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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 remodelling in response to injury, disease, or infection of the central nervous system (CNS). Although this remodelling was first described over a century ago, uncertainties and controversies remain, regarding the contribution of reactive astrocytes to CNS diseases, repair, and ageing. 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/bad, neurotoxic/neuroprotective or A1/A2. We advocate, instead, that research on reactive astrocytes include assessment of multiple molecular and functional parameters, preferably in vivo, multivariate statistics, and determination of impact on pathological hallmarks in relevant models. These guidelines may spur the discovery of astrocyte-based biomarkers, and astrocyte-targeting therapies that abrogate detrimental actions of reactive astrocytes, potentiate their neuro- and glio-protective actions, and restore or augment their homeostatic, modulatory, and defensive functions.

1. Introduction

'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 central nervous system (CNS) have been noted, characterised, and conceptualised 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* [i.e. 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 neuroanatomists.[2] In particular, hypertrophy of astrocytes was recognised very early as an almost universal sign of CNS pathology;[3] "*The protoplasmic glia elements* [i.e. astrocytes] *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 elements.[4] Thus, a historical consensus was formed that changes 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 accepted.[5] 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 remodelling in response to pathologic conditions. The role of reactive astrocytes in forming a scar-border to seal the nervous tissue against penetrating lesions was recognised, with distinct stages being visualised.[5] 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 concepts.[6] 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 we take position on controversies, regarding the impact of astrocytes in CNS diseases and ageing. Finally, we discuss the need for new names to grasp astrocyte heterogeneity, and we outline a systematic approach to unravelling 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.

2. 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 remodelling, 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 recruitment,[6] 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 remodelling, 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, functions, and distinct impact on diseases, while its "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, due to a systemic pathology (e.g., 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 disturbances,[8] as happens in astrocytopathies resulting from mutated alleles of astrocytic genes (e.g. *GFAP* in Alexander disease),[9] as well as from direct viral infections or exposure to toxic substances that specifically damage astrocytes (e.g., 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 (e.g. Huntington's disease, HD), or disease-risk polymorphisms in genes highly expressed in astrocytes (e.g., *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 section 7, below.

3. 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, up-regulation 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 (e.g., when there is synapse loss, minor demyelination, and extracellular amyloid-β oligomers). However, while the degree of GFAP up-regulation in reactive astrocytes often parallels the severity of the injury,[6] 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 cells[15] 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 trauma,[6] and in the spinal cord of a mouse model of amyotrophic lateral sclerosis (ALS), probably due to cleavage of GFAP by caspase 3.[18] Expression of *GFAP* is also modulated by physiological stimuli such as physical activity,[19] exposure to enriched environments,[19] and glucocorticoids,[20] and it fluctuates with circadian rhythms in the suprachiasmatic nucleus.[21] 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-positive cells as local recruitment or proliferation of astrocytes. We recommend to use markers of proliferation (Ki67, PCNA and BrdU

incorporation, Table 2), and to combine 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 numbers,[22] provided that their expression is stable. Finally, there are discrepancies between observed 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 remodelling, it is not an absolute marker of reactivity, nor does it strictly correlate with the extent thereof, or indicate altered functions of reactive astrocytes.

4. 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, since they remain in their territorial domains.[23] 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 2 are not optimal to assess complex astrocyte morphology, as they tend to produce widespread and diffuse staining.[24] In addition, the expression of some of these proteins may change in reactive astrocytes ([22], Table 1) and some might be expressed by other cell types in specific brain regions.[13] Animal models expressing fluorescent proteins in the astrocyte cytosol or membrane through astrocyte-specific transgenesis, or gene transfer with viral vectors,[25] circumvent the limitations of immunohistochemical analysis. Further, dye-filling methods can be used to visualize whole astrocytes in mice[23], as well as in human brain samples from surgical resections (Fig. 1b).[24] Thorough visualisation is necessary because astrocytes undergo distinct morphological changes other than hypertrophy in pathological contexts, including elongation, process extension towards injury site, and some 3D domain overlap.[26] 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 AD[27] and ALS,[18] and in patients with multiple sclerosis (MS).[28] Detailed analyses of astrocyte arborization in CNS diseases and injuries are however pending, given that the fine perisynaptic and perivascular astrocytic processes can only be revealed with super-resolution, expansion, or electron microscopy. Finally, clasmatodendrosis (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, and in the aged brain.[29] 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 artefact derived from *post-mortem* autolysis with no pathophysiological bearing, as suggested by Cajal.[32] In summary, GFAP upregulation and hypertrophy are useful, but insufficient markers of astrocyte reactivity that need to be complemented by additional markers (Table 1, Box 1).

5. 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 endeavour 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 over-expression of receptors, membrane proteins,[33, 34] cytoskeleton proteins,[35] acute-phase proteins,[36] heat-shock proteins,[37] and transcription factors[38-40] in astrocytes or ablation of proliferative scar-border forming astrocytes,[41] 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,[38, 42, 43] worsen[35] outcomes, and no change[44] 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 pathology,[45] (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, (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 injury,[47] traumatic brain injury,[30], spinal cord injury,[48, 49] and motor neuron injury[50] but detrimental in AD models.[42, 43] That is, STAT3-mediated transcriptional programs may contribute to malfunctional 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 malfunctional astrocytes, which, in addition to losing homeostatic functions, may also gain detrimental functions, thus exacerbating ongoing pathology.[6] Numerous mixed scenarios of malfunctional 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 signalling via neuroactive mediators, exert multifarious effects on synaptic function and plasticity, neural-network oscillations, and, ultimately, on behaviour.[51, 52] In pathology, various functional changes emerge. Astrocyte Ca^{2+} dynamics and network responses become aberrant in mouse models of HD,[53] AD,[54] and ALS,[55] possibly contributing to cognitive impairment and neuropathology.[43, 53, 56] Reactive microglia may shift astrocyte signalling from physiological to pathological by increasing production of tumour necrosis factor α, thus altering synaptic functions and behaviour.[57] Functions lost or altered in reactive astrocytes include neurotransmitter and ion buffering in mouse HD models,[58] communication via gap junctions in the sclerotic hippocampus of epileptic patients,[59] phagocytic clearance of dystrophic neurites,[60] and

metabolic coupling by glycolysis-derived D-serine[61] and lactate[62] in mouse AD models. The excessive release of GABA by reactive astrocytes in AD[63] and Parkinson's disease[64] may be a case of gain of detrimental function. Another example may be the so-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 MS.[33] Finally, a classical gain of beneficial function is the restriction of immune cell infiltration in open injuries by scar-border forming reactive astrocytes.[7]

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 cues.[14] 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 pre-selected according to cell-surface markers.[71] Such transcriptomic profiling specifically shows that reactive astrocytes adopt distinct molecular states in different disease models,[48, 66-70] CNS regions,[70] and in brain tumours.[71] These studies also suggested complex functional changes in reactive astrocytes, including novel regenerative functions,[70] proliferation, and neural stem cell potential,[68] as well as loss of homeostatic functions.[66] 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 study[66] and its follow-up,[72] 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 theories.[73] 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 human HD[74] and AD[75, 76] brains, 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, for, 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/nucleus RNAseq (sc/snRNAseq) studies in mouse models and human brains of chronic neurodegenerative diseases have unravelled numerous stage-dependent transcriptomic states in HD,[74] AD,[75, 78] and MS[40], that do not clearly comply with A1/A2 profiles. In addition, advanced statistics using multi-dimensional data and co-clustering approaches reveals that the "A1" and "A2" transcriptomes represent only two out of many potential astrocyte transcriptomes

segregating along several latent variables.[79] 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 metabolism,[11] alteration in the contents of the extracellular vesicles released by astrocytes,[80] reduced autophagy, [81] or altered STAT3 signalling.[82] hiPSC-derived astrocytes are also amenable to study responses to viral infection[83] and to specific stimuli.[84] 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 subject 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 reactive.[84] 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, ageing-related neurodegenerative diseases should be modelled with astrocytes derived from cells from aged subjects, but, in this case, the epigenetic rejuvenation intrinsic to the reprogramming of adult cells arises as a confounding factor to be controlled for.

6. Are ageing astrocytes reactive or senescent?

Healthy brain ageing is not pathological and may be defined as an adaptive evolution of global cell physiology over time.[85] Aged human brains display only mild and heterogeneous changes in astrocyte morphology or GFAP levels.[86] Studies in rodents document region-dependent, often contradictory changes in ageing 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-positive astrocytes.[87-89] Notably, ageing 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 ageing mouse brain.[85, 92] Thus, although the data suggest complex changes in ageing astrocytes, the evidence is not yet sufficient to qualify astrocytes as being *bona fide* reactive during physiological ageing. 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 ageing astrocytes across different CNS regions in humans, and comparison of physiologically aged and reactive astrocytes in various pathological conditions, is 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 markers $p16^{INK4A}$, increased β-galactosidase activity, and secretion of cytokines,[93] because the core definition of senescence (i.e., 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 ageing in astrocytes.

7. 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 mice.[78] 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 dyshomeostatic, while they may be successful homeostasis-preserving adaptations to disease.

We stress that the expression in full or in part of a pre-determined 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/wounded astrocytes" should be reserved for astrocytes with unequivocal morphological signs of damage (e.g., 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 (e.g. hypertrophic, atrophic), molecular markers (Table 1), 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.

8. Towards 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 signalling 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

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 basal[94] 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 being overlooked, particularly in snRNAseq. Translation from sc/snRNAseq data to *in situ* immunohistochemical detection and functional validations is far from trivial, because the molecular profiles of astrocyte clusters/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 phenotypes.[74] 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 FACS and cell-surface markers identified in screens.[71] 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 with *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 subsets.[40]

Signalling

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 microglia[72] 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 PD, to cite another example of *in vivo* inappropriate modelling. To complicate things further, the outcome of activating a signalling pathway may depend on the upstream stimuli[82] or priming caused by previous exposure to other stimuli,[97] perhaps through epigenetic control.[40] 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 biomaterials[99] are available tools to modify pathological signalling in reactive astrocytes for therapeutic purposes. Optogenetics[25] and Designer Receptor Exclusively Activated by Designer Drugs (DREADD)[25] are potential tools to manipulate reactive astrocytes, or restore their aberrant Ca^{2+} signalling observed in mouse models of neurodegenerative diseases.[53-55] However, it is unknown whether, and how, the changes in $Na^{+}/K^{+}/Cl^{-}/Ca^{2+}$ fluxes and second messengers triggered by these approaches[25] modulate signalling cascades driving phenotypical changes of reactive astrocytes (e.g., JAK-STAT and NF-κB pathways).[6]

Humanizing research

Although some basic functional properties of astrocytes have been shown to be evolutionarily conserved between humans and rodents,[100] 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 *post-mortem* samples and biopsies ([59], Fig. 1b), hiPSC-derived astrocytes, which can be generated with a fast protocol in 2D layers,[104] or integrated in 3D systems such as spheroids and organoids,[105-108] are rapidly becoming commonplace in basic research[11, 82] and therapy development.[109] 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 neurotrauma.[31] Biomarkers of reactive astrocytes in human disease will be indeed 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 (e.g. 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

Computerised 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 (e.g., -omics) can be transformed into mathematical maps by artificial intelligence,[118] 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/3D networks (Fig. 1c).[119, 120] Artificial intelligence can identify patterns of Ca^{2+} signalling 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.

9. 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 remodelling 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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Author contributions

AL, ASP, AVerkhratsky, AVolterra, CE, CS, EG, GC, GCP, JPO, and MVS participated in the initial discussion and drafted the outline. CE and EG prepared the Tables, and CE, EG, and MVS the figures. AL, ASP, AVerkhratsky, CE, CS, EG, GCP, and JPO wrote parts of the manuscript. EG and AVerkhratsky assembled a joint text with the help of CE and MVS. The manuscript was then edited by AL, ASP, AVolterra, CS, GC, GCP, and JPO. The rest of the authors fact-checked, improved accuracy, and provided content that was integrated by CE, EG, AVerkhratsky, and MVS, and validated by AL, ASP, AVolterra, CS, GC, GCP, and JPO. The manuscript was circulated several times among all the authors until no mistakes or inaccuracies were detected, and no disagreement was expressed by any author. All authors have approved the final version of the manuscript.

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Figure legends

Figure 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. Bar size: 15 µm. Unpublished image from Drs. Sirko and Götz.

b. Human cortical protoplasmic astrocytes in a surgical specimen injected with Lucifer yellow (arrow, injection site) that traverses the gap junctions into neighbouring astrocytes. Bar size: 45 µm. 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] Colours indicate AQuA events occurring in a single 1-sec frame of a 5-min movie. Bar size: 10 µm.

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. Bar size: 20 µm. Adapted from [121].

e. ScRNAseq in the remission phase of a mouse MS model reveals several transcriptional astrocyte clusters. These astrocyte sub-populations may be validated with spatial transcriptomics, as shown in f in an AD model. Adapted from [40].

f. Distribution of 87 astrocytic (green), neuronal (red), microglial (yellow), and oligodendroglial (blue) genes as shown with *in situ* multiplex gene sequencing in a coronal section from a mouse AD model. The method 'reads' barcodes of antisense DNA probes that simultaneously target numerous mRNAs. Bar size: 800 µm. Boxed area is magnified in bottom image, showing $6E10^+$ amyloid-β plaques (white, arrows). Adapted from [96].

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**) (e.g. 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 (**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/sample) obtained in different contexts (**b**), depicted in different colours. 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) falls in the V1-3 space. The analysis can be n-dimensional, but for visual clarity, we show a 3-dimensional 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/plasma (neurotrauma biomarker)	122
Nestin	Intermediate filament	↑ mRNA & protein	AD, AxD, MS, spinal cord injury, 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 signalling – Transcription factors						
NFAT	Transcription factor	↑ mRNA, protein, nuclear translocation	AD, TBI, PD	Hu, Ms	Links Ca^{2+} signalling 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 signalling, 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						
EAAT1 & 2	Glutamate transporters	↓ mRNA, protein and uptake	ND	Widespread	May be also detected in some neurons	53, 132
KIR4.1	K^+ channel	↓ mRNA & protein	Widespread	Hu, Ms	May or may not translate into alteration of K^+ buffering	58

Abbreviations used: AD: Alzheimer's disease: ALS: amyotrophic lateral sclerosis; AxD: Alexander disease; BDNF: Brain-derived neurotrophic factor; CSF: cerebrospinal fluid; HD: Huntington's disease; Hu: human; LOAD: late onset AD; MS: multiple sclerosis; Ms: Mouse; ND: neurodegenerative disease; NO: nitric oxide; PET: positron emission tomography; PD: Parkinson's disease; Rt: rat; SCI: spinal cord injury; TBI: traumatic brain injury.

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 Section 3), 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 the table will be part of signatures defining disease-specific or core markers of reactive astrocytes, as well as astrocyte-based fluid biomarkers (see Section 8). 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.

Table 2. Potential functional assessments for reactive astrocytes		
Function/Phenomenon	**Potential readouts**	**Ref**
Ca^{2+} signalling 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** **Glutamate uptake and conversion**	Detection of neuroactive factors using fluorescent sensors and *in vivo* two-photon imaging Quantification of neuroactive factors in extracellular milieu and CSF (FRET, HPLC, CE-LIF, fluorescent sensors like GluSnFR, enzymatic kits)	25
	Analysis of glutamate currents (electrophysiology) and/or transporter content (immunoblot, immunostainings)	109, 132
	Metabolism of ^{13}C-labeled substrates (GC-MS & HPLC)	133
Astrocyte inter-cellular connectivity	Diffusion of permeant dyes in astrocyte networks (patch-clamp & imaging), FRAP	59
Vascular coupling **Maintenance of BBB integrity**	Assessment of vascular responses after Ca^{2+} uncaging or optogenetic stimulation of astrocytes (two-photon imaging, optical intrinsic imaging, MRI)	134
	Assessment of BBB permeability with detection in the parenchyma of blood proteins or dyes (Evans blue, Dextrans)	135
Signalling **Transcription factor activation**	Standard biochemical assays. Signalling manipulation by DREADDs Transcription factor translocation and DNA binding assays, chromatin immunoprecipitation, reporters	25, 109, 136
Production of synaptogenic and neurotrophic factors, ECM, cytokines, chemokines	Synapse quantification *in vivo* and upon exposure to astrocyte-conditioned media *in vitro* Proteomics/metabolomics of astrocyte-conditioned media and acutely sorted astrocytes Multiplex ELISA assays, immunostainings	72, 97
Interactions with neurons, oligodendrocytes, OPC and microglia	*In vivo/ex vi*vo analyses, co-cultures or exposure to conditioned media and assessment of function/survival	58, 72, 82
Glycolysis **Fatty-acid oxidation** **Lactate production** **Glycogen metabolism** **Mitochondrial respiration**	Metabolism of $^{3}H/^{14}C/^{13}C$/- labelled energy substrates (GC-MS, radioactive assays, NMR)	133, 137
	Glucose, pyruvate, lactate and ATP quantification with genetically-encoded fluorescent sensors and *in vivo* two-photon imaging	138, 139
	Lipid-droplet and fatty-acid staining with BODIPY dyes	140

	NADH imaging (FLIM)	141
	Activities of electron transport chain complexes Extracellular acidification, oxygen consumption (Sea Horse, voltametry)	141
	Quantification of glycogen granules by EM or immunostainings	142, 143
NO-ROS production/detoxification	NO/ROS imaging with intra/extracellular fluorescent sensors or probes Immunostaining for oxidized residues Activity of antioxidant enzymes with commercial kits	33, 144
Endolysosomal system	Detection of phagocytosed materials (array tomography, EM, 2 photon microscopy) Uptake of myelin debris or labelled synaptosomes	60, 72, 145
	Autophagic flux	81, 146
	Exosome production	80, 147
	Proteasome/lysosome proteolytic activity (fluorescent probes)	148
Proliferation	BrdU incorporation Ki67, PCNA, cyclin labelling (calculation of a proliferative index, i.e. % of positive cells in the population) Characterization of astrocyte progeny by fate mapping	149, 150
Scar-border formation	Morphometric/functional analyses (e.g. composition, permeability to immune cells)	131
Abbreviations used: BBB: blood-brain barrier; BrdU: bromodeoxyuridine; CE-LIF: capillary electrophoresis with laser induced fluorescent detection, CSF: cerebrospinal fluid; DREADD: designer receptor exclusively activated by designer drugs. ECM: Extracellular matrix; 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; NO: nitric oxide; NMR: nuclear magnetic resonance; OPC: oligodendrocyte progenitor cells; PCNA: proliferating cell nuclear antigen; ROS: reactive oxygen species.		

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.

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 (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

4. 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.
5. The specifics of the astrocytes under study should be spelled out in titles, abstracts, and results of articles (e.g., X-positive astrocytes in Y region showed Z phenomenon).
6. Multivariate and clustering analysis of molecular and functional data will facilitate the identification of distinct phenotypes of reactive astrocytes (Fig. 2).
7. Local, regional, temporal, subject/patient, and sexual heterogeneity of reactive astrocytes should be studied (Fig. 2).
8. The discovery and validation of plasma/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.

Figure 1

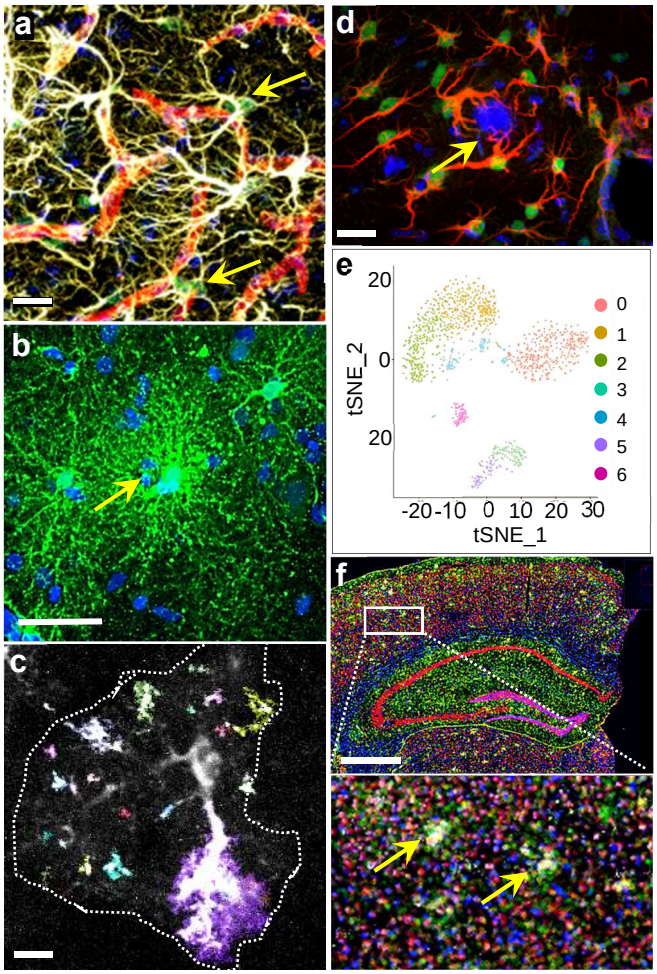

Figure 2