Review

Immortalized hippocampal astrocytes from 3xTg-AD mice, a new model to study disease-related astrocytic dysfunction: a comparative review

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Abstract

Alzheimer's disease (AD) is characterized by complex etiology, long-lasting pathogenesis, and cell-type-specific alterations. Currently, there is no cure for AD, emphasizing the urgent need for a comprehensive understanding of cell-specific pathology. Astrocytes, principal homeostatic cells of the central nervous system, are key players in the pathogenesis of neurodegenerative diseases, including AD. Cellular models greatly facilitate the investigation of cell-specific pathological alterations and the dissection of molecular mechanisms and pathways. Tumor-derived and immortalized astrocytic cell lines, alongside the emerging technology of adult induced pluripotent stem cells, are widely used to study cellular dysfunction in AD. Surprisingly, no stable cell lines were available from genetic mouse AD models. Recently, we established immortalized hippocampal astroglial cell lines from amyloid-β precursor protein/presenilin-1/Tau triple-transgenic (3xTg)-AD mice (denominated as wild type (WT)- and 3Tg-iAstro cells) using retrovirus-mediated transduction of simian virus 40 large T-antigen and propagation without clonal selection, thereby maintaining natural heterogeneity of primary cultures. Several groups have successfully used 3Tg-iAstro cells for single-cell and omics approaches to study astrocytic AD-related alterations of calcium signaling, mitochondrial dysfunctions, disproteostasis, altered homeostatic and signaling support to neurons, and blood-brain barrier models. Here we provide a comparative overview of the most used models to study astrocytes *in vitro*, such as primary culture, tumor-derived cell lines, immortalized astroglial cell lines, and induced pluripotent stem cell-derived astrocytes. We conclude that immortalized WT- and 3Tg-iAstro cells provide a non-competitive but complementary, low-cost, easy-to-handle, and versatile cellular model for dissection of astrocyte-specific AD-related alterations and preclinical drug discovery.

Key Words: Alzheimer's disease; astrocytes immortalization; astroglial Alzheimers's disease model; blood-brain barrier; calcium signaling; central nervous system homeostasis; disproteostasis; endoplasmic reticulum-mitochondria contacts; induced pluripotent stem cell-derived astrocytes; protein synthesis

Introduction

The progressive increase of Alzheimer's disease (AD) incidence in the older adult population underlines the need for new disease-modifying therapeutic strategies to contrast neuronal degeneration (Merlo et al., 2021; Cummings, 2022). Alterations of Ca^{2+} homeostasis, mitochondrial dysfunction, oxidative stress, dysproteostasis, and cell-cell communication are characteristic of cellular dysfunction in AD. The neuronal contribution has been widely documented, and numerous neuronal *in vitro* models have been described, such as neuronal cell lines, primary neuronal cultures, and recent induced pluripotent stem cell (iPSC)-derived models (Chang et al., 2020). However, in the last 20 years, the role of astroglial cells in AD pathology, with the involvement of the mechanisms mentioned above, has received growing attention (Verkhratsky et al., 2012, 2021; Lim et al., 2016b).

Astrocytes are the main homeostatic cells in the central nervous system (CNS), whose activity plays a crucial role in neuronal functions. Astrocytes provide metabolic support, ions, and neurotransmitter modulation and control extracellular environment and synaptic and blood-brain barrier (BBB) functions (Lim et al., 2016a; Adamsky and Goshen, 2018). Reports of recent decades brought a better understanding of their pathological contribution to neurodegenerative diseases. Indeed, astrocytes undergo complex yet brain region-specific and disease-stage-dependent alterations. Functional imaging studies on humans and works on animal models suggest that in early, preclinical, and prodromal disease stages, astrocytes become hypotrophic and asthenic (Verkhratsky et al., 2012, 2021). The asthenia is accompanied by a decrease in glucose utilization, reduction of glial fibrillary acidic protein (GFAP) immunoreactivity, and decrease in cell volume, suggesting reduced homeostatic support to neurons and other cells in the CNS. Another astrocytic disease state is represented by astrogliosis, characterized by hypertrophic changes, such as increased GFAP expression, microglial activation, and neuroinflammation. Astrogliosis is characteristic of advanced stages of many neurological and neurodegenerative diseases, including AD (Alberdi et al., 2013; Lim et al., 2016b, 2021; Verkhratsky et al., 2021; Sims et al., 2022). It involves transcriptional, proteomic, and functional remodeling of astrocytes with still unclear meaning for disease outcomes (Boulay et al., 2017; Garcia-Esparcia et al., 2017; Sakers et al., 2017; Escartin et al., 2021; Sims et al., 2022). These findings emphasize an urgent need for comprehensive research studies on astrocytes to dissect these cells' contribution to AD progression and discover new therapeutic targets (Cummings, 2022).

Detailed investigation of astrocytic cellular pathophysiology in AD requires the development of cellular models which combine several apparently incompatible features, such as i) maintenance of cell-type specificity; ii) faithful reproduction of AD-specific cellular pathophysiology; iii) stability and scalability of cultures to be suitable for a range of assays from single cell to high throughput analyses, and iv) suitability for the inter-laboratory logistics, cryopreservation, and transportation. Several *in vitro* astroglial models have been used to study AD cellular dysfunction, such as primary astroglial cultures, tumor-derived astroglial cell lines, immortalized astrocytes, and iPSC-derived astrocytes. Surprisingly, no stable cell lines from mouse AD models were available. To fulfill this gap, we have recently generated immortalized hippocampal astroglial cell lines from amyloid-β (Aβ) precursor protein/presenilin-1/Tau triple-transgenic (3xTg)-AD mice (denominated as wild type (WT)- and 3Tg-iAstro cells) (Rocchio et al., 2019). iAstro lines were generated by retrovirus-mediated transduction of simian virus 40 (SV40) large T-antigen and propagated without clonal selection, thereby maintaining the natural heterogeneity of primary cultures. Here we discuss the main

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features, advantages, and limitations of iAstro models compared to currently available *in vitro* astrocytic models (Ruffinatti et al., 2018; Dematteis et al., 2020; Tapella et al., 2022). Using search keywords "AD immortalized astrocyte models or *in vitro* AD astrocyte model or AD cell model," we reviewed the literature on the currently available AD *in vitro* astroglial models using PubMed and Scopus resources. Although providing general information about astrocytic cellular models, this review is mainly focused on models suitable for studying AD-related pathology. We suggest that immortalized WT- and 3Tg-iAstro cells provide a low-cost, easy-to-handle, and versatile cellular tool for the dissection astrocyte-specific AD-related alterations.

Astroglial *In Vitro* Models, Advantages and Disadvantages: Primary Cultures, Cell Lines, Immortalized Cell Lines and Induced Pluripotent Stem Cells

Primary astroglial cultures

Primary astrocytic cultures can be obtained from embryos or postnatal dissection of specific brain areas, such as the cortex, cerebellum, hippocampus, and others (Lange et al., 2012; Hertz et al., 2017). They provide a useful tool for studying astroglial physiology or pathology and are considered the gold standard for *in vitro* studies (Eichberg et al., 1976; Hansson, 1983; Fages et al., 1988; Fresu et al., 1999; Bernascone et al., 2010). They are reliable and can be isolated from different tissues and species, but they also present disadvantages due to the continuous need of laboratory animals and the rather limited number of passages, which limits the total amount of cells. The latter limitation is particularly critical for cultures from specific brain areas such as the hippocampus, entorhinal cortex, thalamus, amygdala, substantia nigra, etc. Moreover, primary cultures present a high degree of intra- and inter-cultural heterogeneity, leading to high variability between cultures and laboratories, which hampers the reproducibility of the results.

Astroglial cell lines

Tumor-derived and spontaneously transformed cell lines

Several cell lines were generated from brain tumors or through spontaneous transformation cell lines of embryonic origin in non-tumor tissue. Many of these lines are available in the American Type Culture Collection cell bank (**Table 1**). Astroglial cell lines are generated from mice and rats from the cerebellum, diencephalon, cortex, and embryos and are described as astroglial healthy cellular models. Researchers widely use human cell lines derived from brain glioma tumors as an astrocyte model due to their ease of use and inexpensive management. However, compared with primary cultures, astroglial cell lines display a general downregulation of astrocyte-specific markers (Galland et al., 2019).

Immortalized astroglial cell lines

An alternative to tumor-derived and spontaneously *in vitro* transformed cell lines is represented by artificially immortalized astrocytes. They can be generated through the transduction of primary cultures, from different brain regions of healthy animals, with SV40 large T-antigen and/or human telomerase reverse transcriptase (**Figure 1**). Human immortalized astrocytes were also generated from healthy human tissues, such as cortical, midbrain, and fetal brain cells (**Table 2**).

Once established, they can be manipulated and maintained in stable cell lines (**Figure 1**). Immortalized cells, derived from healthy tissues rather than tumors, maintain an astrocyte-like phenotype with the expression of specific astroglial markers. In the last decades, different immortalized astroglial cell lines have been described, and their use in both *in vitro* and *in vivo* (Sonoda et al., 2001; Dai et al., 2010) studies has been reported. An et al. (2004, 2010) rat-immortalized astrocytes overexpressing galanin to reduce neuropathic pain in a rat model of neuropathic pain injury by subarachnoid transplantation of the cells as lead carriers. Behrstock et al. (2000) generated immortalized mouse astrocytes expressing glutamate decarboxylase (GAD) and γ-aminobutyric acid-secreting as a tool to deliver γ-aminobutyric acid in specific brain regions. The expansion of trinucleotide repeats is the genetic cause of many neurodegenerative disorders. Claassen and Lahue (2007) studied the role of trinucleotide repeat in a human immortalized astroglial cell line. Dai et al. (2010) used normal human immortalized astrocytes to study the role of FoxM1B molecule in cellular transformation and tumor formation, produced by intracranial injection of FoxM1B-expressing immortalized normal human immortalized astrocytes, and displayed glioblastoma multiforme phenotypes. Studies from Frisa's team (Frisa et al., 1994; Frisa and Jacobberger, 2002) used immortalized astrocytes to study the effect of population density on gene expression. Furihata et al. (2016) generated different astrocytic lines to study cell-cell communication and BBB as a tool for drug delivery. Kitamura et al. (2018) generated an immortalized human astrocyte cell line, termed HASTR/ci35 cells, as an *in vitro* model for drug development studies. Roy et al. (2006) characterized beta-adrenergic receptors in a clonal cell line (C1) immortalized from cerebral cortical astroglial cells of FVB/N mice. Sacchettoni et al. (1998) studied the glutamate-dependent γ-aminobutyric acid modulation. Xu et al. (2008) found the therapeutic transplantation potential of immortalized astrocytes in chronic neuropathic pain. Morikawa et al. (2005) and Raofi et al. (2000) generated immortalized astrocytes from apolipoprotein E (*APOE*) knock-in mice for studying mechanisms that underline the isoform-specific effects of *APOE* in the CNS. *APOE2*, *APOE3*, and *APOE4* expressing cell lines were established, and clones were selected for secreting apoE in high-density lipoprotein-like particles at a similar level with primary astrocytes. The authors showed that astrocyte-secreted apoE3 and apoE4 are equally well associated with Aβ, and only a small fraction of Aβ formed sodium dodecyl sulfate-stable

Table 1 | Tumor-derived and spontaneously transformed cell lines list modified from American Type Culture Collection cell lines

Cell line	Species	Tissue	Cell type	Diseases
C8-D1A (astrocyte type I clone)	Mouse	Cerebellum	Astrocytes	Normal
C8-S (astrocyte type II clone)	Mouse	Cerebellum	Astrocytes, type II	Normal
C8-D1A (astrocyte type I clone)	Mouse	Cerebellum	Astrocytes, type III	Normal
DI TNC1	Rat	Diencephalon	Astrocytes, type II	Normal
CTX TNA2	Rat	Cortex	Astrocytes, type I	Normal
Swiss SFME (serum free mouse embryo)	Mouse	Embryo	Astrocytes	Normal
BALB SFME (serum-free mouse embryo)	Mouse	Embryo	Astrocytes	Normal
IDH1 mutant U-87 isogenic-Luc2	Human	Brain	Epithelial-like	Glioma
IDH1 mutant-U-87 isogenic cell line	Human	Brain	Epithelial	Glioma
U-87 MG	Human	Brain	Epithelial	Likely glioblastoma
U-118 MG	Human	Brain	Mixed	Glioblastoma, grade IV
U-87 MG dCas9-KRAB	Human	Brain	Epithelial	Likely glioblastoma
Hs 683	Human	Brain	Epithelial	Glioma
U-87 MG-Luc2	Human	Brain	Epithelial	Glioma
T98G [T98-G]	Human	Brain	Epithelial	Glioblastoma multiforme

All animal origin cell lines were from https://www.atcc.org/cell-products/animal-cells#q=astrocyte&t=productTab&numberOfResults=24, and all human origin cell lines were from https://www.atcc.org/cell-products/human-cells#q=glioma&t=productTab&numberOfResults=24.

Table 2 | Immortalized astroglial cell lines

i-Astrocytes	Species/tissue	Type	Clone/ population	References
Generic immortalized astrocytes from healthy/non-diseased tissue				
Immortalized astrocytes (IAST)	Rat cortex	SV40 large T	Clonal	An et al., 2004, 2010
Astrocytic cell line BAS8.1	Mouse cortex	SV40 large T	Clonal	Behrstock et al., 2000
Mouse cortical astrocytes	Mouse cortex	SV40 large T	Clonal	Frisa et al., 1994; Frisa and Jacobberger, 2002
Immortal astrocyte (C1)	Mouse cortex	SV40 large T	Clonal	Lin et al., 1997; Raofi et al., 2000
C8 cells (also designated C8S)	Mouse cerebellum	Spontaneously immortalized astrocyte cell line	Clonal	Alliot and Pessac, 1984; Sacchettoni et al., 1998
Normal human astrocytes (NHAs)	Human	Human telomerase catalytic component (hTERT)	Clonal	Sonoda et al., 2001; Dai et al., 2010
Immortal human astrocytes	Human midbrain or cortical tissues	hTERT	Clonal	Roy et al., 2006
HASTR/ci35	Human	SV40 large T, hTERT	Clonal	Furihata et al., 2016; Kitamura et al., 2018
SVG-A cells	Sub-clone of SVG human fetal cells	SV40 large T	Clonal	Major et al., 1985; Claassen and Lahue, 2007
Alzheimer's disease astrocytic immortalized cell models				
Immortal primary astrocytes	Mouse; *APOE* knock-in	SV40 large T	Clonal	Morikawa et al., 2001, 2005
WT- and 3Tg-iAstro	Mouse hippocampus	SV40 large T	No clonal selection	Rocchio et al., 2019; Dematteis et al., 2020; Tapella et al., 2022

APOE: Apolipoprotein E; hTERT: human telomerase reverse transcriptase; SV40 large T: simian virus 40 large T-antigen.

complexes with apoE (apoE3 > apoE4), suggesting that these lines may be useful to study apoE related pathways in AD.

Immortalized astrocytes from 3xTg-AD mice represent a reliable tool for studying astroglial AD dysfunction

Surprisingly, no commercially available or reported astrocytic cell lines were derived from a genetic mouse model of AD. The only astrocytic cell line suitable to study *APOE*-related aspects of AD has been generated by Morikawa et al. (2001, 2005) and is described in the previous paragraph. However, the *APOE* lines have been clonally selected to express high levels of *APOE* isoforms. Instead, no clonal selection using "a priori" chosen characteristics should be performed to represent AD-related cellular alterations. In other words, the cell population should maintain the intra-cultural heterogeneity of the primary culture. To exploit this rationale and create an AD-related astrocytic cell model, we have used retroviral delivery of SV40 large T-antigen into primary hippocampal astrocytes from 3xTg-AD mice, a well-characterized AD mouse model. A high rate of retroviral transduction permitted us to avoid clonal selection and to maintain immortalized astrocytes as populations with the natural heterogeneity of primary cultures (**Figures 1** and **2**; Rocchio et al., 2019).

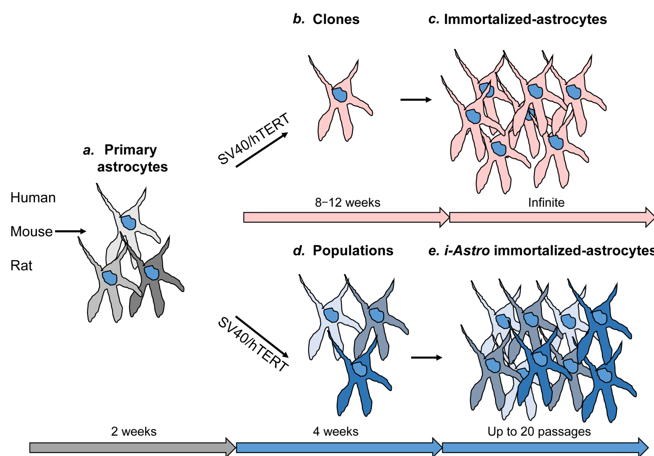

Figure 1 | Schematic representation of the immortalization procedure of primary astroglial cultures, from rat/mouse brain areas or of human origin, by infection with SV40 large T antigen or hTERT and clonal or population selection of immortalized astroglial lines.
hTERT: Human telomerase reverse transcriptase; iAstro: immortalized astrocytes; SV40 large T: simian virus 40 large T-antigen.

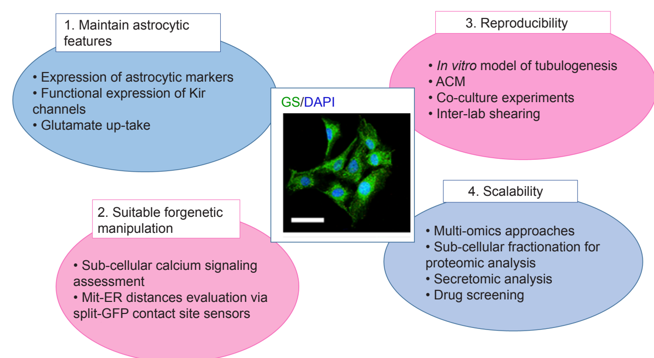

Figure 2 | Schematic representation of main features of immortalized iAstro cells.
In each balloon are reported practical examples of the most relevant advantages from recent reports (1–4). Scale bar: 50 µm. ACM: Astrocytes conditioned media; DAPI: 4′,6-diamidino-2-phenylindole; GS: glutamine synthetase; Kir: inwardly rectifying K^+ channels; MERCS: mitochondria-endoplasmic reticulum contact site; Mit-ER: mitochondrial-endoplasmic reticulum; Split-GFP: split green fluorescent protein-based contact site sensor.

Six immortalized cell lines have been established from WT- (WT-iAstro#1–6) and 3xTg-AD (3Tg-iAstro#1–6) mice from independent primary astrocytic cultures. Before immortalization, primary astroglial cultures were first depleted of microglial cells by magnetic-assisted cell sorting using anti-CD11b-conjugated microbeads to avoid microglial contamination and to obtain a population of highly purified astrocytes. Transduced astrocytes were grown in G418, amplified, and stabilized for 7 passages before characterization. The cells were cryopreserved at the 7th–9th passages. Interestingly, the proliferative potential of iAstro cells is not unlimited: after about 20 passages, the proliferation rate slows down, and the morphological heterogeneity of cultures increases. Thus, iAstro cells can be defined as partially immortalized cells or cells with an extended lifespan. We, therefore, recommend using co-passaged WT- and 3Tg-iAstro cells between 7 and 18 passages. In the following paragraphs, the key features and advantages of iAstro cell lines will be illustrated (**Figure 2**).

3Tg-iAstro cells maintain astrocytic phenotype and AD-related molecular alterations

To be considered a reliable model for studying AD-related astroglial dysfunction, immortalized iAstro cells should maintain cell type-specific characteristics of primary AD astrocytes such as astroglial markers, gene, and protein expression profiles. Therefore, the expression of astroglial markers (GFAP, glutamine synthetase, aldehyde dehydrogenase 1 family member L1, aquaporin 4) and morphology have been assessed and found to be comparable with primary cultures, with no macroscopic differences between WT- and 3Tg-iAstro cell lines (Rocchio et al., 2019). In AD animal models and human brains, during AD pathogenesis, as described in the Introduction, astrocytes undergo complex yet brain region-specific and disease-stage-dependent alterations (Lim et al., 2016b). Therefore, one could expect to find morphological differences between WT- and 3Tg-iAstro cells. However, iAstro cells are prepared from highly purified astrocytic cultures, and their morphological similarity in resting non-stimulated conditions may be explained by the absence of interaction with other cells of the CNS. Nevertheless, 3Tg-iAstro cells recapitulate gene expression alterations of 3xTg-AD primary astroglial cultures and present multiple biochemical and functional differences compared with WT-iAstro cells (see below).

WT-iAstro expresses functional inwardly rectifying K^+ channels activity

Inwardly rectifying K^+ (Kir) channels, expressed in astrocytes, is crucial for potassium buffering and maintenance of neuronal excitability. In patch-clamp experiments, we showed that both WT- and 3Tg-iAstro maintain a Kir channel functional expression similar to that of the primary astrocytes, although there were no differences in Kir currents between WT- and 3Tg-iAstro cells (Rocchio et al., 2019).

iAstro retains the ability to up-take glutamate from media

Astrocytes play a crucial role in extracellular glutamate homeostasis: they eliminate glutamate from the synaptic cleft, preventing excitotoxicity. High-affinity glutamate transporter, glutamate transporter 1, is the most expressed in hippocampal astrocytes *in vivo. In vitro*, hippocampal astrocytes reduce the expression of glutamate transporter 1 and upregulate glutamate-aspartate transporter, which becomes the predominant glutamate transporter in cultured astrocytes (Tapella et al., 2022). Glutamate-aspartate transporter expression and activity have also been found in iAstro cells (Rocchio et al., 2019).

Transfectability of iAstro cells: crucial for single-cell and population Ca^{2+} signals and mitochondrial-endoplasmic reticulum interactions measurements

An important feature of iAstro cells is their high transfectability, compared to primary cultures, which allows the expression of heterologous proteins, genetically encoded sensors, or reporters, allowing a detailed analysis of intracellular alterations and signaling.

Astrocytes respond to extracellular stimuli by generating Ca^{2+} signals. They engage metabotropic receptors linked to Ca^{2+} release from internal stores or calcium entry from the extracellular milieu through the plasma membrane. We and others have shown that both AD neurons and astrocytes show alteration in calcium signals (Grolla et al., 2013; Lim et al., 2013, 2014, 2021). The deregulation of Ca^{2+} signaling has been confirmed and further investigated using iAstro cells. We analyzed cytosolic, endoplasmic reticulum (ER), and mitochondrial Ca^{2+} signals, using Fura-2 and aequorin-based sub-cellular calcium measurements (Lim et al., 2016a; Dematteis et al., 2020; Tapella et al., 2022). The deficit of the ER-mitochondrial Ca^{2+} transfer has been correlated with the strength of the ER-mitochondrial interaction using SPLICS, a split-green fluorescent protein contact site sensor (Vallese et al., 2020). We detected an increased interaction between ER and mitochondria in 3Tg-iAstro cells, representing an emerging novel mechanism of cellular dysfunctions in neurodegenerative diseases, including AD (Dematteis et al., 2020; Lim et al., 2021; Tapella et al., 2022).

3Tg- and WT-iAstro cultures can be scaled up to allow the isolation of subcellular structures and multi-omics analyses

The increasing development and popularity of omics approaches have underlined the growing need for applying these techniques in neuroscience research. We performed proteomic analysis of WT- and 3Tg-iAstro cells using whole-cell lysates and subcellular fractions. While whole-cell proteomics can be done using a relatively small amount of material (2×10^6 cells equivalent to one 10 cm culture dish), proteomic analysis of sub-cellular fractions requires a significantly higher amount of material. Mitochondrial fractions enriched with ER-mitochondria contact sites have been isolated from as many as 100 10-cm culture dishes, exploiting the advantage of scalability. The results of this analysis allowed us to generate the hypothesis of ER-mitochondria contact site-related bioenergetic deficit and protein kinase RNA-like endoplasmic reticulum kinase-independent inhibition of protein synthesis (Dematteis et al., 2020; Tapella et al., 2022). Another point of application, in which reproducibility is of paramount importance, is represented by using an astrocyte-conditioned medium or co-culture of iAstro cells with other cell types. We used these approaches to study the mechanisms of the reduced support to neurons and failure to maintain the endothelial integrity in an *in vitro* model of the BBB (Kriaučiūnaitė et al., 2021; Tapella et al., 2022).

Proteomic analysis of iAstro secretome suggests that 3Tg-iAstro astrocyte-conditioned medium has reduced amounts of neurogenic and neuroprotective proteins such as secreted protein acidic and rich in cysteine and heat shock protein 90. Moreover, 3Tg-iAstro cells fail to secrete cell adhesion molecules and components of the extracellular matrix (Tapella et al., 2022).

It should be acknowledged that iAstro cells share many characteristics with other astrocytic cell lines. Nonetheless, key differences such as i) non-clonal populational maintenance of lines; ii) their origin from a genetic AD mouse model, and iii) faithful recapitulation of astrocytic and AD-related cell pathology make them an outstanding model for their potential in studying the astrocyte-specific alterations in AD.

iPSCs derived astrocytes

Since the groundbreaking discovery of the possibility to reprogram adult somatic cells in stem cells with consequent differentiation into virtually any cell type, iPSC technology has become the leading edge field in cell biology and medicine. As an unprecedented opportunity for regenerative medicine, personalized cell therapy, and diagnostics, iPSC technology presents key advantages compared with conventional human and animal cellular models. All major brain cell types can be differentiated from subject-/patient-derived iPSCs, thus allowing deciphering of the cell-specific involvement and contribution to the disease pathogenesis. In addition, increasingly complex co-culture systems and three-dimensional models incorporating multiple brain cell types have been recently developed, providing great improvement for studying intercellular interactions (**Figure 3**).

The impact of iPSC technology on the study of AD pathogenesis are well covered by a growing number of comprehensive contributions (Hernández-Sapiéns et al., 2020; Barak et al., 2022; Hasan and Trushina, 2022; Verheijen et al., 2022). Here we limit the discussion to the AD-related features reproduced with iPSC-derived astrocytes, while **Table 3** provides a comparative overview of the different astroglial models used in preclinical research.

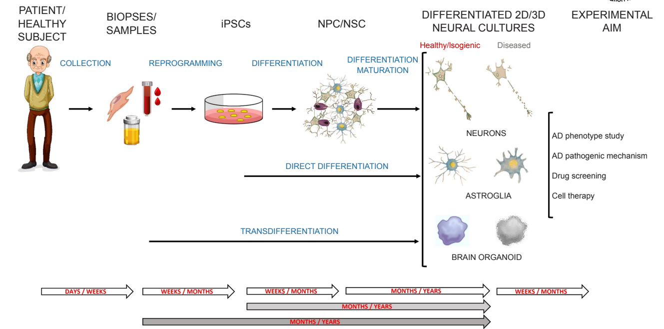

Figure 3 | Schematic representation of iPSC-derived neuron and glia to study AD pathology.
Created by https://www.freepik.com/. AD: Alzheimer's disease; iPSC: adult-induced pluripotent stem cells; NPC: neural progenitor cell; NSC: neural stem cell.

Many studies have been published in the last few years on iPSC-derived astrocytes from AD patients. Sienski et al. (2021) and de Leeuw et al. (2022) used iPSC to study *APOE* allele and its role in lipid homeostasis, and Lin et al. (2018) used clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) to study the modulation of pathological outcomes in iPSC-derived neurons and astrocytes from AD patient after convertion of *APOE4* into *APOE3*. *APOE* genotype determines a neurotrophic effect of human iPSC-derived astrocytes when cocultured with neurons and seems to have a role in the endocytosis pathway involving PICALM (Zhao et al., 2017; Narayan et al., 2020). Bassil et al. (2021) set up an automated platform to generate a human cell-based AD model *in*

Table 3 | Advantages and disadvantages of *in vitro* astroglial cell models to study Alzheimer's disease or neurodegenerative diseases

Models	Benefits	Drawbacks
Primary cultures		
Astroglial primary cultures	· Genetically stable · From different species and brain areas · Better respect astroglial phenotype	· Use of animals · Inter-cultural variability · Low number of passages · Limited amount of cells (from small brain areas) · Less suitable for genetic manipulations · Not suitable for cryopreservation and logistic
Stable cell lines		
Astroglial cell lines	· Low handling costs · Unlimited proliferative potential · Suitable for genetic manipulation · From animals and humans, and different brain areas · Unlimited amount of cells · Cryopreservation and logistics	· Generally de-differentiated cells with poor expression of astroglial makers · Transformed cells with activated cancerogenic signaling
Immortalized astroglial lines with clonal selection	· Selected for a priory-defined criteria · Express specific markers · Low handling costs · High-to-unlimited proliferative potential · Suitable for genetic manipulation · Unlimited amount of cells · From animals and humans, and different brain areas · Unlimited amount of cells · Cryopreservation and logistics	· Express immortagens · Clonal selection
Immortalized astroglial lines without clonal selection (iAstro cells)	· No clonal selection, maintain natural heterogeneity of primary cultures · Express most astrocytic markers at levels comparable with primary cultures · Low costs for generation and handling · Can be generated on demand each time when needed · Can be generated from disease model animals · Suitable for genetic manipulation · Unlimited amount of cells · Cryopreservation and logistics · Correlation with *in vivo* alterations in parent mouse models	· Express immortagens · High but not unlimited proliferative potential
iPSC-derived astrocytes		
iPSC-differentiated astrocytes	· Human origin (healthy or disease-carrying) · Subject-specific · Express most astrocytic markers · Suitable for genetic manipulation · Suitable for cell therapy, personalized medicine, and diagnostics	· Generated using genetic reprogramming · High generation and handling costs · Long work-flow process · High inter-and intra-culture heterogeneity · Ethical and privacy issues

iAtro: Immortalized astrocytes; iPSC: adult-induced pluripotent stem cells.

vitro by adding soluble Aβ_{42} species on iPSC-derived neurons, astrocytes, and microglia. This AD model recapitulates critical hallmarks of human AD pathology, including severe synapse loss, followed by phosphorylated Tau induction and resulting in neuronal loss, which cannot be reproduced in mouse models overexpressing mutant *APP* and/or *PSEN*. Consistently with the human pathology, this iPSC-based model recapitulates Aβ plaque formation, including surrounding phosphorylated Tau-positive dystrophic neurites and microglia in close contact with plaques (Bassil et al., 2021). Astrocyte stimulation with Aβ_{42} alters GFAP expression in this setting, consistently with astrogliosis. Astrocytic cultures obtained from AD patients' iPSC have been successfully adopted to define the cell-specific main alterations induced by the pathology. Indeed, AD astrocytes showed cell-autonomous morphological atrophy and altered basal astrocyte secretome profile, regardless of whether they carried *FAD* or *SAD* mutations (Jones et al., 2017). AD-related mutations impair the iPSC-derived astrocyte ability to promote the survival, maturation, and function of cocultured human neurons, possibly by affecting astrocytic Ca^{2+} homeostasis and reactive oxygen species production and altering the cytokine and Aβ_{42} release (Kuijlaars et al., 2016; Oksanen et al., 2017; Zhao et al., 2017; Konttinen et al., 2019).

To understand the toxic effect of blood extracellular vesicles of astrocytic origin from AD patients, Nogueras-Ortiz et al. (2020) used iPSC-derived astrocytes and neurons as a reliable tool. iPSC-derived astrocytes were used to demonstrate that Aβ degradation is increased when cocultured with microglia (Rostami et al., 2021). The pathway of glutamate synthesis involving the branched-chain amino acids, leucine, isoleucine, and valine have also been investigated in iPSC-derived astrocytes, and Salcedo et al. (2021) demonstrated that it is impaired in cells from AD patients.

iPSC-derived astrocytes can be prepared as single-cell cultures or embedded in three-dimensional structures, commonly defined as cerebral organoids (Fan et al., 2019; Barak et al., 2022), allowing the understanding of multicellular crosstalk (Kuijlaars et al., 2016). When included in three-dimensional culturing systems, astrocytes showed dramatic improvement in development and maturation compared to two-dimensional monolayer cultures, providing opportunities to study glial contribution and cell-specific alterations of development and function in neurological disorders (Zhang et al., 2016; Quadrato et al., 2017; Sloan et al., 2017). Interestingly, this culture condition enabled the investigation of the effect of cell-specific AD-related mutations by the generation of chimeric cerebral organoids containing neurons and astrocytes with different genotypes, namely *APOE3* or *APOE4*. By this approach, it was shown that astrocytic *APOE4* was sufficient to significantly promote lipid droplet formation and cholesterol accumulation in neurons, while co-occurrence of astrocytic and neuronal *APOE4* was required to elevate neuronal phosphorylated Tau levels (Huang et al., 2022).

The mitochondrial peptidase pitrilysin metallopeptidase 1 loss of function was also studied in cerebral organoids and recapitulates AD features such as the accumulation of protein aggregates, Tau pathology, and neuronal death. Single-cell RNA sequencing showed mitochondria dysfunction and altered transcriptional signatures in astrocytes (Pérez et al., 2021).

iPSC-derived organoids have also been adopted to investigate the detrimental effect on BBB leakage in AD. Chen and colleagues mimicked the serum exposure consequence of BBB breakdown in AD patient brains by treating brain organoids with human serum. In this setting, the authors observed a recapitulation of disease-specific alterations *in vitro*, including the increase of Aβ and phosphorylated Tau levels and the immune response stimulation in astrocytes (Chen et al., 2021). Activated astroglia was shown to exert a protective effect in a fully human iPSC-derived BBB model against cytokine-induced BBB dysfunction (Mantle and Lee, 2018).

Personalized medicine is one of the most promising fields of application for iPSC. Cell lines can originate from a single patient and be used to set a personalized therapy and/or a drug screening directly on the affected cells and tissues (Jones et al., 2017; Sullivan and Young-Pearse, 2017). AD-patient iPSC-derived cells were used, indeed, to study the druggability of a variety of AD-related pathways, including i) cholesterol metabolism (van der Kant et al., 2019), ii) membrane-type 5 matrix metalloproteinase, which has been highlighted as a new key player in APP-processing (Arnst et al., 2021), iii) *APOE* gene isoforms (Brookhouser et al., 2021; Jun et al., 2022) expression or iv) secretion of apoE protein modulated by an U.S. Food and Drug Administration-approved 5-hydroxytryptamine 3 antagonist (Ondansetron; Shinohara et al., 2019), v) fatty acid oxidation that can be reduced by the synthetic peroxisome proliferator-activated receptor delta agonist GW0742 (Konttinen et al., 2019).

In spite of undisputed advantages, iPSC-based models still present several features which limit their widespread usage and comprehensive investigation. Among them, the main limitations are i) high cost of generation and maintenance of iPSC and control lines; ii) months-long processes for their establishment and differentiation; and iii) high degree of heterogeneity among differentiated cultures, hampering comparison of the result and standardization of protocols between labs. Some of these limitations can be overcome, e.g., the timing of iPSC generation, maintenance, and differentiation can be shortened by cryopreservation and consequent usage of committed differentiating iPSCs. Single-cell RNA sequencing can help to overcome intra-cultural heterogeneity (Doss and Sachinidis, 2019). Genetic differences can also represent a major hindrance to experimental analysis since they may mask or exacerbate a phenotype, which calls for the need of isogenic controls. Thus, tools for manipulating gene expression and generating mutations in iPSC and their derivatives, like the CRISPR/Cas9 system, have been exploited to introduce or correct specific mutations without altering the remaining genetic background (Cong et al., 2013; Paquet et al., 2016; van Essen et al., 2021).

Conclusions and Perspectives

The impact of cell type-specific pathology in AD pathogenesis can be studied at different levels using human subjects and animal or cellular AD models. Despite the undisputed translational value of human material and model animals, the dissection of cell-autonomous and non-cell-autonomous dysfunctions, signaling pathways, and key molecules responsible for the dysfunction requires simplified models which can easily be adopted for interdisciplinary investigations. In this frame, astrocytic cell lines generated from a specific brain region of an AD animal model may be particularly useful. Additional motivation for the use of cellular models in preclinical studies is given by a recent resolution of the Joint Research Center of the European Commission, which urges the acceleration of the transition to innovation without using animals in research. The generation of *in vitro* astroglial AD models will hopefully help overcome animal models' unmotivated use and limitations.

Except for human iPSC-derived astrocytes, whose implication in AD field has been extensively covered; the spectrum of cellular models currently available for studying astrocyte-specific AD-related cell pathology includes i) tumor-derived and spontaneously transformed astrocytic cell lines, many of which are commercially available (**Table 1**); ii) artificially immortalized astrocytes from different brain regions with the consequent clonal selection according to a priori defined criteria (some of the lines are commercially available); iii) partially immortalized astrocytes from an AD mouse model, propagated without clonal selection, exemplified by WT- and 3Tg-iAstro cells from hippocampi of 3xTg-AD mice. In this panel, surprisingly little attention has been made to the generation and usage of conditionally immortalized cell lines from genetic animal models of different diseases. In part, this may be caused by the conventional immortalization workflow, which traditionally includes the selection of single clones according to defined criteria. Such a selection eliminates "natural" disease phenotype, characteristic of primary cultures, making these lines useless for studying disease-related cellular dysfunctions. Instead, highly efficient viral transgenesis (in terms of percentage of transduced cells), with consequent propagation of transduced cells without clonal selection, makes it possible to maintain a "natural" cell phenotype that faithfully respects that of primary astrocytic cultures. We have demonstrated the feasibility of this approach on the example of WT- and 3Tg-iAstro cells generated from primary hippocampal astrocytes of 3xTg-AD mice. In several reports and inter-lab collaborations, we confirmed that 3Tg-iAstro cells maintain many physio-pathological features of AD astrocytes while being extremely versatile in terms of logistical convenience and culture scalability. An important advantage of conditionally immortalized astrocytes is represented by very contained costs of generation and handling, making these lines affordable for many labs worldwide.

Additionally, due to the reduced time scale of the immortalization process, which does not require the selection and characterization of clones, the lines can be generated each time upon demand within 4–6 weeks. This approach can be pursued for the generation of immortalized astrocytes from any genetic animal model and, potentially, from adult human primary astrocytes, which, we believe, will greatly accelerate basic and preclinical research and drug discovery processes. Importantly, the hypothesized niche for use and applications in biomedical research for immortalized astrocytes is not overlapping but complementary to the iPSC technology. We envisage that the illustrated immortalized astrocytes technology will cover the highly demanded need in the low-cost, easily handled, versatile, and representative cell model for studying disease-related cellular pathology, identifying targets for drug development, and routine drug screening.

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