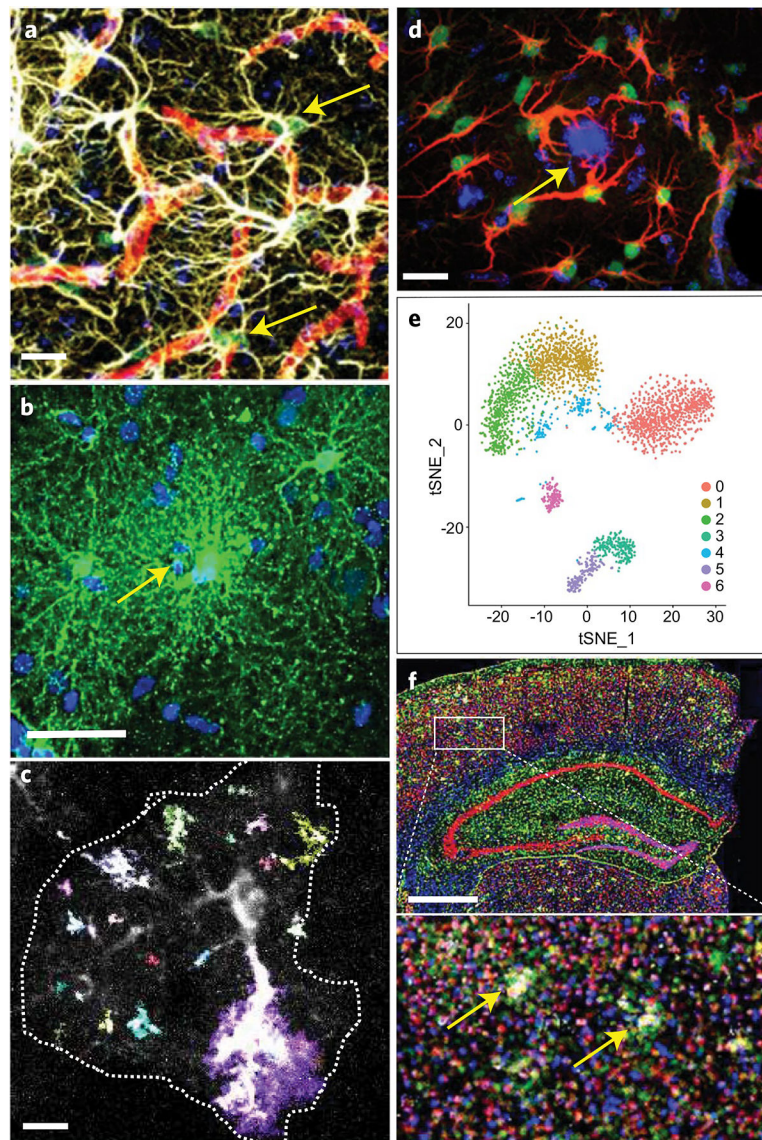


Fig. 1 |. Multivariate assessment of reactive astrocytes.

a, Reactive astrocyte proliferation in the vicinity of blood vessels assessed by co-staining for BrdU (green; arrows), DAPI (blue), GFAP (white), and CD31 (red) after stab injury of the mouse cortex. Scale bar, 15 μ m. Unpublished image from authors S.S. and M.G. **b**, Human cortical protoplasmic astrocytes in a surgical specimen injected with Lucifer yellow (arrow, injection site) that traverses the gap junctions into neighboring astrocytes. Scale bar, 45 μ m. Courtesy of Drs. Xu, Sosunov, and McKhann, Columbia University Department of Neurosurgery. **c**, Event-based determination of Ca^{2+} responses in a GCaMP6-expressing astrocyte (surrounded by a dashed line) in mouse cortical slices using astrocyte quantitative analysis (AQUA)¹²⁰. Colors indicate AQUA events occurring in a single 1-s frame of a 5-min movie. Scale bar, 10 μ m. **d**, Activation of the transcription factor STAT3 (green) assessed by nuclear accumulation in GFAP+ reactive astrocytes (red) surrounding an amyloid plaque (blue, arrow) in a mouse AD model. Scale bar, 20 μ m. Adapted from ref. ¹²¹, Society for Neuroscience. **e**, ScRNAseq in the remission phase of a mouse model of MS reveals several

transcriptional astrocyte clusters. These astrocyte sub-populations may be validated with spatial transcriptomics, as shown in **f** in a model of AD. Adapted from ref. ⁴⁰, Nature Publishing Group. **f**, Distribution of 87 astrocytic (green), neuronal (red), microglial (yellow), and oligodendroglial (blue) genes with in situ multiplex gene sequencing in a coronal section from a mouse model of AD. The method ‘reads’ barcodes of antisense DNA probes that simultaneously target numerous mRNAs. Scale bar, 800 μm . Boxed area is magnified in bottom image, showing 6E10⁺ amyloid- β plaques (white; arrows). Adapted from ref. ⁹⁶, Cell Press.

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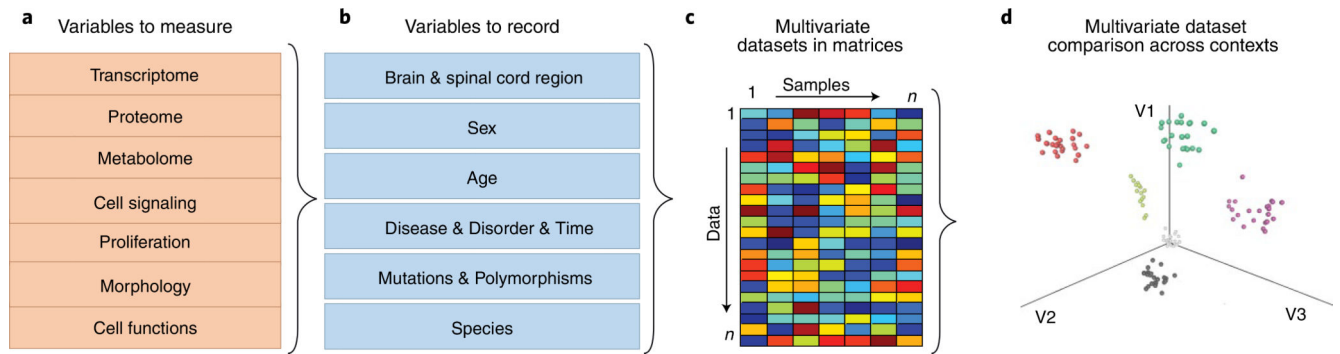


Fig. 2 |. Workflow for the identification of key variables shaping astrocyte reactivity using multidimensional analyses.

a, Variables to measure in individual experiments. Although at present it is unrealistic to measure all in the same experiment, it will in most cases be possible to measure at least two or three. **b**, Variables to record in individual experiments. In some experiments, all or most of these variables are kept constant and are not compared, but they should all be recorded to allow for future comparison across experiments and studies. **c**, Individual studies will generate multidimensional datasets of reactive astrocytes that can be organized in matrices containing all outcome measures of variables assessed in **a** (for example, -omics data, functional measurements). One matrix may be generated for each condition listed in **b** using data obtained in **a**. Determining whether such states are equivalent to fixed categories rather than temporary changes due to the dynamic nature of cell functioning requires cross-comparison among studies or longitudinal studies, paired with statistical analyses, as in **d**. **d**, Multidimensional data analysis and clustering statistics of weighted scores from datasets (**a**) across different contexts (**b**) represented in matrices (**c**) allow identification of functional vectors (**V**) driving astrocyte reactivity in different contexts. A high score and a low score in each vector represent gain and loss of function, respectively. The graph shows a hypothetical plot of simulated multivariate datasets from **a** (each dot represents one dataset or sample) obtained in different contexts (**b**), depicted in different colors. Astrocytes with shared features segregate together along three axes according to the predominance of the function represented in each vector. A state is defined by where the dataset(s) are placed in the V1–3 space. The analysis can be n -dimensional, but for visual clarity, we show a 3D scenario.

Table 1 |

Potential markers of reactive astrocytes

Marker	Known function	Type of change	Conditions observed	Species	Comments	Ref.
Cytoskeleton						
GFAP	Intermediate filament	↑ mRNA & protein	Widespread. Not in some trauma models	Widespread	Released by injured astrocytes. Cleavage product found in CSF and plasma (neurotrauma biomarker)	122
Nestin	Intermediate filament	↑ mRNA & protein	AD, AxD, MS, SCI, TBI	Hu, Ms	Also a marker of progenitor cells	123
Synemin	Intermediate filament	↑ mRNA & protein	AD, AxD, astrocytoma, TBI	Hu, Ms	Normally expressed in a subset of astrocytes during development	124
Vimentin	Intermediate filament	↑ mRNA & protein	Widespread	Widespread	Also expressed by endothelial cells, vascular smooth muscle cells, and immature astrocytes	125
Metabolism						
ALDOC	Glycolytic enzyme	↑ protein	SCI, TBI	Hu, Ms	Released by injured astrocytes. Fluid biomarker for neurotrauma	30,31
BLBP/ FABP7	Lipid transport	↑ protein	AD, MS, TBI	Hu, Ms	Also a marker of immature astrocytes. Released by injured astrocytes. Fluid biomarker for neurotrauma	31,60
MAO-B	Catecholamine catabolic enzyme	↑ protein	AD, ALS, PD	Hu, Ms	PET radiotracers available. Also expressed by catecholaminergic neurons	63,64,117
TSPO	Mitochondrial lipid transporter	↑ mRNA & protein	AD, MS, ischemia	Hu, Rt, Ms	PET radiotracers available. Also induced in reactive microglia. Expressed by vascular cells	126
Chaperones						
CRYAB	Chaperone activity	↑ mRNA & protein, ↑ secretion	AD, AxD, epilepsy, HD, MS, TBI	Hu, Ms	Reduces protein aggregation	74,95
HSPB1/HSP27	Chaperone	↑ mRNA & protein	AD, AxD, epilepsy, MS, tauopathies, stroke	Widespread		95,127
Secreted proteins						
C3	Complement factor	↑ mRNA & protein	ND, prion disease, septic shock	Hu, Ms	Also expressed by microglia	72
CHI3L1/ YKL40	Unclear function	↑ mRNA & protein, ↑ secretion	Widespread	Hu, Ms	Increase in CSF is a prognostic biomarker in LOAD and MS	79,115
Lcn2	Iron trafficking protein	↑ mRNA & protein	AxD, MS, septic shock, ALS, stroke	Widespread		66
Serpina3n/ACT	Serine protease inhibitor	↑ mRNA	AD, septic shock, stroke	Hu, Ms	Secreted to extracellular matrix	66
MT	Metal binding	↑ mRNA & protein	HD, PD, AD	Hu, Ms	Antioxidant effects	74
THBS-1	Synaptogenic factor	↑ mRNA & protein, ↑ secretion	Axotomy, MS	Hu, Ms	STAT3-regulated. Has beneficial synaptogenic effects	50
Cell signaling - transcription factors						

Marker	Known function	Type of change	Conditions observed	Species	Comments	Ref.
NFAT	Transcription factor	↑ mRNA, protein, nuclear translocation	AD, TBI, PD	Hu, Ms	Links Ca ²⁺ signaling with reactive transcriptional changes	38,128
NTRK2/ TrkB IL17R	Receptors	↑ mRNA and/or protein	Epilepsy, MS (white matter)	Hu, Ms	Trigger non-canonical pathological BDNF-dependent signaling, and/or NF-κB activation and NO production	33,109
S100B	Ca ²⁺ binding protein	↑ protein and release	Widespread	Widespread	Released upon injury. Fluid biomarker	129
SOX9	Transcription factor	↑ mRNA and/or protein	ALS, stroke, SCI	Hu, Ms	Nuclear staining. Also present in ependymal cells and in neurogenic niches	130
STAT3	Transcription factor	Phosphorylation, nuclear translocation	Widespread	Widespread	Also expressed in neurons and other cell types	49,50,131
Channels - Transporters						
EAXT1 & 2	Glutamate transporters	↓ mRNA, protein and uptake	ND	Widespread	May be also detected in some neuronal cells	53,132
KIR4.1	K ⁺ channel	↓ mRNA & protein	Widespread	Hu, Ms	May or may not translate into alteration of K ⁺ buffering	58

This table lists potential markers for reactive astrocytes in different pathological contexts in human diseases and animal models. The list is not meant to be exhaustive; other markers exist and more will be added over time. These proteins can be used to further characterize the reactive state of astrocytes, although note that, like GFAP (see 'GFAP as a marker'), none of these proteins should be used as a single or universal marker of reactive astrocytes, nor for the time being do they identify a specific type of reactive astrocyte. Plausibly, markers in this table will be part of signatures defining disease-specific or core markers of reactive astrocytes, as well as astrocyte-based fluid biomarkers (see 'Toward astrocyte-targeting therapies'). Importantly, few of these markers are astrocyte-specific; therefore, additional methods to identify or isolate astrocytes and remove contamination by other cell types will be in order. AxD, Alexander disease; BDNF, brain-derived neurotrophic factor; CSF, cerebrospinal fluid; Hu, human; LOAD, late-onset AD; Ms, mouse; ND, neurodegenerative disease; NO, nitric oxide; Rt, rat; SCI, spinal cord injury; TBI, traumatic brain injury.

Table 2 |

Potential functional assessments for reactive astrocytes

Function or phenomenon	Potential readouts	Ref.
Ca ²⁺ signaling in single cells Ca ²⁺ -based network dynamics	Ca ²⁺ imaging with chemical or genetically encoded Ca ²⁺ indicators	25,52,55,119,120
Ionic homeostasis	Measurement of ionic currents and membrane potential (electrophysiology). Direct measurement of extracellular K ⁺ levels	58,132
Glutamate, GABA, D-serine and ATP release	Detection of neuroactive factors using fluorescent sensors and in vivo two-photon imaging.	25
Glutamate uptake and conversion	Quantification of neuroactive factors in extracellular milieu and CSF (FRET, HPLC, CE-LIF, fluorescent sensors like GluSnFR, enzymatic kits)	
Astrocyte inter-cellular connectivity	Analysis of glutamate currents (electrophysiology) and/or transporter content (immunoblot, immunostainings)	109,132
Vascular coupling Maintenance of BBB integrity	Metabolism of ¹³ C-labeled substrates (GC-MS and HPLC) Diffusion of permeant dyes in astrocyte networks (patch-clamp and imaging), FRAP	133 59
Signaling	Assessment of vascular responses after Ca ²⁺ uncaging or optogenetic stimulation of astrocytes (two-photon imaging, optical intrinsic imaging, MRI)	134
Transcription factor activation	Assessment of BBB permeability with detection in the parenchyma of blood proteins or dyes (Evans blue, Dextran)	135
Production of synaptogenic and neurotrophic factors, ECM, cytokines, chemokines	Standard biochemical assays. Signaling manipulation by DREADDs. Transcription factor translocation and DNA binding assays, chromatin immunoprecipitation, reporters	25,109,136
Interactions with neurons, oligodendrocytes, OPC and microglia	Synapse quantification in vivo and upon exposure to astrocyte-conditioned media in vitro. Proteomics and metabolomics of astrocyte-conditioned media and acutely sorted astrocytes.	72,97
Glycolysis	Multiplex ELISA assays, immunostainings	
Fatty-acid oxidation	In vivo and ex vivo analyses, co-cultures or exposure to conditioned media and assessment of function and survival	58,72,82
Lactate production	Metabolism of ³ H-, ¹⁴ C-, and ¹³ C- labeled energy substrates (GC-MS, radioactive assays, NMR)	133,137
Glycogen metabolism	Glucose, pyruvate, lactate and ATP quantification with genetically-encoded fluorescent sensors and in vivo two-photon imaging	138,139
Mitochondrial respiration	Lipid-droplet and fatty-acid staining with BODIPY dyes NADH imaging (FLIM)	140 141
NO-ROS production and detoxification	Activities of electron transport chain complexes. Extracellular acidification, oxygen consumption (Sea Horse, voltammetry) Quantification of glycogen granules by EM or immunostainings	141 142,143
	NO-ROS imaging with intra- and extracellular fluorescent sensors or probes. Immunostaining for oxidized residues. Activity of antioxidant enzymes with commercial kits	33,144

Function or phenomenon	Potential readouts	Ref.
Endolysosomal system	Detection of phagocytosed materials (array tomography, EM, two-photon microscopy). Uptake of myelin debris or labeled synaptosomes	60,72,145
	Autophagic flux	81,146
	Exosome production	80,147
	Proteasome and lysosome proteolytic activity (fluorescent probes)	148
Proliferation	BrdU incorporation. Ki67, PCNA, cyclin labeling (calculation of a proliferative index, that is, the percent of positive cells in the population). Characterization of astrocyte progeny by fate mapping	149,150
Scar-border formation	Morphometric and functional analyses (for example, composition, permeability to immune cells)	131

The table depicts assays that can be performed in astrocytes to characterize their functional properties. References and functions are not exhaustive and aim to illustrate the existing methodology by providing recent protocols for each approach. Although most references concern studies in healthy or reactive astrocytes, some additional tools relevant to reactive astrocytes are listed as well. Assays can be performed in human neurosurgical samples, *in vivo*, or in acute brain slices of animal models and/or *in vitro* (pure cultures, mixed cultures, organoids). Note that some assays require specific equipment and skills or the physical isolation of astrocytes to measure astrocyte-specific functional parameters. No reference is provided for enzymatic assays that are commercially available. BBB, blood-brain barrier; BODIPY, boron dipyrromethene; BrdU, bromodeoxyuridine; CE-LIF, capillary electrophoresis with laser induced fluorescent detection; ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; FLIM, fluorescence lifetime imaging microscopy; FRAP, fluorescence recovery after photobleaching; FRET, Förster resonance energy transfer; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; NADH, reduced form of nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; OPC, oligodendrocyte progenitor cells; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species.