




REVIEW

Astrocytogenesis: where, when, and how [version 1; peer review: 2 approved]

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Abstract

Astrocytes are the most abundant cell type in the central nervous system and have diverse functions in blood–brain barrier maintenance, neural circuitry formation and function, and metabolic regulation. To better understand the diverse roles of astrocytes, we will summarize what is known about astrocyte development and the challenges limiting our understanding of this process. We will also discuss new approaches and technologies advancing the field.

Keywords

Glia, Astrocyte, Gliogenesis, Neurodevelopment

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Introduction

The central nervous system (CNS) is composed of multiple highly specialized cell types, including neurons, astrocytes, oligodendrocytes, microglia, and endothelial cells, each with different functions that are highly adapted to local circuitries¹⁻³. Astrocytes are the most abundant glial cells in the CNS, representing between 20 and 40% of total cells in the brain^{4,5}. Astrocytes were first described by Rudolf Virchow as a homogenous connective tissue supporting nervous system elements. Later, Ramon y Cajal and others investigated the cellular structure of the brain and observed that different brain regions contain morphologically distinct neuronal and glial cell types.

Over the past century, astrocytes have emerged as a bridge to the outside world for neurons with diverse physiological roles in neural development, neural circuit function, neurotransmission, blood–brain barrier formation, and metabolic support. Astrocytes form the neurovascular unit between neurons and endothelial cells, and their end-feet structure maintains brain homeostasis by regulating water, amino acid, and neurotransmitter uptake. Astrocytes also have the ability to monitor the ongoing local activity of synaptic circuits with their elaborate processes ensheathing synapses and forming tripartite synapses. Astrocytes express neurotransmitter receptors that sense synaptic activity and respond to it by elevating astrocytic Ca^{2+} and secreting neuroactive molecules back to synapses. Despite these known roles, how astrocytes develop and mature to form functional neurovascular circuits to carry out these diverse functions remains relatively unknown¹⁻⁴.

Previous studies have shed light on how neurons and oligodendrocytes develop in a stepwise fashion, where they specify, undergo terminal differentiation, and enter the postmitotic stage. Since the initial observation by Ramon y Cajal, hundreds of types of neurons have been identified and functionally characterized, while astrocytes are still broadly classified as either protoplasmic or fibrous. The lack of markers and tools to access the precursor and intermediate stages of astrocyte development has hindered characterization of their development and heterogeneity. Further complicating their characterization, astrocytes are more plastic than neurons and proliferate after specification. However, with their critical and diverse functions that actively regulate neuronal function, it is essential to understand where, when, and how astrocytes are generated during development.

Stages of astrocyte development

Neural stem cells (NSCs), or radial glia, generate astrocytes through complex intrinsic and extrinsic cellular processes. This sequential series of events results in mature astrocytes that actively participate in CNS physiology. Conserved mechanisms regulate gliogenesis in the spinal cord and brain, although the process begins early, at embryonic day 11.5 (E11.5), in the spinal cord and later, at E18, in the brain. Whereas spinal cord astrocytes are derived from the ventricular zone (VZ), forebrain astrocytes are from the ventricular–subventricular zone (V-SVZ)⁶. Below, we discuss recent progress toward understanding astrocyte development and maturation, beginning with patterning and specification, proliferation, and maturation.

Astrocyte specification and developmental patterning

During CNS development, neurons are specified from NSCs before glial cells, and radial glia serve as a scaffold for this process. Signaling pathways and dynamic transcription factor expression control these cell fate decisions. Previous studies have focused on the spinal cord VZ, where the timing of the gliogenic switch in NSCs is clearly defined. In the VZ, neurogenesis ceases at E11.5 and gliogenesis commences at E12.5, and transcription factors sex-determining region Y-box 9 (Sox9) and nuclear factor-I A (NFIA) play critical roles during this developmental interval. Sox9 and brain-specific homeobox/POU domain protein 2 (Brn2) regulate NFIA induction and glial specification⁷. NFIA is both necessary and sufficient for embryonic gliogenesis⁸, and the association between Sox9 and NFIA regulates genes essential for astrocyte migration and maturation⁹ (Figure 1). In neocortical development, zinc finger- and BTB domain-containing protein 20 (Zbtb20) was shown to promote astrocyte specification while suppressing the production of oligodendrocyte precursors (OPCs), and knockdown of either NFIA or Sox9 suppresses Zbtb20 activity¹⁰.

Notch signaling is another important regulator of cell differentiation. In early development, Notch activation maintains the NSC pool in addition to inducing NFIA. NFIA maintains the continued inhibition of neurogenesis through induction of the Notch effector Hes5, further clarifying the role of Notch signaling in early gliogenesis⁸. NFIA associates with astrocytic gene promoters such as *GFAP* to disrupt their methylation through association with DNA methylating dissociation of the DNA methylating enzyme 1 (Dnmt1)^{11,12}. Loss of function of Notch effectors such as Hes5 leads to loss of glia, and gain of function produces more glia; however, Notch activity alone is insufficient to drive precocious astrocytic differentiation; thus, Notch signaling is permissive and not instructive for gliogenesis¹³ (Figure 1).

During early development, the embryonic spinal cord is patterned throughout the dorsoventral axis by a combination of morphogens—sonic hedgehog (Shh), BMPs, and Wnts—which regulate the combinatorial expression of homeodomain transcription factors¹⁴. These patterning principles govern what type of progenitor cells will be generated from.

The first evidence of patterning during astrocyte development emerged in 2005, when Muroyama *et al.* showed that the basic helix-loop-helix (bHLH) transcription factor *Scl/tal1* (stem cell leukemia, Tal) promotes astrogenesis via a cross-antagonistic relationship with the oligodendrocyte transcription factor *Olig2*¹⁵. At the p2 and pMN domain boundary, the interaction between *Scl* and *Olig2* allowed p2 domain to generate astrocyte precursors while the pMN domain produced oligodendrocyte precursors¹⁵.

When expressed in different VZ domains, the transcription factors Pax6 and Nkx6.1 yield three distinct astrocyte subtypes (VA1, VA2, and VA3, dorsal to ventral) that arise from the p1, p2, and p3 progenitor domains, respectively¹⁶. These populations are further demarcated by differential expression of Reelin and

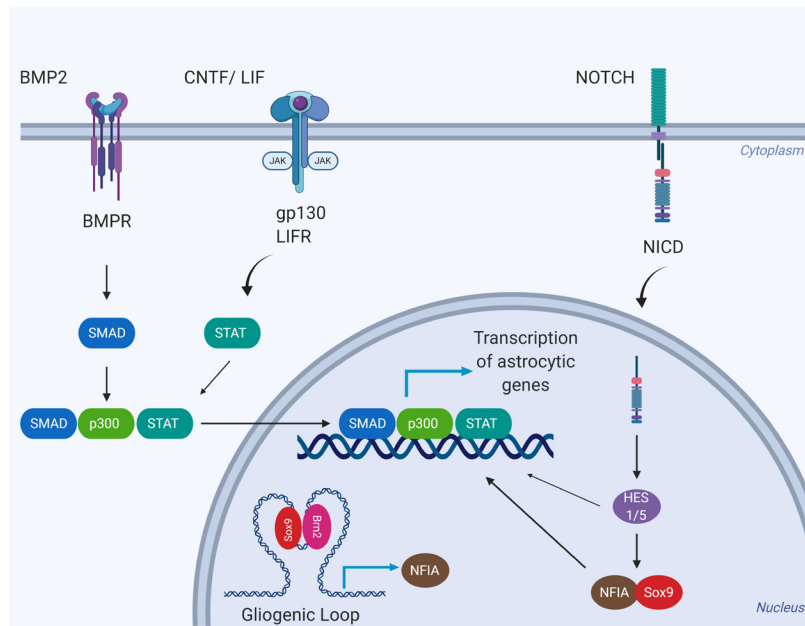


Figure 1. Molecular signaling pathways involved in astrocytogenesis. Neural stem cells (NSCs) express astrocytic genes in response to several signaling molecules, including bone morphogenic protein (BMP) families, the leukemia inhibitory factor/ciliary neurotrophic factor (LIF/CNTF), and Notch pathway. BMPs signal primarily through SMAD, whereas LIF/CNTF activates the JAK/STAT pathways. The active SMAD–STAT complex bridged by p300 goes directly into the nucleus, binds to the promoter, and activates astrocytic genes such as *GFAP* and *S100*. Another important pathway that regulates astrocytogenesis is the Notch pathway. Notch ligands will activate Notch receptors and activate the expression of Hes genes, *Hes 1/5*. Hes1, Hes5, and activated forms of Notch receptors induce the expression of astrocytic genes and glial promoting transcription factor nuclear factor-I A (NFIA). In early development, a pre-formed gliogenic loop serves to facilitate the association between sex-determining region Y-box 9 (Sox9) and brain-specific homeobox/POU domain protein 2 (Brn2), which also drives expression of NFIA. NFIA is both necessary and sufficient for the induction of astrocytic genes.

Slit. Tsai *et al.* used lineage tracing with Cre recombinases to specifically target these domains and demonstrated that astrocytes arise from different segmental domains throughout the brain and spinal cord¹⁷. When specific astrocyte populations were ablated with a Cre-dependent diphtheria toxin A (DTA) approach, the authors observed that neighboring astrocytes from other progenitor domains were unable to migrate, suggesting that there are region-specific neuron–astrocyte interactions following developmental patterns. This domain-specific identity is also observed in forebrain astrocytes, which showed dorsal-ventral restriction depending on the developing region they derived from, as a result of strictly radial migration¹⁷. Overall, these studies illuminate the transcriptional code determining astrocytes' regional identity and suggest that patterning also may lead to functional diversity.

Before terminal differentiation, astrocyte precursors migrate from VZ and SVZ along the radial glial processes. Newborn astrocytes continue to divide locally after migration. Initial studies identified these targeted precursor astrocytes with *Glast*¹⁸, a glutamate aspartate transporter whose induction coincides with the gliogenic switch. A recent study identified new glial markers for the intermediate stages of astrocyte development using a *Glast* reporter mouse, including *Asef*, *Gpr3711*, *Mfge8*, and *Tom111*¹⁹. *Asef* is a guanine exchange factor that has a role in cell migration, and functional studies *in vivo* have shown that *Asef* is required for AQP4 expression on the end-feet of spinal cord astrocytes¹⁹.

Our understanding of the stepwise specification and migration of astrocytes is improving with newly developed tools for cell lineage mapping, spatial and temporal profiling, and functional studies. However, several important questions remain. For instance, which other factors trigger the gliogenic switch in NSCs is an ongoing area of investigation. Furthermore, owing to the lack of markers for astrocyte precursors, it has been difficult to dissect the signaling mechanisms responsible for astrocyte specification and migration. Whether we can use the recently identified and functionally characterized intermediate markers to address these remains an open question.

Astrocyte differentiation

The differentiation trajectory of astrocytes in the postnatal mouse brain remains somewhat controversial^{10,20,21}. In one study, Nagao *et al.* identified astrocyte-lineage restricted progenitors using astrocyte lineage-specific marker *Zbtb20* along with markers that label both astrocyte and oligodendrocyte precursors, *Sox9* and *Olig2*¹⁰. Using immune co-staining, the group identified the presence of *Zbtb20*⁺/*Sox9*⁺/*Olig2*⁺ triple-positive cells in the mouse postnatal day 3 (P3) neocortex and demonstrated that *Zbtb20*⁺/*Olig2*⁺ cells were devoid of *Sox10*, a marker of OPCs¹⁰. However, in other studies, several groups have identified progenitor cells that exhibited bipotent signatures, expressing cell markers of both the astrocytic (*Aldh1l1*, *AldoC*, *Aqp4*, and *Slc1a3*) and oligodendrocytic (*Pdgfra*, *Olig1*, and *Olig2*) lineages by microarray, immunofluorescent co-staining, and single-cell sequencing^{20,21}. This inconsistency may stem

from the current use of a single marker (for example, Aldh1l1 only or GFAP only) to identify astrocytes. Similar to the hematopoietic system, specific lineages may be better characterized by combinatorial codes of multiple cell markers. Recent advances in single-cell sequencing technology should enable us to discover and validate combinations of cell markers that better demarcate the intermediate stages of the astrocyte lineage.

Astrocytes express canonical markers before initiating terminal differentiation. One of the markers expressed at a later stage is glial fibrillary acidic protein (GFAP), which provides structural stability and motility to astrocytes. Several groups generated *GFAP-Cre* mouse lines to target astrocytes, but these lines also target neural progenitor cells^{11–13,18,22}. Although GFAP is commonly used as a marker, it is weakly expressed in protoplasmic astrocytes in rodents. Other currently used astrocytic markers include calcium-binding protein S100²³, glutamate transporter GLAST²⁴, aldolase C²⁵, CD44²⁶, glutamine synthase (GS), and fatty acid-binding protein FABP7. Aquaporin 4 (AQP4) and connexins 30 (Cx30) have been used as astrocyte end-feet markers^{27,28}. These markers are less specific than GFAP, as all of them also can be expressed in neurons, oligodendrocytes, or ependymal cells¹⁴. Glutamate transporter, EAAT2, also known as GLT-1, is expressed in astrocytes and neurons, although 80% of total EAAT2 protein is expressed in astrocytes in the hippocampus²⁹. These markers can be used in a combinatorial approach. For example, Miller *et al.* used a specific promoter region of *GLT-1* to generate a reporter mouse that targets gray matter astrocytes in the cerebral cortex, which interestingly are absent in the hippocampus³⁰. The authors further analyzed the transcriptome of this astrocytic population and determined its unique molecular profile, identifying a pathway-specific to it with the expression of the genes *norrin* and *LRG6*, which have roles in dendritic spine maintenance in this population³⁰. (Also, see the “Molecular maturation” section).

Recent genetic profiling studies identified aldehyde dehydrogenase family 1, member L1 (ALDH1L1), a metabolic enzyme, as the most homogeneously expressed astrocyte marker throughout the brain. Aldh1l1 was reportedly used to stain cortical astrocytes, whereas hippocampal astrocytes are widely stained with GFAP³¹. Morel *et al.* also used a combinatorial approach with the *Aldh1l1-GFP* transgene reporter mouse with the EAAT2-tdtomato mouse line and identified a tdT-eGFP⁺ astrocyte population that is selectively localized at layers I and II in the cortex³¹. Furthermore, this population of cells showed increased resting membrane potential and resistance and reduced potassium channel Kir4.1 expression³¹. The recently developed *Aldh1l1-CreER* transgenic mice should allow astrocyte-specific manipulations³².

Transcription factors NFIA and SOX9 are also non-stage-specific astrocytes markers, although NFIA also is expressed in oligodendrocyte precursor cells and some neurons. On the other hand, Sox9 is not expressed in neurons and recently was identified as an astrocyte-specific marker in the adult brain. Henceforth, Sox9 may be an important tool to access astrocytes in the adult brain^{32–34}.

GFAP expression is used as an indicator of astrocyte maturation and has given significant insight into the mechanisms regulating astrocyte differentiation. Previously, it was used to identify the JAK-STAT pathway and BMPs and Notch signaling as central players controlling astrocyte differentiation from precursor cells^{35,36}. Recently, a large-scale interchromosomal interaction study identified *Brahma-related gene 1* (BRG1), an ATP-dependent chromatin remodeling factor, as clustering with the *GFAP* gene and regulating GFAP expression³⁷.

In addition to these general astrocyte markers, astrocyte markers for anatomically distinct populations have been identified in recent studies. Molofsky *et al.* used the *Aldh1l1-GFP* transgene reporter mouse to identify distinct molecular differences between dorsal and ventral astrocytes and identified Sema3a, an axon guidance protein, as being highly expressed in ventral astrocytes³⁸. Ventral region motor neurons α -MN failed to maintain axon initial segment orientation following loss of astrocytic Sema3a, affecting their survival and function. That study was the first to show how positional cues by diverse astrocytes maintain specific circuitry³⁸. Inwardly rectifying potassium channel, Kir4.1, also was shown to be enriched in astrocytes of the spinal cord ventral horn, which support the survival and function of motor neurons³⁹. Another study compared adult striatal and hippocampal astrocytes and identified μ -crystallin Crym as a striatal astrocyte-specific marker². Although the functional significance of this protein is unknown, it is the first marker that defines a region-specific astrocyte population in the brain. These studies indicate that the molecular and anatomical properties of astrocyte subpopulations may yield insight into their function.

Although our knowledge of astrocyte biology continues to expand, how mature astrocytes are formed and differentiate to carry out their diverse roles remains unclear. Historically, GFAP has been used to understand how external inputs affect astrocyte maturation, even though it is not the most comprehensive astrocyte marker in rodents. In the future, identifying region-specific astrocyte markers may allow us to study specific circuits. In addition, we have not yet identified a developmental endpoint for astrocytes. In the CNS, terminally differentiated neurons and oligodendrocytes are postmitotic while GFAP-expressing astrocytes retain their ability to proliferate (discussed in further detail in the next section).

Astrocyte proliferation

Neurogenesis is complete in most regions of the brain at birth, and the same number of neurons is maintained throughout life⁴⁰. On the other hand, concurrent with brain growth during this period, the number of glial cells increases six- to eight-fold during the first three weeks of postnatal development⁴¹. Given the diverse functions of astrocytes, which facilitate maturation of the neuronal network during this critical developmental period⁴², it is important to understand where, when, and how astrocytes proliferate to reach their final population size.

Over the past few decades, it has become clear that cortical astrocytes are generated from four main sources in successive

yet overlapping chronological order^{43–45}. Each stage of development takes place in a specific anatomical region; astrocyte differentiation first occurs from radial glia in the VZ of late embryonic-perinatal brain (1), followed by astrocytes in the cortex of postnatal brain (2), progenitor cells in the SVZ of postnatal and adult brain (3), and NG2 cells in the cortex of postnatal or adult brain or both (4). However, as discussed above, astrocytes in the developing spinal cord are generated mainly from radial glia and astrocyte progenitors derived from radial glia at an earlier developmental stage. Below, we will focus on developmental astrocytogenesis from the VZ and local proliferation in the cortex, which are major sources of astrocytogenesis and together contribute to about 80% of cortical astrocytes⁴⁵. Meanwhile, we will briefly describe the process of astrocyte proliferation in the spinal cord. For SVZ and NG2 cell-derived astrocytes in the cortex, please see reviews^{43–45}.

Astrocyte generation in the ventricular zone

Radial glia are bipolar cells with their soma residing in the VZ of the embryonic CNS. Radial glia extend and anchor one branch to the ventricular wall and the other to either the pial surface or blood vessels^{46–50}. During the late embryonic stage, radial glia

are the major source of astrocytogenesis, and their bipolar structure provides a scaffold for the migration of newborn astrocyte precursor cells⁵⁰.

Astrocytogenesis from radial glia in the developing cortex occurs in two waves. In the first wave, glia progenitors or glioblasts are derived from the asymmetrical division of radial glia. Glioblasts are proliferative glia progenitors found between the late embryonic (E16–E18) and perinatal stage in the mouse cortex^{50,51}. Upon generation, these glia progenitors migrate radially from the VZ/SVZ and undergo several rounds of proliferation on their way out, giving rise to multiple clusters of astrocytes in the same cortical column of the postnatal cortex^{52–54}. The second wave, which occurs at the terminal stage of differentiation, results from the direct transformation of radial glia. Between the late embryonic and early perinatal stage, radial glia detach their anchorage from VZ and lift their soma toward the pial surface, resulting in unipolar transitional radial glia (tRG). These tRG undergo terminal differentiation to give rise to protoplasmic and fibrous astrocytes in the gray matter and white matter of cortex, respectively (Figure 2a). Using retroviral-mediated lineage tracing, organotypic slice culture, and confocal

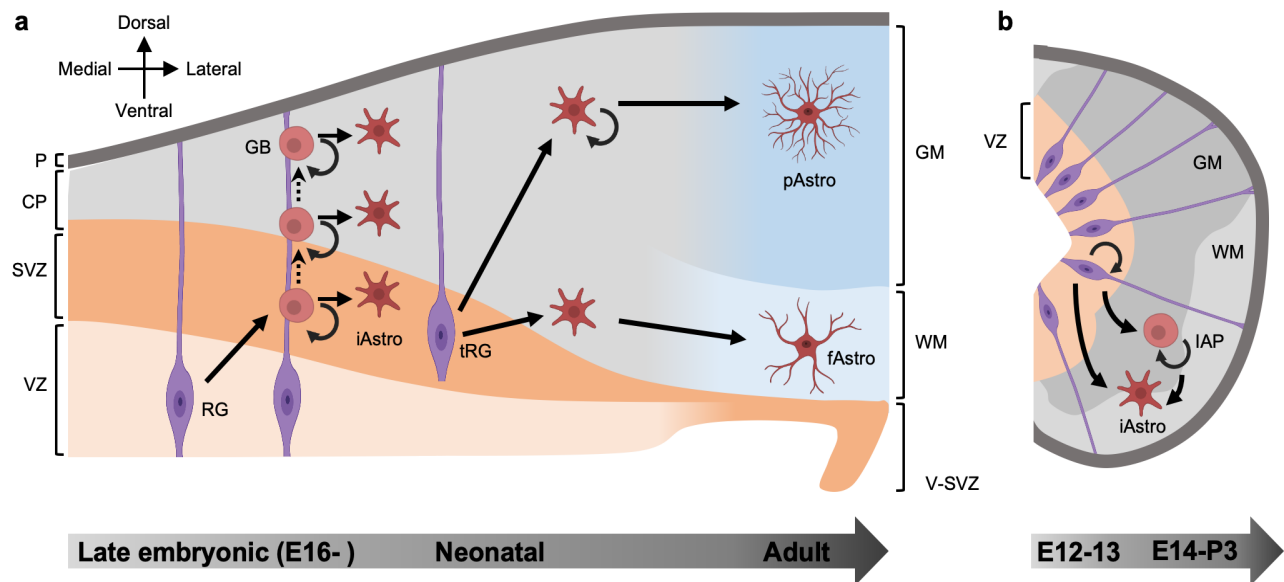


Figure 2. Astrocytogenesis from the ventricular/subventricular zones and outer cortical layers in the mouse developing central nervous system (CNS). (a) In the developing cortex, radial glia (RG) first give rise to glioblasts (GBs) during the late embryonic to perinatal period. Glioblasts undergo several rounds of division while migrating out along radial glia, resulting in clusters of astrocytes in the developing cortex. At the terminal stage of radial glial differentiation, radial glia detach from the ventricular zone and form unipolar transitional radial glia (tRG), which give rise to protoplasmic and fibrous astrocytes in the gray matter and white matter of the cortex, respectively. During the early postnatal period, differentiated astrocytes in the outer cortical layer undergo symmetric division and generate daughter astrocytes that exhibit astrocytic morphology and functions. (b) In the developing spinal cord, radial glia first proliferate during embryonic day 12 (E12) to 13, giving rise to radial glial pool which differentiates into astrocytes between E14 and postnatal day 3 (P3). Alternatively, radial glial cells differentiate into intermediate astrocyte precursors (IAPs), which proliferate during E14 to P3 and undergo terminal differentiation, ultimately giving rise to astrocytes. The progression from embryonic stage to adult is shown from left to right below each panel. Straight arrows indicate differentiation or maturation from one cell type to another. Circular arrows indicate proliferation. Dashed arrows indicate migration. CP, cortical plate; fAstro, fibrous astrocyte; GM, gray matter; iAstro, immature astrocyte; P, pia mater; pAstro, protoplasmic astrocyte; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter.

time-lapse imaging on rat brain slices at E16, Noctor *et al.* demonstrated direct evidence that individual radial glial cells transform into astrocytes at the terminal stage of differentiation⁴⁹. The presence of tRG-derived astrocytes has been seen across several different species, including monkey, ferret, human, and rodents, suggesting an evolutionarily conserved mechanism that controls terminal differentiation of radial glia into astrocytes^{46–50}.

Parallel to findings in the developing cortex, astrocytes in the spinal cord are also derived from radial glia, albeit earlier, between E14 and P3^{17,55,56}. Using *Aldh1L1-GFP* labeling, Tien *et al.* found two types of proliferative astrocyte precursors that exhibit distinct morphology⁵⁶. Radial glia in the VZ were the first precursors identified, and the second were astrocyte progenitors called intermediate astrocyte precursors (IAP)⁵⁶. These IAPs are generated first from radial glia before migrating out of the VZ into the mantle zone, where they undergo several rounds of division before terminally differentiating into spinal cord astrocytes (Figure 2b).

Overall, these studies demonstrate that a conserved mechanism exists between the embryonic cortex and spinal cord, wherein radial glial cells first give rise to lineage-restricted glia progenitors, which then undergo multiple rounds of division to give rise to astrocytes in the postnatal CNS.

Astrocyte proliferation in the outer cortical layer

Early lineage-tracing studies with VZ/SVZ progenitors identified clusters of astrocytes in the postnatal cortex, indicating local proliferation of cells during or after migration or both^{51,52,57}. Although the field long hypothesized that these clusters derived from glioblasts^{50,51,56}, one study found that local proliferation of terminally differentiated astrocytes in the outer cortical layer (I–IV) of the early mouse postnatal brain (P0–P2) may have contributed to some of these clusters⁵⁸ (Figure 2a). These parental cells exhibited astrocyte characteristics, including morphology, gap junction connectivity, and expression of astrocyte markers; however, they underwent symmetrical division to give rise to two functional daughter astrocytes. This symmetrical division was at its highest rate before P6 and decreases over time, resulting in the generation of 50% of total astrocytes at P28⁵⁸. Consistently, Moroni *et al.* identified, in the rat postnatal cortex, astrocytes that expressed differentiated astrocyte markers S100b or *Aldh1L1* that co-expressed proliferation marker *Ki67*⁵⁹. The proliferation rate of these cells increased from P1 to P10 and decreased thereafter⁵⁹. The consistent timeline between mouse and rat suggests a conserved mechanism regulating the proliferative potential of these differentiated astrocytes. Conversely, in the adult SVZ, lineage tracing demonstrated that adult SVZ-derived astrocytes are mostly postmitotic with no signs of local proliferation⁶⁰. These studies suggested that astrocytes derived from different sources may have different proliferative potential once differentiated. Henceforth, it will be important to delineate the mechanisms that endow postnatal astrocytes with their unique proliferative potential.

Despite the above discoveries, several unanswered questions remain in the field. For example, since not all differentiated astrocytes proliferate⁵⁸, it will be important to uncover which mechanisms

drive the transition between dividing to non-dividing astrocytes. Interestingly, Ge *et al.* demonstrated that dividing astrocytes exhibit slight changes in their membrane properties compared with surrounding non-dividing astrocytes, suggesting that dividing astrocytes retain or regain proliferative activity, which could result from local environmental cues or cell-intrinsic molecular mechanisms or both⁵⁸. Indeed, several previous studies identified clusters of astrocytes residing around specific structures of the brain, including blood vessels, the pial surface, and corpus callosum, or specific layers of the cortex^{58,61}, indicating the presence of local environmental cues. As of today, we still know very little about the cell-intrinsic mechanisms underlying the local proliferation of differentiated astrocytes. Recent studies identified that YAP (yes-associated protein), a transcription co-factor of the Hippo signaling pathway, is required for the proliferation of astrocytes in postnatal neocortex via cooperation with the BMP-SMAD signaling pathway⁶². However, more detailed study on the *in vivo* brain is needed. With new single-cell sequencing techniques, we should be able to delineate the transcriptomes of these dividing astrocytes and thus identify signaling pathways and associated environmental cues that alter gene expression to confer these dividing astrocytes with proliferative activity.

Studies during the embryonic stage have delineated gliogenesis during development, but our understanding of postnatal astrocytogenesis is still in the early stage. Answering the above questions should shed light on the mechanisms controlling postnatal astrocyte proliferation, which may be further applied in different contexts, including reactive gliosis and glioma.

Astrocyte maturation

For more than 100 years, astrocytes have been divided into two main subtypes—protoplasmic or fibrous—on the basis of differences in their cellular morphologies and location. During the late phase of astrocyte proliferation, astrocytes undergo morphological and molecular maturation to develop their characteristic “spongiform” morphology and tiny distal processes called perisynaptic astrocytic processes (PAPs). Protoplasmic astrocytes of the gray matter have PAPs with several stem branches, giving rise to fine branches that ensheath the neural synapses and form direct contact with blood vessels. Fibrous astrocytes of white matter have an elongated morphology and are in contact with myelinated axonal tracts and nodes of Ranvier⁶³. Concurrent with morphological maturation, both astrocyte types begin to express functional proteins, including channels and receptors in their membrane, and secrete synaptogenic factors. Below, we summarize the current understanding of morphological and molecular astrocyte maturation and discuss their functional implications.

Morphological maturation

Early studies in postnatal rats delineated the stages of astrocyte morphogenesis in the hippocampus by using intracellular dye filling in fixed brain slices⁶⁴. At P7 and P14, astrocytes appear smaller and less ramified, and a dozen long processes stick out from soma and end with filopodia-like structures. The territory of these astrocytes is not well defined, and long extending branches often invade the “territory” of neighboring astrocytes. By P21, most of the filopodia-like structures disappear and fine distal

processes appear, resulting in more ramified astrocytes with clear territories and processes with limited overlap among neighboring cells. Concurrently, from P7 to P14, astrocyte morphology becomes more homogeneous, and by P28, spongiform, highly ramified protoplasmic astrocytes are abundant (Figure 3a).

As astrocyte morphology matures, a specialized structure called PAP also appears at the distal ends of astrocytes. PAPs are extremely fine (<50 nm) distal processes positioned near synapses^{65,66}. This arrangement allows communication between astrocytes and synapses and originally formed the basis of the concept of the “tripartite synapse”^{64,67,68}. To facilitate crosstalk with the synapse, PAPs exhibit high surface-to-volume ratios, providing ample space for the expression of channels, transporters, and receptors in the membranes, including glutamate transporters GLAST and GLT1⁶⁹, potassium channel Kir4.1⁷⁰, and glutamate receptors mGluR 3 and 5⁷¹ (see the “Molecular maturation” section). With this specialized anatomical arrangement and protein localization, PAPs maintain homeostasis of the local environment at the synaptic cleft^{69,70} and also actively crosstalk with synapses and regulate their function^{68,71}.

Given the close relationship between astrocyte processes and synapses, it is not surprising that neurons or neuronal activity or both participate in astrocyte morphogenesis and PAP plasticity^{41,72–82}. Previous studies demonstrated that astrocytes exhibit reduced territory and neuropil infiltration in dark-reared animals, providing evidence for the involvement of neuronal activity in astrocyte morphogenesis^{73,74}. Several subsequent studies demonstrated that the neurotransmitter glutamate is one molecular mechanism underlying this activity-dependent morphogenesis^{75–77}. For example, using a VGluT1 KO mouse model in which VGluT1⁺ synaptic activity is silenced, Morel *et al.* found that excitatory synaptic activity is required for the growth of astrocyte territory and for perisynaptic astrocyte processes to ensheath synapses⁷⁵. Mechanistically, this crosstalk is mediated by an astrocytic glutamate receptor mGluR5-dependent signaling pathway and downstream intracellular Ca²⁺ activity⁷⁵. The above findings suggest that the local environment guides astrocyte maturation, resulting in astrocyte processes that facilitate neighboring synapses.

Interestingly, a similar mechanism influences the structural plasticity of PAPs in the adult brain. Using time-lapse imaging on organotypic brain slices and anesthetized adult mouse, Bernardinelli *et al.* found that PAPs are highly motile at a time scale of several minutes⁷⁶. This structural plasticity is regulated by synaptic activity and is dependent on astrocyte metabotropic glutamate receptor mGluR1/5. Moreover, astrocytic Ca²⁺ signaling, presumably downstream of mGluR1/5, is both necessary and sufficient for PAP motility. In that study, changes in PAP motility enhanced astrocytic coverage of the synapse and spine stability⁷⁶. Currently, whether glutamate also regulates PAP maturation and motility during development remains unknown. However, the conserved function of glutamate in controlling astrocyte morphology during development and PAP plasticity in adulthood suggests that glutamate might also play a role in PAP development during the postnatal period.

Neurons also regulate astrocyte morphogenesis and PAP plasticity via contact-dependent mechanisms, including neuroligin–neurexin⁷³, Notch signaling⁷², and EphA4/ephrine-A3^{79,80,83}. In the case of neuroligin–neurexin interaction, direct contact between astrocytic neuroligins and presynaptic neurexin is required and sufficient for astrocyte morphogenesis and PAP development *in vitro* and *in vivo*⁷³. Moreover, the morphological effect of astrocytic neuroligins is associated with the development of local excitatory synapses. In the case of EphA4/ephrine-A3, neuronal EphA4 and astrocytic ephrin-A3 interact to maintain the proper structure of spine morphology and also are required for proper expression of glutamate transporters in the astrocytic PAPs that surround the spine^{79,80,83}. Together, the above studies reveal a contact-dependent reciprocal mechanism between astrocytic synapses and PAPs and demonstrate that astrocyte morphogenesis and synaptic formation and function are tightly linked components that coordinate during the critical period of postnatal synaptogenesis.

Although the biological consequences of neuron–astrocyte interactions are well characterized, the downstream mechanisms by which their contact results in morphological changes or PAP plasticity are still limited. Interestingly, there is a plethora of evidence demonstrating the involvement of actin filaments and their regulators in astrocyte morphological changes, including Arp2/3, N-WASP, small GTPase Rho and Rac, and the effector ROCK, though mostly *in vitro*^{84–87}. Henceforth, testing whether similar mechanisms exist under physiological conditions *in vivo* upon neuron–astrocyte contact and mediate astrocyte morphological maturation during development would be necessary.

Molecular maturation

As astrocytes mature, their transcriptional profiles change dramatically to exhibit stage-specific signatures^{14,19,21,34,88,89}. The genes that are induced during astrocyte maturation can be categorized into three groups on the basis of their locations: (1) membrane-bound proteins, including GLT1, Cx43, Cx30, Kir4.1, and Aqp4; (2) cytosolic proteins, including GFAP, S100b, AldoC, and GS; and (3) secretory proteins, including Thbs1, Gpc4, Gpc6, Hevin, and SPARC^{28,42,90–101} (Figure 3b).

The membrane-bound proteins necessary for astrocytic maturation include channels and receptors, and this group of genes is enriched in subcellular structures such as PAPs and end-feet. Expression of these genes offers astrocytes their characteristic functions during postnatal development. For example, the glutamate transporter GLT1 mediates glutamate uptake and regulates glutamate availability in the synaptic cleft, thus regulating synaptic transmission^{102,103}. Cx43 and Cx30 form gap junctions to connect neighboring cells, allowing the exchange of molecules (water, glucose, metabolites, and neurotransmitters) and ions (Ca²⁺, K⁺, and Na⁺) over long distances^{104,105}. Kir4.1 is the major potassium channel of astrocytes and plays an essential role in buffering extracellular potassium built up during action potential. Aqp4 is the most abundant water channel in the brain and is important for water balance and also contributes to synaptic plasticity and learning/memory in the CNS^{27,106}. During the morphological maturation of astrocytes, neurons often induce these genes^{72,75,107–109}.

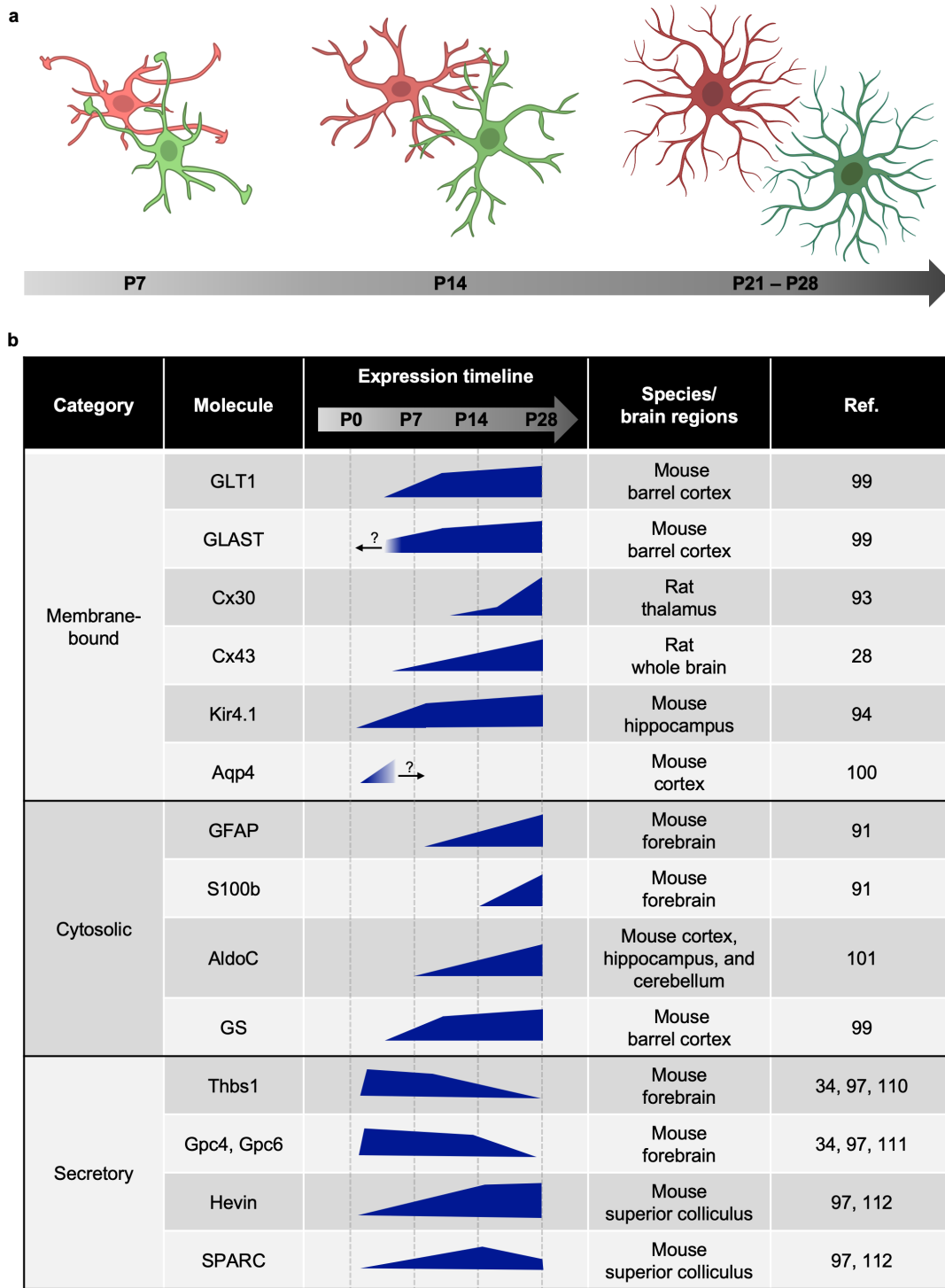


Figure 3. Maturation of astrocytes during postnatal development demonstrated by progressive changes of morphology and gene expression. (a) Morphology of astrocytes matures in a stepwise manner. At postnatal day 7 (P7), astrocytes exhibit few branches and the overall morphology is simple and territories are small. Long protrusions are often seen extending into the territories of the neighboring astrocytes. The boundaries of territories are not well-defined yet. At postnatal day 14 (P14), the number of branches become greater and astrocyte territories become bigger. More branch points grow out of existing branches, resulting in more complex morphology. Long protrusions are seen less, and the boundaries of territories start to emerge. At postnatal day 21 to day 28 (P21–P28), astrocytes develop their characteristic “spongiform” complex morphology, and there is minimal overlap between neighboring astrocytes and clear boundary of each territory. **(b)** Genes that are induced during astrocyte maturation. Blue polygons indicate expression levels of the genes during development. Question marks indicate that the expression level beyond the developmental stage is unknown.

The second group of genes is composed of enzymes and cytoskeleton proteins that serve as markers of mature astrocytes. However, each one of these genes labels only a portion of astrocytes, and there is some overlap in expression with other cell types. Comprehensive characterization and immunohistochemical validation of the timeline and astrocyte specificity of these markers during development remain open avenues of investigation. Furthermore, whether and how cytosolic proteins serve as members of combinatorial codes in different brain regions are interesting areas for future research. (Also, see the “Astrocyte differentiation” section.)

Astrocytic neuroactive molecules that regulate synaptogenesis make up the genetic signature of the third and final stage of astrocyte maturation. These molecules include *Thbs1*, *Gpc4*, *Gpc6*, *Hevin*, and *SPARC*, each of which regulates a different phase of synaptogenesis. These genes are concurrently expressed in astrocytes at corresponding developmental stages. For example, *Thbs1*, which is required for the formation of silent synapses, and *Gpc4/6*, which is required for inducing post-synaptic active synapses, have the highest expression between P0 and P14 in developing astrocytes and are down-regulated thereafter. On the other hand, *SPARC*, an inhibitor of hevin and a negative regulator of synaptogenesis, is elevated only around P14 and remains expressed thereafter^{34,110–112}. The temporal control of these synaptogenic genes suggests a well-defined mechanism that regulates different phases of synaptogenesis.

Despite the above findings, most studies to date have focused on neuron–astrocyte crosstalk via secretory molecules and membrane proteins (that is, glutamate, BDNF, mGluR, Eph–Ephrine, neuroligin–neurexin), and very little investigation of the upstream transcriptional mechanisms governs their expression. Thus, how transcription programs reciprocally respond to neuronal signaling is an important area of future research.

Borrowing a concept from neurons, astrocytes may respond to external environmental cues and tailor their course of maturation to the local environment via transcriptional regulation. With more advanced technology, including single-cell sequencing, super-resolution microscopy, *in vivo* live imaging, membrane-bound optical sensors for neurotransmitters and ions,

fluorescence resonance energy transfer techniques, and three-dimensional culture systems that reproduce astrocyte morphology *in vivo*, we should be able to increase our understanding of the mechanisms that mediate crosstalk between neurons and astrocytes, and that facilitate maturation of astrocytes into the right subtype, at the right time and place.

In the future, it will be interesting to answer the following questions: (1) Do any cell types other than neurons (for example, neighboring astrocytes, endothelial cells, oligodendrocytes, or oligodendrocyte precursors) contribute to astrocyte maturation? (2) How does transient Ca^{2+} , an indicator of mature astrocyte activity, develop over this postnatal period? (3) Does Ca^{2+} activity also mediate astrocyte–neuron crosstalk to facilitate the co-maturation of PAPs and synapses? When addressing these questions, we should bear in mind that astrocytes are very responsive cells that preserve certain progenitor properties even in their mature form, a characteristic supported by recent studies looking into the transformation of reactive astrocytes in response to injury and trans-differentiation into neurons *in vivo*^{113,114}. Thus, the astrocytic developmental program may be defined by proliferation, differentiation, and maturation over time, and their final refinement is regulated by the local environment, which gives rise to the optimal number, function, and morphology of astrocytes.

Closing remarks

Since Virchow first discovered astrocytes more than a hundred years ago, much progress has been made in our understanding of the processes by which they are specified, migrate, proliferate, and mature. However, there remain significant gaps in our characterization of these processes and our knowledge regarding molecular mechanisms underlying astrocyte phenotypes. Some crucial outstanding questions include (1) which upstream signaling pathways regulate the proliferation of astrocytes in the postnatal cortex? (2) Do transcription factors serve as immediate early genes that govern astrocyte maturation in accordance with the local synaptic environment? (3) Is Ca^{2+} activity mature during the period of astrocytic morphological and molecular maturation? With the recent explosion of knowledge and tools in the glial field, the future is bright, and we are looking forward to a better understanding of astrocyte development and the mechanisms underlying it.

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