

Review

# The Relevance of Astrocytic Cell Culture Models for Neuroinflammation in Neurodegeneration Research

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**Abstract:** Astrocytes are the predominant glial cells that provide essential support to neurons and promote microenvironment changes in neuropathological states. Astrocyte and astrocytic-like cell culture have substantially contributed to elucidating the molecular pathways involved in key glial roles, including those relevant to neurodevelopment, brain physiology and metabolism, which are not readily accessible with traditional approaches. The in vitro methodology has also been applied to neuroinflammatory and neurodegeneration contexts, revealing cellular changes involved in brain dysfunction. Astrocytes studies in culture started with primary cell approaches using embryonic and postmortem tissue. Further developments included newborn rodent primary cells, cell lines and immortalized astrocytes, which resulted in homogeneous cell-type preparations grown on flat surfaces. To overcome some in vitro shortcomings, tridimensional bioprinted models and organoid culture enabled the mimicking of tissue cellular arrangements and, above these achievements, complex astrocyte cell culture can be generated from induced pluripotent stem cells (iPSCs) to model diseases. These unprecedented breakthroughs allowed the development of platforms to test new therapies in brain cells derived from human material noninvasively obtained from live patients. In this work, we reviewed the most studied astrocytic cell models for discussing limitations, advantages and reliable experimental readouts for neuroinflammation in neurodegeneration research.

**Keywords:** alternative model; astrocyte; cell lines; cell culture; inflammation; innate immune response; neurodegenerative disease



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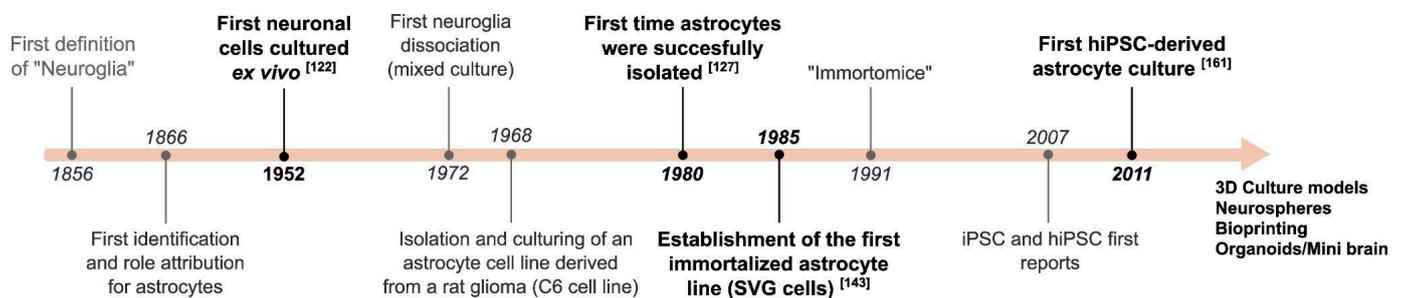
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## 1. Introduction

Glial cells are non-neuronal cells initially described during the 19th century as connective brain elements, which were not thought to be cellular but capable of keeping the brain tissue together [1–3]. The name derives from their adhesive (glue-like) properties. Initially, the development of knowledge on glial cells was overshadowed by the research on neurons because these are the electrically excitable cells that convey neurotransmitter-based signals in distinct brain circuits [4]. One outcome of this neuronal-centered perspective is that a simplified supporting and protective role has been attributed to the glia for a long period. Astrocytes are well known for providing metabolic and trophic signals to neurons, while forming a blood barrier when associated with neurovascular unity. Oligodendrocyte function is traditionally understood as axon insulation to promote saltatory fast conduction. Microglia are usually defined as immune cells that protect neurons from potential pathogens [5]. All these glial cell types have more elaborate and complex roles in the CNS, and astrocyte research has consistently shown that these cells are versatile, essential to normal brain function and can play important roles in brain diseases by impacting the function of neurons, oligodendrocytes and microglia.

The notion of glia heterogeneity became clear when the star-shaped cells were named astrocytes [6] and, later, cells with other morphologies and characteristics were identified

as oligodendrocytes and microglia [7–12]. Golgi made important initial descriptions of astrocytes, including the end-feet glia-vascular contacts. Later, Cajal further developed an investigation technique that employed the gold chloride-sublimate staining, which is specific for both protoplasmatic (gray matter) and fibrous (white matter) astrocytes. These and other developments resulted in particular attention to astrocytes during those years [13–20]. Once it was understood that astrocytes represent a distinct cell type, one of the challenges was to isolate these cells to study them apart from the other neural cells (see Figure 1 for a timeline). Here, we review how *in vitro* experimental approaches with astrocytes provide the means to dissect and test hypotheses on cell-autonomous mechanisms, and we evaluate real-time cellular events in different conditions, including physiological and inflammation models related to neuropathologies. While addressing these aspects, we also present examples of how these models have been applied in studying conditions related to neurodegeneration to illuminate their potential contributions to understanding neuroinflammation and broader neurological disorders.



**Figure 1.** Timeline of major developments in astrocytic cell culture. Major breakthroughs and milestones related to astrocyte cell culture are given in chronological order.

Microglia and astrocyte reactivity is frequently associated with neurodegenerative diseases, including those that feature protein misfolding and aggregation. Although inflammation is not considered an initiating factor in neurodegenerative diseases, it has been found that neuroinflammation occurs earlier than protein aggregation. In addition, the literature suggests that sustained and deregulated inflammatory responses involving microglia and astrocytes contribute to disease progression. The relationship between inflammation and neurodegenerative diseases have been substantially reviewed elsewhere [21–23]. Therapeutic approaches aiming to modulate neuroinflammation in neurodegenerative diseases depend on mechanistic research that dissects precise components of cellular responses, which is currently investigated in astrocytes through several approaches.

At the time of writing this paper, almost 77,000 articles in the national library of medicine ([pubmed.gov](https://pubmed.gov) or [www.ncbi.nlm.nih.gov/pubmed/](https://www.ncbi.nlm.nih.gov/pubmed/); accessed 21 February 2024) were retrieved with "astrocyte" as the search term, nearly 8000 articles when the term "cell culture" was conditionally added to the search as the obligatory (AND) term and almost 562 articles were retrieved when combining these two terms with "inflammation". Unfortunately, we are unable to list all potential and relevant work here. When using MeSH terms "((astrocyte[MeSH Terms]) AND (cell culture[MeSH Terms])) AND (inflammation[MeSH Terms])", the search returned 50 results that were prioritized for selection in this review along with other search strategies. According to official protein/gene symbol notation, we adopted the use of all uppercase nonitalicized letters in abbreviations for proteins (without special characters or hyphen), all uppercase letters italicized for human gene and transcript and only first uppercase and italicized letters for rodent genes/transcript.

## 2. A Brief Overview of Astrocytes General Features and Physiological Roles

The descriptive approach in the astroglial biology field predominated until the development of purified astrocyte cultures and astrocytic cell lines. However, one of the earliest perceptions from morphological studies led to an important hypothesis that glia drive the connections between themselves and blood vessels, in addition to their participation in

cellular interactions that occur during brain development [24–29]. Intriguingly, a recent study estimated that 99.8% of all gray matter astrocytes are connected with blood vessels, thereby reinforcing their role in metabolic supply [30].

Revised concepts on the importance of glia in brain functions have been claimed in recent decades, in part due to the popularization that such cells, mainly astrocytes, outnumber human neurons by one order of magnitude. However, detailed literature review and modern cell count techniques, including unbiased stereology and isotropic fractionation, showed that this is not the case. Glia do not extensively outnumber neurons in general, being less than 2:1 in the human cortex and around 3:1 in the spinal cord [31–33]. While cell number is not equivalent to cell importance, it is relevant to understanding how neuron-to-glia ratio changes in different species (a higher proportion of astrocytes according to increased brain size), regions (e.g., ~12:1 in the posterior gray matter of the thoracic human spinal cord) and during development and pathological states [34]. Hence, we are tempted to suppose that astrocyte functions in nutrient supply and energetic metabolism should be critical in species with larger brains, which could explain the higher proportion of astrocytes. However, it is not clear what the precise role is for different astrocyte tissue densities in normal physiology.

Astrocytes play critical roles in CNS homeostasis because, in addition to supporting neuronal function, these cells also influence neurotransmission and other glial cells. The physiological activation of the astrocytes involves calcium propagation (calcium waves), a mechanism that allows cellular communication with other astrocytes through gap junction channels; or pre- and postsynaptic neurons, forming the tripartite synapse by regulating neurotransmitters glutamate and GABA uptake and synthesis [16,35–45]. In addition, astrocytes are implicated in brain tissue waste drainage by fluid exchange from the cerebrospinal fluid (CSF) compartment to the interstitial fluid through astrocytic aquaporin 4 (AQP4), which is essential for normal function of the glymphatic system [46], a newly described waste clearance system. Toxic residues, such as A $\beta$ -amyloid peptides and the Alzheimer's disease (AD) pathology-associated tau protein, for example, are removed from the brain by the glymphatic system. Local homeostasis at Ranvier nodes, the site of saltatory conduction of action potentials, and oligodendrocyte normal functions, also involves astrocytes that modulate blood flow through vasoactive nitric oxide (NO) and arachidonic acid, and provides energetic metabolites glucose and lactate. Astrocytes projections called end-feet form remarkable associations with the vascular endothelial cells, determining the formation and maintenance of the blood–brain barrier (BBB). These interconnections are included in the structuring of the outermost glial limiting membrane (glia limitans), which insulates the brain parenchyma from the vascular and subarachnoid compartments [47–49].

Molecular markers immunostaining successfully allow the identification of astrocytes. Although these cells represent a heterogeneous group, they consistently express glial fibrillary acidic protein (GFAP). This intermediate filament protein is involved in cell motility and structural stability, and is highly expressed during brain damage and other insults. Other markers include another intermediate filament protein, nestin and brain lipid binding protein (BLBP) in the case of immature astrocytes. Mature cells express calcium-binding protein S100 $\beta$ /S100B, excitatory amino acid transporters EAAT1/GLAST-1/SLC1A3 and EAAT2/GLT-1/SLC1A2, glutamine synthetase (GS), aldehyde dehydrogenase ALDH1L1, AQP4 and gap junction components connexin 30 (Cx30/GJB6) and Cx43/GJA1 [50–60]. These molecules are indeed useful for identifying astrocytes, and their functions are well documented in key functional aspects of astrocytic cells. We highlight that these markers may not be used unequivocally for every occasion. *GFAP* gene expression is usually observed in white matter fibrous astrocytes, and its expression in radial glial progenitor cells makes it less specific [61]. *S100B*, in contrast, has shown to be predominantly expressed in gray matter protoplasmic astrocytes, and in differentiating oligodendrocytes [62].

Astrocyte markers should ideally reflect their relevant molecular functions and can be selected and/or used in combination to increase specificity and label most types of astrocytes in different contexts. A recent study profiled astrocyte density and molecular

signatures in different murine brain regions using fluorescent labeling driven by *Aldh111* promoter, which suggested that neuron-to-astrocyte ratios change because of neuronal density, and not because of astrocyte cell numbers. Although astrocyte density is quite homogenous and correlated with endothelial cell numbers, transcriptional signatures point to the existence of molecular features and functions specific to brain regions [63]. In the future, cell markers, alongside neuroanatomical and functional information, will have to be combined for proper evaluation of the varied spectrum of astrocyte physiology. These recent developments on astrocyte heterogeneity will also impact the design, interpretations and perception of limitations for studies conducted in vitro.

### 3. Reactive Astrocytes and Pathological States

The role of astrocytes in neuropathologies has been intensively studied, including neuroinflammation, neurodegenerative diseases and neurodevelopmental disorders. The mechanisms by which astrocytes intensify or cause detrimental effects to brain elements include: (1) loss of tissue homeostasis control; (2) disease progression by transduction of inflammatory signals into a changed cellular microenvironment; and (3) secretion of molecules that damage or affect other brain cells. Much of the knowledge on astrocytes behavior in pathological states is related to a reactive phenotype, which will be discussed in the following paragraphs.

Escartin et al., in a review article signed by leading research groups, discussed that the terminology for astrocytes in pathological states is not well defined [64]. Indeed, the confusing terminology in the literature, and the lack of validation of in vitro findings using in vivo models, render the comparisons between studies difficult and challenging. According to that review work, special attention should be given to the following terms: “reactive astrocyte”, “astrocytosis”, “reactive gliosis”, “astrogliosis”, “astrocyte activation” and similar. Following the terminology consistency recommendation, in contrast to “astrocyte activation”, which refers to more physiological contexts, we will apply the terms “reactive astrocytes”, “reactive astrogliosis” and “astrocyte reactivity” when referring to a pathological state, or a model that simulates a pathological context.

In vitro studies designed to mimic disease states are also capable of displaying astrogliosis, including increased chondroitin sulfate proteoglycans (CSPGs), which is the hallmark of this process, along with other features of reactive astrocytes [65–69]. In severe pathological states, it is well recognized that intense astrogliosis takes place during brain injury, infection and neurodegenerative processes in general. The increased astrocyte density and other relevant changes in pathological states, including cell shape, size and function, determine profound changes in brain metabolism and reactive defense mechanisms, such as the glial scar discussed below [64,70].

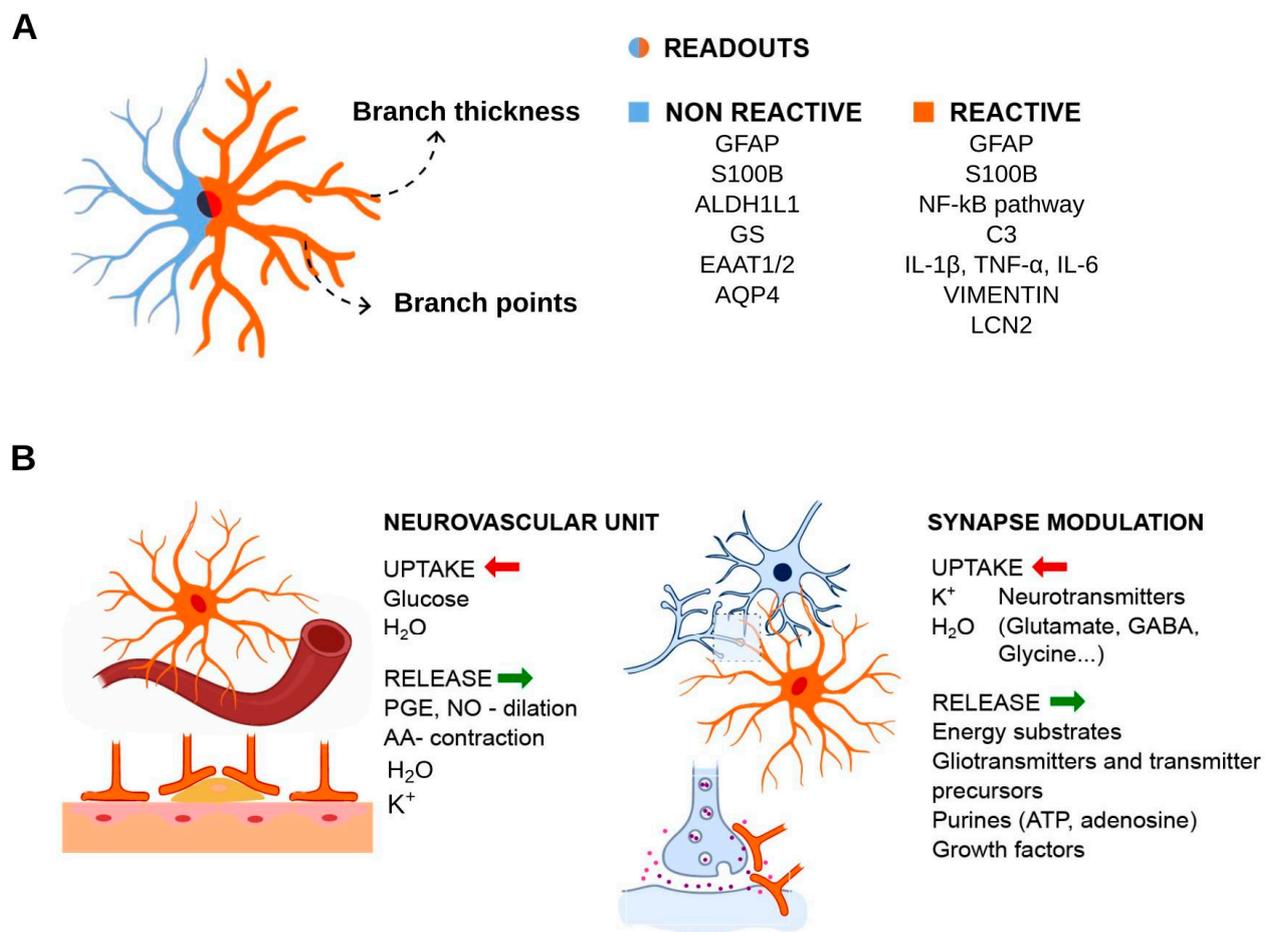
Reactive astrogliosis and certain astrocytes phenotypes can contribute to progressive neuronal damage as a consequence of a proinflammatory state (called A1-, or A1-like astrocytes, in former literature) and from the loss of the protective and prosurvival functional activities typical of A2-like cells (nomenclature also employed here to match common terminology). The bias toward the “reactive” astrocyte can be triggered by varied stimuli such as injury, pathogens, toxic molecules and so on [71–74]. It is beyond the scope of this review to discuss reductionism of cell state interpretations, and whether astrocytes reactivity leads solely to the secretion of inflammatory mediators and the increase in potentially harmful molecular species. As one may expect, this concept also claims that these cell changes are detrimental to neuronal elements, due to a decreased release of beneficial and neuroprotective astrocyte-derived factors/metabolites (reviewed in [64,75–77]). While we refrain from a simplified view of astrocyte states, we observe that the literature frequently interprets astrocyte responses as a dichotomized polarization. This limitation is often present in cell culture studies as well.

The complete understanding of reactive astrocytes and astrogliosis depends on the observation and analysis of other components of the brain environment. In contrast to the experimental setups that aim to approach the entire scenario, cell culture techniques

have contributed to understanding the cell-autonomous mechanisms by which astrocytes modify their functions under noxious stimuli. Different paradigms have shown that astrocytes participate in neurotoxic events; for instance, mediating neuronal cell death upon exposure of A $\beta$ -amyloid peptide from AD, or by secreting proteins that reduce neuronal survival in models of neurodegeneration or neurodevelopmental diseases, such as Fragile X syndrome, Rett syndrome and Down syndrome [78–80]. For more information on the different neurodegenerative diseases in which astrocytes play critical roles, we suggest dedicated reviews [81–83].

#### 4. Experimental Readouts for Reactive Astrocytes in Inflammation Context

As summarized in Figure 2, the analysis of astrocyte reactivity relies greatly on the activity/expression of astrocyte markers, a few metabolic enzymes and transporters, pro-inflammatory molecules and components of the extracellular matrix (ECM).



**Figure 2.** Meaningful metabolic, gene expression and morphological changes in astrocytes. (A) Typical readouts related to astrocyte identification and those associated with reactive astrocytes. (B) Physiological astrocyte functions in barrier formation and tripartite synapse.

In experimental settings for neuroinflammation research, pathogen-associated molecular patterns (PAMPs), such as Gram-negative bacteria lipopolysaccharide (LPS), have been widely employed to engage *toll*-like receptors (TLRs) and promote proinflammatory gene expression in the brain or in cell culture. Shortly after TLRs characterization in the very early 2000s, it was demonstrated that the effects of LPS infusion in the brain depend on its cognate receptor TLR4 [84]. Lenhardt et al. corroborated this when reporting that astrocytes and oligodendrocytes primary cultures do not express significant amounts of TLR4; instead, only microglia cells express it and are a required factor to promote damage induced by

LPS [85]. In a dedicated review a few years later, Saura questioned the literature regarding NO production by astrocytes stimulated with LPS, arguing that small microglial contaminations are often present in astrocyte primary culture preparations, which accounts for a dramatic effect in response to LPS [86]. In consequence, NO and other proinflammatory molecules may not be produced by astrocytes.

Even though contaminating microglia is an alarming issue in cell culture, several studies continued to report evidence that astrocytes are fully capable of mounting innate immune responses through the activation of different signaling pathways [87]. These findings were contested by experiments conducted with primary cultures treated with liposomal clodronate, which reduced microglia and blunted proinflammatory cytokines interleukin 1  $\beta$  (IL-1 $\beta$ ; protein symbol: IL1B) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ; protein symbol: TNF) upon IL6 stimulation [88]. In addition, microglia depletion with a CSF1 receptor inhibitor (PLX-5622) successfully reduced microglia content in astrocyte/microglia mixed cultures. These microglia-depleted cultures were incapable of producing TNF when stimulated with LPS, but maintained partial induction of IL6 [89]. Another CSF1R inhibitor (PLX-3397) was also able to deplete microglia from primary astrocyte culture [90], and is a promising tool, along with other CSF1R inhibitors, to accurately evaluate astrocyte responses in inflammatory conditions.

While microglia is naturally in contact with astrocytes, one must note that microglia in standard culture conditions lose their phenotype [91]. Stimuli that trigger contaminating microglia reactivity and proliferation pose a difficult problem regarding the evaluation of genuine astrocytes responses. This contributes to the finding that microglia secreted factors (C1q, TNF and IL1A) cause robust reactivity of astrocytes [92]. Overall, these observations indicate that IL6 is a suitable cytokine to evaluate astrocyte reactivity and has been known to have functions related to astrocytes for quite a long time [93]. Other transcripts have been identified as pan reactive (*Gfap* and vimentin/*Vim*), neuroinflammatory-specific (A1/A1-like), or ischemic-specific (A2/A2-like), discriminating phenotypes and highlighting complement C3 as a good marker of A1/A1-like cells in neurodegenerative diseases [92]. Also of note, oncostatin M receptor (*Osmr*), ceruloplasmin (*Cp*) and lipocalin 2 (*Lcn2*) could be sensitive markers of astrocyte reactivity; in fact, these transcripts show major induction in brain tissue infused with LPS (as well as other transcripts classified as A1- or A2-specific), and *Osmr* induction presents a relative selectivity for astrocytes [94,95]. A well-studied mechanism by which astrocytes respond to inflammatory stimuli, pathological and/or aging conditions is the release of extracellular vesicles (EVs), promoting neuroprotection and neuronal survival in response to anti-inflammatory cytokines, such as IL10 [96,97]. On the other hand, under pro-inflammatory stimuli, such as IL1B or TNF, secreted EVs can promote inflammatory signal transmission, as well as neuronal plasticity and modulation [97–99]. The presence of the inflammasome NLRP3 and NLRC4 expression was observed in both human and mouse astrocytes [100] and NLRP2 only in humans [101]. Especially when related to certain disorders, such as demyelination, multiple sclerosis (MS), AD, amyotrophic lateral sclerosis (ALS) and traumatic brain injury [72,100,102–104], culture models were extremely important to some of these findings.

Other readouts were studied in the context of brain damage or neurodegenerative diseases, including CSPG production in response to transforming growth factor beta (TGFB1/2/3) signaling, which resulted in glial scar formation [105–107]. Loss of AQP4, which impacts cerebrospinal fluid homeostasis, is also a relevant index of pathological changes in astrocytes relevant to barrier function [108,109]. Morphological changes, calcium signaling, and metabolic analytes (ATP levels, reactive oxygen species, synthesis and uptake of neurotransmitters) present changes in models of AD, Parkinson's disease (PD) and ALS [75,110].

## 5. Astrocytic In Vitro Models

When selecting an in vitro model, it is critical to consider the advantages and limitations to provide plausible answers to the investigated biological question. The most used

cell culture models are cell lines, primary cell culture, immortalized astrocytes (IA), differentiated induced pluripotent stem cells (iPSCs), 3D culture, bioprinting and organoids/mini-brains. We reviewed the most common astrocytic cell models and evaluated their limitations and advantages (see Table 1), along with the reported outcomes (Table 2). In addition, we show some examples of studies conducted with astrocyte primary culture in Table 1. The outcomes from the analysis of different astrocytic cell culture types, especially in the case of convergent results, provide powerful means to establish specific roles of the most abundant glial cell type in neuroinflammation and neurodegeneration research.

**Table 1.** Comparison between the different cell culture techniques for astrocyte research.

Culture Type Cell Line	Main Advantage	Limitations
Primary culture	Low cost and simple methodology. Disseminated methodology that is comparable to literature.	Usually employs serum (not obligatory) and is susceptible to induction of reactive phenotypes. High percentage of cellular senescence with passages; low proliferation; difficult to scale up; prone to contamination.
Cell lines	Easy to obtain (many are commercial); expandable; allow several passages; easy to manipulate; reduces animal usage	Cancer cell phenotype; expression profile differs from in vivo astrocytes.
Immortalized astrocytes	Intended to keep primary cell characteristics; less cell death and increased resilience to passages, freezing/thaw; easier to manipulate	Similar to primary cell culture; genomic integration that promotes cellular changes
iPSC-derived astrocyte	Relevant to disease studies (derived from patient); can be grafted; more similar to in vivo; reduces animal usage; good proliferation; allows differentiation into astrocyte types; serum-free	High cost; long-term culture; can present mutations
Bioprinting	Robotized; standardized dimensions; improves ECM	Expensive; delicate constructs; demands bioink characterization
3D culture/organoids	Allows the analysis of complex interactions between different cell types; mimics in vivo conditions; cell heterogeneity	High cost; difficult to reproduce results, cell death in the organoid core; difficult nutrient diffusion

**Table 2.** Readouts in different astrocyte culture models (neuroinflammation studies).

Astrocyte-Related Readouts	Primary Culture	Cell Lines	Immortalized Astrocytes	iPSC-Derived Astrocyte	Bioprinting	3D Culture and Organoids
GFAP, S100B, Vimentin	[88–92,111,112]	[113,114]	[115–117]	[118–120]	[121–123]	[124] (GFAP only), [125]
C3, AQP4, ALDH1L1, CD44	[92,111,112]	-	[115,117]	[118–120]	-	[126]
GS, GLT-1, GLAST	[111]	-	[115,117]	[118]	-	[126]
Metabolic assays (e.g., glutamate clearance/uptake, lactate and glucose uptake)	[111]	-	[115]	-	-	-
Pro-inflammatory (e.g., NFκB, TNF-α, IL-1α, IL-1β, IL-6)	[87,88,91,93]	[113,114,127]	[116,128]	[119]	[123]	[126]
Anti-inflammatory (e.g., IL-10)	-	-	-	-	-	[126]
Oxidative stress (e.g., ROS production, iNOS, DDIT3/CHOP *)	-	[113,114,127]	[116]	-	-	-
Intracellular calcium	-	[113,114]	-	-	-	-

\* DDIT3/CHOP: C/EBP homologous protein 10.

### 5.1. Primary Cell Culture

Brain cell culture procedures started with studies of neuronal development, perhaps because nerve cell biology was one of the most favorable subjects to be investigated under

the microscope in the early 20th century. In fact, the hanging drop technique developed by Ross Harrison in 1910 showed for the first time that it was possible to culture neurons outside the body. This breakthrough highly influenced similar applications for numerous cell types (Figure 1), and subsequent improvements like culture flasks, culture chambers and, more recently, microfluidic devices and others refined neuroscience studies *in vitro* [129]. Human and other mammalian neuroglia observation in cell culture dates from the 1930s, and possibly earlier, as reviewed in a study by Mary Jane Hogue [130]. However, conditions that made it possible to enrich astrocytes were developed later. For instance, dissociated human brain cells from a fetus gave rise to astrocytes and radial glia cells formerly known as spongioblasts [131,132]. This preparation was successfully used to investigate astrocyte transformation by an oncogenic DNA virus, for example [133]. In the 1970s and early 1980s, it was shown that a newborn rodent brain could be used to obtain macroglia [134], and McCarthy and Vellis successfully separated oligodendrocytes from astrocytes by driving sheer forces through shaking to obtain a purified primary cell culture expressing GFAP with typical astrocyte morphology [135–137]. These purified preparations represented key methodologies to characterize metabolic and electrophysiological properties of the astrocytes, including the transport of excitatory neurotransmitter glutamate ([82], reviewed in [83]) which is converted to glutamine because mature astrocytes in culture express glutamine synthase (GS) [138–140]. There is also *in vitro* evidence that astrocytes are metabolically involved in ionic and osmotic homeostasis in the brain [141]. Subsequently, it was found that glutamate cotransport with sodium in astrocytes stimulates aerobic glycolysis, consequently with lactate production, which mediates metabolic coupling between neuronal activity and glucose consumption by the brain [142]. With that in mind, at least four relevant biochemical parameters to brain function can be measured from cell culture material due to astrocyte metabolism: glucose consumption, lactate production, glutamate uptake and glutamine synthesis. Primary culture of combined cell types can also help disclose mechanisms of cell–cell interactions, like those involved in the formation of glia limitans, which is important to BBB [143]. In addition, the study of astrocyte function in neurodegenerative diseases has been improved with primary culture techniques. MS, an autoimmune disease mediated by the demyelination of the CNS, is an example of a degenerative disease where astrocytes play both inflammatory and protective roles, secreting chemokines and promoting cell migration through the BBB, but also favoring CNS remyelination by promoting the differentiation of oligodendrocyte precursor cells. These mechanisms have been well studied by different groups utilizing not only *in vivo* models [144] but also primary cultures of astrocytes and co-cultures with microglia [100,145,146].

Much of the discussion on primary cell culture, microglial contamination and neuroinflammatory analysis has been presented in the Experimental Readouts for Reactive Astrocytes in an Inflammatory Context Section (see above). In fact, primary cell culture of astrocytes is the most used model *in vitro* to evaluate how these cells respond to inflammation.

Although it is not possible to promptly translate *in vitro* findings into physiological astrocyte function in brain tissue, primary cell culture data not only enabled exploring new mechanisms and cellular features, but also provided proof of concept that is very difficult to show *in vivo*. It should be noted that much of the knowledge discussed in this section was reported before the massive development of genetic tools available today to work *in vivo* (transgenics, gene editing, viral delivery, conditional and cell-specific DNA recombination, etc.). Primary culture from transgenic and knock-out rodent models increased the possibilities to expand our knowledge on gene function in astrocytes.

## 5.2. Cell Lines

Cell lines are essential for the development of the cell biology field. The advantages of using cell lines are numerous: stable and reproducible preparations within certain passage numbers; cells are handled easily and in a short time they expand to large quantities, sparing numerous animals from being used in experimental research; and the cells are usually available in cell banks so there is a widespread use and it is easier to compare

results worldwide. Several studies related to astrocytes have been conducted with cell lines, helping to unveil their cellular functions in the CNS [147–151]. As we will discuss, there are more limitations concerning the use of cell lines in terms of representation of brain astrocyte functions in vivo.

One of the most used cell lines for astrocyte research is C6 rat astrogloma cells. These cells were isolated from rat glioma induced by N-nitrosomethylurea with verified relative preservation of the expression of typical astrocyte markers such as GFAP, glutamate transporter, S100B and GS [152]. The expression of genes typical of brain tumors such as PDGFb, IGF-I and EGFR was also observed in C6 cells [153]. The ambiguous nature of these cells, sharing both astrocytic and tumoral origins, clearly limits the interpretation of data that could be relevant to astrocyte physiology, and that accounts for the fact that C6 are also used in cancer research for glioma and glioblastoma, including new potential drug testing.

The advantages and limitations of the cell lines must be carefully considered while planning the experiment design, because chromosome stability, cell growth, metabolism, oxidative stress resistance and susceptibility to cell death are impacted by the cancerous transformation [152,154]. These limitations may not be present for other types of cell culture listed below.

C6 cells are responsive to LPS and capable of inducing *Il6*, *Tnf*, GFAP, ciclo-oxygenase 2 (PTGS2/COX-2) and inducible nitric oxide synthase (NOS2), although at a very high concentration (10 µg/mL) [113]. In addition, the PD-inducing neurotoxin 1-methyl-4-phenyl 1,2,3,6 tetrahydropyridine (MPTP), or ferrous sulfate that induces oxidative stress, also increases proinflammatory molecules [114,155]. These studies were conducted by the same research group, and it will be interesting to verify the capacity of astrocytic cell lines to respond to and promote inflammatory reactivity in comparison to other models. In this direction, it was shown that Aβ<sub>25–35</sub>-amyloid peptide is able to induce the expression of proinflammatory molecules in C6 cells [127].

### 5.3. Immortalized Astrocytes (IAs)

To complete studies involving many treatments, replications and validations, in addition to combining results acquired over years, it is important to preserve the cells' morphologic, proliferative and metabolic patterns between passages. This is usually difficult to achieve with primary cell culture, which presents limited cellular replication and capacity to preserve gene expression and morphology [152]. Immortalization is a suitable solution for this problem.

Cell immortalization can be performed through oncogenes expression, namely hTERT (human telomerase reverse transcriptase) and SV40 large T antigen; one promotes telomere extension during replication and the other interferes with cell-cycle control, respectively. Telomeres shortening during each cell-cycle is a consequence of the linear nature of chromosome endings, and hTERT efficiently replenish the lost DNA fragments, while large tumor antigen interacts and inactivates tumor suppressors p53 and retinoblastoma-protein (pRb) [115,156–160].

Since varied cell lines were generated by immortalization, it is important to observe the different strategies employed in each case, which can impact the choice for a particular astrocytic cell. Immortalization with SV40 large T antigen was achieved in cells from a human fetus brain in 1985. Unlike former experiments with SV40 infection [133] that provided cells with limited passage, the employed strategy consisted in transfecting origin defective SV40 DNA into glia cells, so that the virus would not replicate, which resulted in an increased ratio of replication and survival of the cells called SVG [156]. Similar results of immortalization were observed in other subsequent studies; for instance, HSC2/HSC6 cells using temperature-sensitive mutant of SV40 large T antigen [161], A735 cells also using SV40 large T antigen [162], NHA-E6/E7/hTERT cells using telomerase [163], NHA/TS cells using SV40 early region [164] and C8-D1A [165]. These models have been widely

employed, including preclinical studies and BBB modeling [166–168]. It is worth noting that IAs from different species can be acquired through commercial platforms.

Another approach to generate immortalized cells involves obtaining a primary cell culture from transgenic animals designed for this purpose. Liu and colleagues were able to isolate immortalized astrocytes from murine optic nerves [169]. The transgenic strain expresses the temperature sensitive SV40 large T antigen mutant tsA58 under the class I MHC H2kb promoter, leading to ubiquitous oncogene expression upon IFN $\gamma$  exposure [170]. For instance, astrocytes deficient in *Abcd1*-deficiency (X-linked adrenoleukodystrophy model) have been studied using this technology [171]. As will be discussed in the Section 7, these immortalization techniques can produce cells with similar and dissimilar characteristics compared to primary cell culture and, once again, this can impact interpretations and comparisons of the resulting experimental data.

IAs can replicate inflammatory responses observed with other in vitro models. A proteomic study compared the responses of immortalized human fetal astrocytes to high concentrations of IL1B, or TNF, or LPS, and concluded that several proteins involved in inflammation could be induced by these treatments but, unlike the cytokines, LPS failed to activate the canonical nuclear factor (NF)- $\kappa$ B pathway and induce IL6 [128]. The failure in IL6 induction by LPS is actually surprising considering primary cell culture and cell line data, and deserves further investigation. Cytokine treatment on murine IAs were also able to convert the cells to a neurotoxic phenotype that did not depend on inflammatory molecules released by the astrocytes [116], which reinforces the concept that these cells are sensitive to the proinflammatory milieu typical of neurodegenerative diseases. SV40 large T antigen transformed IAs from the hippocampus of 3xTg-AD and wild-type control mice were shown to display comparable features to primary astrocytes, except for diminished levels of GFAP and AQP4 [117]. Few inflammatory readouts were investigated in this study, but curiously NOS2 induction by LPS and TNF was present in these IAs, and astrocytes as a source of NO have been questioned before (see above). Overall, IAs represent a promising tool to conduct large-scale studies for neuroinflammation in combination with neurodegenerative disease models, although validation of cellular responses must be carefully evaluated.

#### 5.4. iPSC-Derived Astrocytes

Takashi and Yamanaka showed that somatic cells could be reprogrammed to pluripotency by the forced expression of four transcription factors: *Pou5f1/Oct-3/4*, *Sox2*, *cMyc* and *Klf4*. This was achieved in complete differentiated fibroblasts, and the resulting cells were called induced pluripotent stem cells (iPSCs), which presented morphofunctional features typical of embryonic stem cells [172]. The method was successfully applied to human cells [173].

One of the first astrocyte cell culture obtained from human iPSCs (hiPSCs) was reported in 2011, when iPS(IMR90)-4 cells reprogrammed from an IMR90 fibroblast were subjected to differentiation, resulting in immature astrocytes [174]. More than 90% of these cells showed expression of GFAP and S100B after 24 weeks in culture. In addition, these cells could propagate calcium waves, uptake glutamate and promote synaptogenesis and BBB formation. In conclusion, these cells presented primary astrocyte culture characteristics that could be maintained for 180 days after several passages and expansion. The predifferentiation of hiPSCs into neuroepithelial cells was a key step to achieve astrocytic cells. In contrast, induced astrocytes (iAstrocytes) could also be directly obtained from fibroblasts through the expression of NFIA, NFIB and SOX9 transcription factors [175]. Several methods were reported to generate iAstrocytes through neural progenitor cells (NPC) like the pioneer methods [174,176–179]. Most methods demanded long culture protocols to generate homogeneous mature astrocytes [174,179], or required sorting to reduce heterogeneity [180,181]. More recently, cell culture media conditions provided the means to reduce the protocol timeline [182].

Due to technical improvements and popularization of hiPSCs generation, this approach has been applied to study neurodegenerative diseases [183] and drug testing [184] through the use of the patients' cells to discover new treatments. In line with this strategy, patients' hiPSC-derived astrocytes have received growing attention in the last decade, which is corroborated by many preclinical studies [185].

The possibility to explore astrocytes from human subjects with minimal invasivity (cell collection) allowed the cellular investigation of mechanisms in the same genetic background of the patient, which is very relevant for sporadic cases of neurodegenerative diseases. However, it is important to evaluate if epigenetic marks and gene expression are compatible with differentiated astrocytes and without residual molecular signatures from the parental cell type [186–188]. Although hiPSC-derived astrocytes represent several advantages for studying brain diseases, interpretation issues may arise depending on the differences between hiPSC lines that can impact growth properties, susceptibility to cell death, effects of long-term culturing and so on. These problems can be circumvented by the use of several age-matched controls and hiPSCs from different patient donors, or the use of controls generated through correction of the disease-associated mutation (see [189] for a discussion). The high costs and laborious procedures involved in iPSC generation and maintenance make it difficult to obtain representative samples from different patients. The CRISPR-Cas9 gene editing methodology allowed significant advances in terms of introducing or correcting mutation, avoiding genetic alterations caused by viral integration into the genome and other artifacts promoted by previous generation strategies [190]. Several companies and nonprofit organizations provide hiPSCs and hiPSC-derived astrocytes, alleviating research laboratories from the burden involved in hiPSC generation and maintenance.

iAstrocytes development has already produced notable results in the field of neuroinflammation [110]. Santo et al. described a method to generate astrocytes from human iPSCs and ESCs, yielding functional astrocytes that are inflammation responsive to cytokines with increased percentage of IL8- and IL6-positive cells, similarly to primary culture [191]. Stratification of MS patients into benign or progressive MS phenotypes allowed the study of iPSC-derived reactive astrocytes and the discovery of neuroprotective properties in inflammatory conditions related to the benign MS phenotype [192]. Another recent article also made use of co-culture assemblies of neurons and astrocytes derived from iPSCs, modeling tau-pathology that features tau hyperphosphorylation and misfolding, resulting in changed neuroinflammatory patterns [118]. iPSCs of a different source, in this case human peripheral blood mononuclear cells, have also been used to model ALS-astrocytes, which shows increased secretion of IL1B, TNF and IL6 [119]. Other examples involved the investigation of inflammatory disruptors of the BBB modeled with iPSC-derived cells, and drug testing against inflammation using human fibroblasts as source material to generate iAstrocytes [193–195].

### 5.5. 3D Culture and Bioprinting

The traditional culturing in flasks and plates presents an obvious limitation compared to *in vivo*: cells grown as monolayers do not replicate the microenvironment found in tissues [196]. It is well documented that many cell types present aberrant cell division rates, show flatter shape and lose their phenotypic identity when cultured in 2D [197]. The development of 3D culture methods circumvented this limitation. This involves assembling a natural or a synthetic polymeric scaffold jointly with the cell suspension. In addition, the employed material can simulate the architecture and soft fabric of the brain. The use of hydrogels in combination with ECM components promote important changes in cell behavior, and provide a molecular microenvironment more compatible with the tissue *in vivo* [198–201].

The 3D culturing technique can be further combined with bioprinting, and the hydrogel mixture to be mixed with cell suspension, in this case, is called bioink. Bioprinting improves the spatial distribution of biomaterial and cellular components, leading to the construction of a synthetic and complex living tissue. The bioprinting process is controlled and

can produce constructs previously determined in size, thickness and shape [199,200,202]. Accordingly, 3D culturing and bioprinting increase the dimension of exploratory evaluations of cell–cell [189,190,203,204], cell–hydrogel [205] and cell–ECM interactions [206] in processes of cell migration [207], shape remodeling [122], differentiation [208,209], neuropathological paradigms [210,211] and so on, with greater chance of compatibility with *in vivo* conditions.

A 3D collagen hydrogel-based culture was successfully employed with astrocytes, thus generating a culture system that consistently generates less reactive state, resembling undamaged brain phenotypes. Upon TGF $\beta$  treatment, these cells presented more ramification morphology and expressed molecules associated with gliosis (elevated GFAP, Aquaporin 4, CSPG, Vimentin and IL-6 secretion) [212]. Different materials have been systematically tested to evaluate *in vitro* conditions that mimic the natural resting state of astrocytes and microglia culture (reviewed in [213]). Although 3D culture is promising for modeling neuroinflammation and neurodegeneration, there is still a lack of systematic studies that compare neuroinflammatory readouts in 3D cultures versus canonical 2D. For instance, SARS-CoV-2 infection and AD has been investigated in 3D cultures [214,215], but neuroinflammatory readouts are not explored in general.

### 5.6. Neurospheres and Brain Organoids (Mini-Brain)

Neural stem cells (NSCs) have been isolated from the embryonic, neonatal and adult rodent CNS, and respond to the mitogenic signal from epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF-2). These cells are capable of asymmetric division, maintaining the stem cell pool, while retaining the ability to differentiate into neurons and macroglia. NSCs from rodents and humans can be cultured in nonadherent conditions, developing spheroids called neurospheres. Neurospheres in adherent conditions and deprived from growth factors differentiate in neurons and astrocytes [80,216,217]. Neurospheres have been used to access cellular properties from progenitor and stem cells, such as proliferation, self-renewal capacity, and multipotency, while their capacity to generate astrocytes has been less explored. It has been argued that experiments involving neurospheres lack consistency between research groups, due to sensitivity to the culturing method used (for review see [218]). Nevertheless, neurospheres can be considered a good approach to study the development of neural cells.

Further improvements of human NSC culture methods allowed the evolution of a methodology called brain organoids (also known as mini-brains). The hiPSCs grown on feeder cells were cultured in nonadherent conditions, and without 3D scaffolds could generate laminated structures called human cortical spheroids that presented functional astrocytes and only excitatory neurons in a reproducible manner [124]. Brain organoids are self-organized structures that are successfully used to model brain diseases (reviewed in [219–221]). The Zika virus (ZIKV) infection is one example of mini-brain modeling for studying fetal virus infection, revealing the dynamics of infectivity in astrocytes [222]. Several methodologies have been developed to include technological upgrades from bioprinting, for instance the inclusion of vascular elements and the use of microfluidic devices [222–226]. Although much effort has been employed to use brain organoids in regenerative medicine, their use as disease models started a new chapter for studying the glia relationship with neuroinflammation. An important implementation is the incorporation of microglia in these organoids, which has been recently achieved [126,227–229].

Since the employment of mini-brain-containing microglia cells is quite recent, the reports of neuroinflammation are related to neuronal/microglial components only. This is in agreement with studying astrocytes disconnected from microglia, which represents most studies conducted so far. Cairns et al. introduced a model of herpes simplex (HSV-1) infection in mini-brains that presented features of AD brains including amyloid plaque-like formations, gliosis, neuroinflammation and decreased functionality. In this study, gliosis and astrocyte reactivity was accessed by increased GFAP, vimentin, LCN2 and serpin family E member 3, while the proinflammatory profile was accessed by the upregulation

of *TNF*, *IL1B*, *IL6* and *IFNG* [125]. ZIKV infection in mini-brains also induced markers of neuroinflammation [222], which were also shown in a model of methamphetamine exposure that promoted astrogliosis and upregulation of GFAP, inflammasome NRLP1, *IL6* and other genes related to inflammation [230]. Altogether, brain organoids offer an advanced platform for testing mechanisms involving astrocytes that are relevant to neuropathologies with inflammatory components.

## 6. Major Differences between Cell Cultures: Implications for Neuroinflammation Associated with Neurodegeneration

Primary astrocyte cell culture development not only paved the way for studying these cells, but also promoted the use of a widespread technique that is relatively easy to standardize in research laboratories. As mentioned, microglia and other cell-type contamination can lead to variable degrees of astrocyte reactivity that are problematic for results interpretation. In addition, the use of fetal calf serum presents the cells with many soluble molecules usually not present in the brain environment. These culturing techniques yield a reactive phenotype, unlike humans *in vivo* (see [195] for discussion). New technical advances in cell purification and synthetic medium formulation can overcome these limitations, although the classical culture methodology will still be used for screening analysis and preliminary data acquisition. This also holds true for cell lines, which are advantageous for large-scale analysis such as drug testing and RNAi or CRISPR-Cas9 screenings.

Regarding cell lines and IA, Galland et al. compared the C6 cell line, IA and primary astrocyte culture, suggesting that C6 and IA show different morphology and biochemical activity compared to primary astrocytes [152]. The authors observed a decrease in astrocyte markers such as GFAP, S100B, ALDH1L and AQP4, in addition to a reduced glutamate metabolism in IA cells, indicating an inferior profile when compared to the other cells [115,158,231–234]. Although these striking differences are present in cell lines, immortalized and C6 cells preserve cell shape plasticity and glucose consumption in response to glutamate [152]. iAstrocytes represent a new chapter in astrocyte *in vitro* studies, and it is important to compare treatment and disease modeling outcomes with traditional models, as has been done by some groups. So far, the possibilities offered by this technology are more prevalent than the drawbacks.

Both primary astrocytes, cell lines, IA and iAstrocytes may benefit from 3D culturing, which can promote a resting state that is similar to the natural microenvironment in the undisturbed brain. Co-cultures of iPSC-derived neural cells also seem promising for modeling diseases and BBB function. The complexity of varied cell populations in the same system pose a problem similar to studies *in vivo*, which is the evaluation of individual cell-type responses and contributions. Since neuroinflammation is quite susceptible to the influence of microenvironment modifications promoted by the different neural cells, the gain of reproducing the natural context demands comes with the cost of sophisticated analysis application. Cell sorting, single-cell RNA sequencing and other single-cell approaches are being used to analyze astrocytes responses and generate detailed information of subpopulations and heterogeneity with unprecedented cutting-edge technologies.

## 7. Conclusions and Future Directions

Cell lines and IAs are important tools in astrocyte research, especially those involving complex molecular/genetic manipulations. One advantage of these cells is their capacity to rapidly expand cellular material and apply large-scale analysis. However, experimental interpretations are quite limited compared to primary cultures, since astrocytes features can be compromised by cell transformation and modify neuroinflammatory responses to a large extent. Also, rodent 2D culturing can be very limited in terms of providing pathological mechanisms, excluding those that are conserved and cell-autonomous, since cells are grown as monolayer and present initial degrees of reactivity, and these are limited to simpler interactions. Co-cultures may circumvent the lack of cell diversity interaction and offer a middle ground compared to more elaborated 3D cell cultures, which recapitulates

natural astrocyte behavior. iAstrocytes represent a new frontier to model neurodegenerative diseases, and neuroinflammation research is gathering important information for both protective or detrimental effects from astrocytes reactivity. Organoids combined with iPSC technology and gene-editing tools represent the highest level of neurodegenerative disease modeling. The combination of these different in vitro resources will be essential to validate findings and evaluate cellular responses individually.

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