Astrocyte identity: evolutionary perspectives on astrocyte functions and heterogeneity

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The development of new animal models, *in vivo* isolation approaches, and improvements in genome-wide RNA expression methods have greatly propelled molecular profiling of astrocytes and the characterization of astrocyte heterogeneity in the central nervous system (CNS). Several recent reviews have comprehensively discussed the molecular and functional diversity of mammalian astrocytes. In this brief review, we emphasize interspecies comparisons and an evolutionary perspective regarding the astro(glia) of vertebrates and invertebrates which are similar in form and function. This analysis has revealed conserved astrocyte transcriptomes in the fly, mouse and human. We also offer opinions about the pattern and origin of astrocyte heterogeneity in the CNS.

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The central nervous system (CNS) is the most complex and sophisticated biological system, controlling physiology and behavior in phylogenetically diverse animal species. As a consequence of this complexity, multiple classes and unique subtypes of electrically excitable neurons cooperate with functionally unique glial cells to control CNS development, physiology and behavior. Whereas numerous studies have documented unique neuronal cell subtypes, only recently have investigators begun to do the same for glial cells of the CNS. In organisms ranging from worms and insects to rodents and humans, the glia to neuron ratio increases with nervous system complexity [1], suggesting that the expansion of glial cells is particularly correlated with advanced CNS functions. Astroglia, in particular, are the most abundant glial cells in the mammalian CNS and they play versatile roles in regulating synaptogenesis, modulating synaptic transmission, maintaining the integrity of the blood-brain barrier, providing trophic and metabolic support, and contributing to patterns of neuronal network activity [reviewed in Refs. [2,3]]. Although astrocytes have been historically regarded as homogenous cell populations, in part due to their non-electrically excitable nature, it has become increasingly evident that astrocytes exhibit significant functional and molecular heterogeneity [4]. As distinct brain regions and neural circuits often contribute to a specific physiological or behavioral function, it is important to understand how interregional astrocyte heterogeneity might influence such functions. The morphological and functional diversity of astrocytes is thus an important topic in glial biology, and studies in this area have been greatly facilitated by the recent development of new animal models and improvements in methods for measuring genome-wide RNA expression.

This review does not intend to provide a comprehensive summary of glial biology or astrocyte heterogeneity, both of which are the subject of recent reviews [5–8]. Instead, we will emphasize interspecies comparisons and an evolutionary perspective regarding (astro)glia diversity. We also offer opinions about the pattern and origin of astrocyte heterogeneity in the CNS.

Insects as genetic models in studying astrocyte heterogeneity

We think it is worthwhile to consider astrocyte diversity not only in mammals but also in invertebrates given the evidence for similarity of form and function in the different species, and the utility of certain insects as genetic models. The adult Drosophila brain contains multiple classes of glia with many subclasses located throughout the brain; certain classes, including astrocyte-like glia (ALG), have morphological and molecular similarities to their mammalian counterparts [9–13,14••]. Similar to mammals, fly astrocytes exhibit developmental tiling (inhabiting largely non-overlapping brain areas) [15], have processes covering a wide anatomical territory that contact many neuronal synapses, and are electrically-coupled by gap junctions, but are not electrically excitable, instead employing Ca^{2+}-dependent signaling mechanisms [16,17••,18•].

Conserved mechanisms for astrocyte differentiation between fly and mammals

Although certain factors including glial cells missing (*gcm*) — which specifies the fly glial lineage [reviewed in Refs. [19,20]] — do not function in mammalian glial development, we think the available evidence nonetheless supports the idea of a common evolutionary origin for vertebrate and invertebrate astroglia [reviewed in Ref. [8]]. This evidence includes the conservation of mechanisms regulating the differentiation and maturation of fly and mammalian glial cells. From *in vitro* and *in vivo* studies in flies and mammals, a variety of intercellular signaling pathways, including Notch, Hedgehog (Hh)/ Sonic Hedgehog (Shh), Fibroblast Growth Factor (FGFs) and Bone Morphogenetic Protein (BMP) signaling have been implicated in astrocyte proliferation, migration and maturation [reviewed in Refs. [6,8,21]]. Hh/Shh signaling from neurons, for example, is known to regulate glial cell precursor proliferation and migration in Drosophila and mammalian eye development [22–24] and is important for functional diversification of astrocyte subtypes in a variety of adult mouse brain regions [6,25]. FGFs and BMPs have been shown to promote survival and/or maturation of immature cultured rodent astrocytes [26,27]. *In vivo* studies demonstrate that FGF promotes Drosophila glial cell migration/differentiation [28] in the developing fly eye as well as the later morphological maturation of astrocytes in the fly visual system and central brain [14[••],29].

Similar gene expression profiles of fly and mammalian astrocytes

The observed similarity of fly and mammalian astrocytes is also reflected in their comparable gene expression profiles [30[••],31[•],32[•],33[•]]. For this review, we analyzed astrocyte-expressed fly, mouse and human genes [31[•],32[•],33[•]], revealed by Translating Ribosome Affinity Purification (TRAP) methods for mouse and fly or FACS analysis for humans. Comparison of the three species indicates that 900 of 2623 astrocyte-expressed fly genes (those with >500 reads) have mammalian orthologs that are also known to be expressed in astrocytes. Figure 1 depicts conservation of astrocyte processes and functions among flies, mice and humans, and Table 1 lists representative orthologs within several interesting functional categories that are detected (FPKM > 1 for mammals) in astrocytes of the three species. Notably, important transcription factors, ion channels, transporters (including mouse GLT1, GLAST and GAT-1) and potential gliotransmission regulators are found in all species. Of interest, it is known that several factors listed in Table 1 are required for normal fly behavior [31[•]]; see footnote to Table 1]. Not shown in Table 1 is a mammalian astrocyte-expressed gene [30[••]] called Acsf4-U26 (formerly Aasdh; [34]). Acsf4-U26 has a fly ortholog but it is also homologous to fly *ebony*. The *ebony* gene encodes a glial non-ribosomal peptide synthetase that conjugates β-alanine to amines; it is important for aminergic neurotransmitter

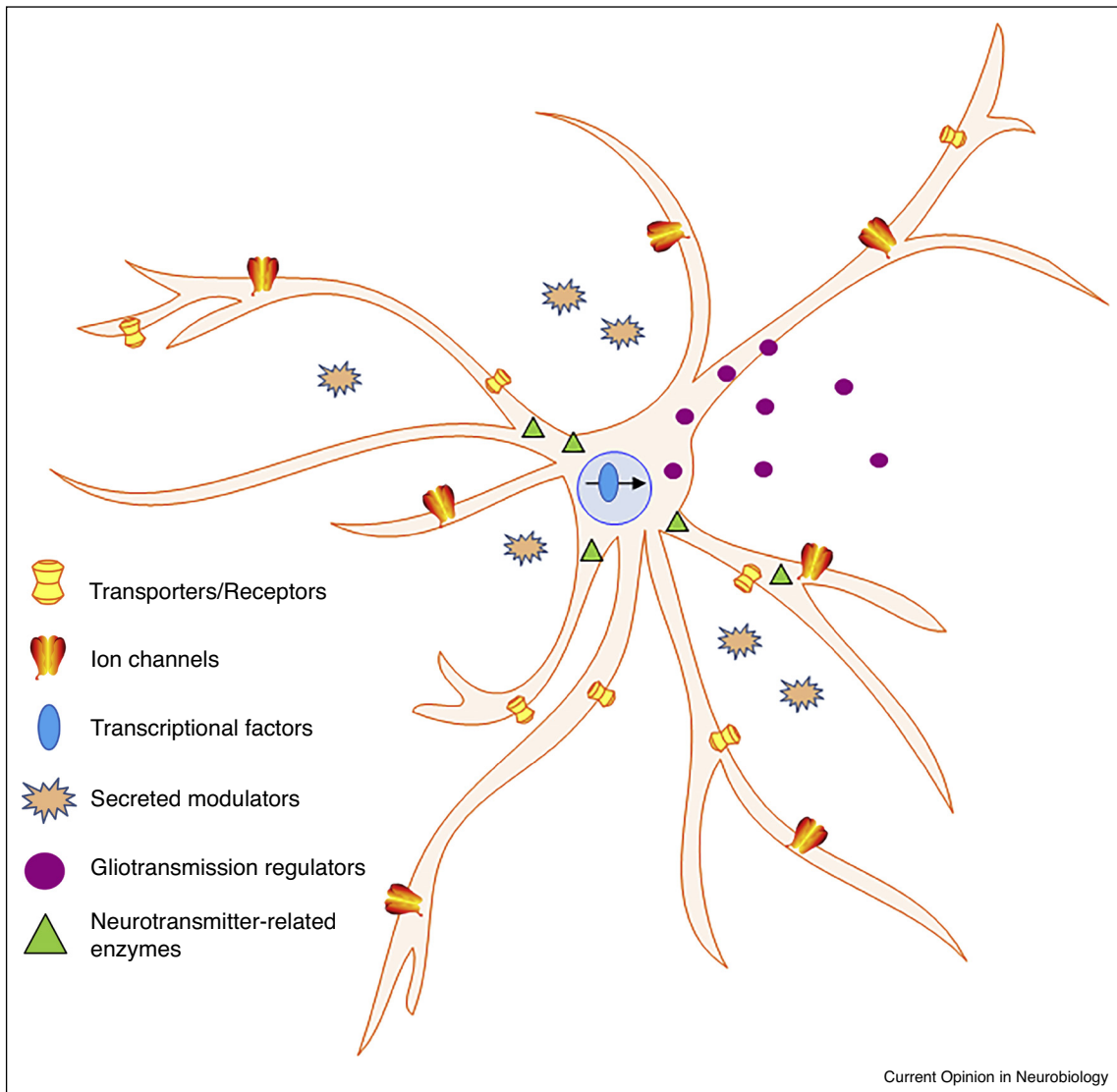recycling [35] and circadian behavior [36]. The Ebony vertebrate ortholog (ACSF4-U26) has β-alanine-activating activity, although the other substrate does not appear to be an amine [34]. Nevertheless, it may have an interesting post-translational function in mammalian astrocytes related to behavior.

Inter-regional and intra-regional mouse astrocyte diversity

There is ample evidence for astrocyte heterogeneity within the mammalian brain [33[•],37,38[••],39[•],40[•],41[••]], whereas such studies have only begun in Drosophila. Mature protoplasmic astrocytes from cortex and hippocampus possess substantially more branch arborization than astrocytes from subcortical regions such as the hypothalamus [42,43]. The overall domain size of cortical/hippocampal astrocytes is also significantly greater than that of hypothalamic astrocytes [42,43]. Astroglial expression levels of the GLAST and GLT1 glutamate transporters are CNS region-dependent [44,45[•],46]. Physiological measures of astrocyte function including gap-junctional coupling [47] and Ca^{2+} responses [38[••],48] also differ in a region-dependent manner. Within the brain and spinal cord, astrocyte heterogeneity exhibits an interesting dorsal to ventral pattern. Dorsal Bergmann glia and ventral velate astrocytes in the cerebellum show divergent expression patterns for a number of peri-synaptic astroglial proteins, such as GLAST, Kir4.1, and GLT1. GLAST, for example, is highly expressed in dorsal Bergmann glia but is almost undetectable in velate astrocytes [49[•]]. Based on the use of TRAP methods, it was shown that astrocytes from dorsal cortex and hippocampus have mRNA expression profiles that are quite different from those of ventral thalamic and hypothalamic astrocytes [33[•]]. Similar findings were observed for two distinct groups of telencephalon astrocytes using a single-cell RNA-seq approach [41[••]]. And, the profiles of mouse spinal cord astrocytes in the dorsal and ventral horns are significantly different Molofsky *et al.* [65].

In addition to interregional variation, astrocytes from a single brain region, for example the cortex, have been shown to display dorsal-to-ventral variations in morphology, physiology, and mRNA transcriptome. The recent study of Lanjakornsiripan *et al.* [40[•]] investigated cortical astrocyte heterogeneity and found layer-specific molecular and morphological diversity for this population of astrocytes. Based on the use of astrocyte expression reporters (Bac *aldh1l1*-eGFP and *eaat2*-tdTomato) mice, it was also recently found that three astrocyte subpopulations exist in the cortex [50[•]]. These subpopulations exhibited distinct cortical locations as well as physiological and molecular properties. Strikingly, expression of Kir4.1, an important astrocyte ion channel, is significantly underrepresented in one cortical astrocyte subpopulation that is located primarily in layers I-II. In contrast, the

Figure 1

Representative conserved gene functions in fly, mouse and human adult astrocytes.

enpp2 and *ptgds* genes are uniquely and highly expressed in layer I-II astrocytes.

Diversity of Drosophila ALG

Astrocyte heterogeneity is also observed in the nervous systems of invertebrates although it has not been explored in detail. Similar to mammals, for example, there is evidence for interregional variability in the morphology and functional diversity of fly glial cells, including astrocyte-like glia (ALG) of the fly optic lobes in the adult visual system. This is a particularly well characterized region of the fly nervous system, consisting of the lamina, medulla, lobula and lobula plate. Within the lamina, for example, ALG associate with fly photoreceptors and their targets, forming visual system columns [12,51•]. Within this region, the shape of glial cells and the number of processes associated with columns varies depending on the depth of cells within the lamina [12], indicative of heterogeneity. ALG are also observed in the optic medulla with multiple subtypes including so-called chandelier and serpentine glia that are resident in the proximal and distal medulla, respectively. Similarly, such glia can be observed in the optic lobula and lobula plate. Although a Gal4 driver known as alrm-Gal4 [10] is commonly employed to label or perturb fly ALG, it has been noted that the optic lobe expression pattern does not include cells of the lamina [52], again highlighting diversity among fly ALGs. The use of additional Gal4 drivers has highlighted significant interregional variability for ALG of the adult fly brain. Kremer *et al.* [51•]

Table 1

Representative conserved orthologs with expression in fly, mouse and human adult astrocytes. The human gene name is shown in the first column

Transcription Factors	Encoded Function(s)
Max	Myc-associated factor; cell proliferation
Sox5,6	CNS development & determination of cell fate
Cbx1,3,5	Histone-binding proteins
Jun/FosB	Major early-gene TFs
Crebl2	cAMP response element (CRE)-binding protein
Transporters/Receptors	
Slc1a3 (Glast); Slc1a2 (Glt-1)	Major astrocyte glutamate transporters
Slc6a1 (fly Gat [a])	Membrane GABA transporter
Gaba-a-associated	Links GABA-A receptors to cytoskeleton
Gabarb1	GABA-B receptor subunit
Aqp4	Aquaporin 4 water channel
Npc1,2 [a]	Cholesterol transporters
ApoD	Transport of sugars and other factors
Egfr	Epidermal growth factor receptor
Ion Channels	
Kcnj10,16	Potassium (K+) channels; K + buffering
Grina	NMDA receptor-associated protein
Vdac1	Anion channel; cell volume regulation
Kctd3	Regulator of cyclic nucleotide-gated channel
Stim1	Membrane Ca^{2+} channel; store-operated Ca^{2+} entry (SOCE)
Secreted Extracellular Proteins	
Tgfβ2	TGFβ receptor ligand
Sparcl1	HEVIN; regulator of syaptogenesis
Sparc [b]	Antagonist of HEVIN; regulates synaptogenesis
Spon1	Axon growth, guidance
Gliotransmission-related	
Snap23,25	Regulators of neurotransmitter release
Synj1	Regulates membrane phosphatidylinositol-4, 5-bisphosphate
Syt11	Ca^{2+}-dependent regulation of synaptic transmission
Vamp2,3,4,7	Synaptic vesicle docking and fusion
Stxbp1 (fly Rop [a])	Regulation of neurotransmitter release
Vti1b	Vesicle trafficking
Neurotransmitter-related Enzymes	
Abat (fly Gabat [a])	GABA transaminase
Glud1	Glutamate/Glutamine regulation
Many Rab family members	GTPase; vesicle trafficking & secretion

Table 1 (Continued**)**

Neurotransmitter-related Enzymes	
Glul (fly Gs1)	Glutamine synthetase
GNAQ	Membrane bound GTPase linked to GPCRs

[a] Deficits cause altered locomotor activity levels or circadian behavior in Drosophila.
[b] A single Sparc is found in the fly genome.

characterized thousands of different Gal4 drivers to identify those expressing in subpopulations of ALG (and other major glial cell classes) that vary in shape and position.

In the fly optic medulla, there is obvious morphological variability in cell and process alignment among ALG [14[••],51[•]], and this variability seems to depend on the depth of cells within the medulla. Based on careful morphometric analysis of medulla ALGs, Richier *et al.* [14[••]] showed that ALG of the medulla (astrocyte-like medulla neuropil glia or mng) can be categorized into at least 4 subtypes that are located in different regions. Their studies used the 'Flybow' genetic system [53] to generate stochastic labeling of astrocytes with combinations of fluors, making it possible to visualize individual cells with distinct positions and morphologies. Importantly, all variants expressed GABA transaminase (GAT), the dEAAT1 glutamate transporter and glutamate synthase 2 (Gs2), indicative of astrocyte-like cell types.

Diversity of clock-containing Drosophila ALG

It is known that glial cells, particularly astrocytes, of mammals and flies are critical for the function of neuronal circuits controlling rhythmic behaviors such as sleep and circadian behavior. In mammals, astrocytes regulate sleep (homeostasis) via adenosine and an adenosine receptor-mediated mechanism [54]. In contrast, wakefulness is modulated by cholinergic stimulation of astrocytes from septal neurons, resulting in glial D-serine release and action on neuronal NMDA receptors [55]. In both mammals and flies, circadian behavior is modulated by astrocytes, which like clock neurons contain PER-based oscillators [reviewed in Ref. [56]]. Perturbation of fly astrocytes results in arrhythmic behavior [36,57,58,31[•]] whereas the clock in astrocytes of the mouse suprachiasmatic nuclei (SCN) actually contributes to the determination of circadian period [59,60]. Thus, it is of interest that glial cells of the fly optic lobes with astrocyte-like characteristics are heterogeneous with regard to expression of PERIOD (PER), an important circadian clock protein [61]. To our knowledge, glial variation in clock protein localization has not been studied in mammals. In Drosophila, however, PER expression is significantly

higher in distal medulla neuropil glia (dmng) than in lamina epithelial glia (lg). In dmng cells, Glial PER oscillates in abundance, and cycling is, at least in part, regulated by release of a peptide neurotransmitter (PDF) from important circadian clock neurons. Thus, similar to mammals, heterogeneity among fly ALG can result from the action of neuronal factors that impact glial physiology.

Mechanisms regulating astrocyte diversity

The dorsal-to-ventral (DV) variation observed for mammalian astrocyte gene expression profiles provides an important clue as to how astrocyte heterogeneity might arise. Early embryonic development shows a clear DV (and posterior to anterior) axis patterning, from which neural progenitors differentiate into distinct neural cell types. Astrocyte fate-mapping experiments in spinal cord and forebrain confirmed that astrocytes are derived in a region-restricted manner from radial glia's domains of origin, similar to neurons [62,63••]. Therefore, astrocyte heterogeneity may arise, in part, from intrinsically defined signals that serve to regulate the early embryonic dorsal to ventral (and posterior to anterior) axis patterning. This notion is further supported by the recent observation that telencephalon and non-telencephalon astrocyte populations, with distinct molecular profiles, occupy different brain areas with very little overlap [41••].

Given the intensive interaction between neurons and astrocytes and the temporal differentiation of these two cell types during development, it is not surprising that neuronal (and other extrinsic) signals also influence-specific molecular and functional properties of astrocytes in each brain region or nucleus during the postnatal astrocyte maturation phase. It was previously demonstrated that loss of VGluT1 neuronal glutamatergic signaling selectively affects the morphological and molecular maturation of cortical, but not hypothalamic astrocytes [43]. The role of neuronal signals in directing localized astrocyte molecular and morphological features is further demonstrated by the selective deletion of *Dab1* in cortical neurons, which disrupts layer-specific neuronal migration during early development [40•]. In neuronal *Dab1* conditional knockout mice, the normal layer-specific branch arborization pattern of cortical astrocytes (more arborization in layers II-III relative to IV) and their molecular expression differences (lower Lef1 and Id1 expression in layers II-III) were abolished [40•]. Additionally, there is differential expression of several transcription factors (*emx2, lhx2,* and *hopx*) in astrocytes of distinct brain regions [33•]. As transcription factor NF1A mediates neuronal JAG1 and DLL1-dependent activation of Notch signaling to promote gliogenesis during early development ([64], Namihira *et al.* [66]), it is conceivable that these differentially expressed transcription factors (*emx2, lhx2,* and *hopx*) may respond to localized and specialized neuronal signals and subsequently contribute to astrocyte heterogeneity during postnatal development.

With regard to neuronal signals regulating heterogeneity, Kremer *et al.* [51•] have demonstrated variability in the thickness of fly ALG processes that is correlated with the particular neuropil they invade. Remarkably, even processes of the same cell can differ in thickness when they infiltrate adjacent neuropils, and this structural diversity is correlated with the density of invaded neuropil in different anatomical regions. This suggests that certain aspects of glial cell morphology may be determined by local synaptic density.

Concluding thoughts

While substantial progress has been made in understanding the molecular heterogeneity of astrocytes, it is important to note that most mRNA profiling studies have employed populations of astrocytes (generally >100 000 cells), isolated using a cell surface marker (i.e. by immunopanning), a genetic fluorescence reporter (fluorescent activated cell sorting) or by association with ribosomes (translating ribosome affinity purification) [31•,33•,32•]. Therefore, these results reflect average mRNA profiles for groups of cells. In contrast, recent developments in single-cell RNA-seq methods are beginning to provide an unprecedented opportunity to examine molecular heterogeneity in individual astrocytes that reside in neighboring functional nuclei or neuronal circuits. Indeed, a recent comprehensive profiling of adult mouse central and peripheral nervous systems, using single cell RNA-seq, has classified seven distinct and regionally-restricted astrocyte subtypes [41••]. In addition to the highly specialized astrocyte subtypes of the cerebellum (Bergmann glia, ACBG), olfactory bulb (ACOB), and dorsal midbrain (Myoc-expressing, ACMB), this study characterized two astrocyte subtypes in the telencephalon (ACTE1 and ACTE2) and two subtypes in non-telencephalon brain regions (mostly diencephalon, ACNT1 and ACNT2) [41••]. The telencephalon/diencephalon differences in astrocyte expression profiles revealed by single-cell RNA-seq is consistent with previous studies using the TRAP approach with populations of astrocytes [33•]. These results further support the notion that astrocyte subtypes may be influenced by developmental patterning. On the other hand, the expression pattern differences between ACTE1 versus 2 or ACNT1 versus 2, identified using single-cell RNA-seq, also suggest the involvement of extrinsic signals, likely the local neuronal environment during postnatal development.

While such techniques have revealed a great deal about conserved genes that are expressed in astrocytes of the fly, mouse and human (Figure 1, Table 1), a large percentage of such genes remain poorly annotated and their molecular functions are unknown. Thus, it is equally important to develop new experimental reagents and approaches to explore the functions of these astrocyte-expressed genes. Such studies will ultimately provide new insights about the diverse roles of astrocytes in the CNS.

Conflict of interest statement

Nothing declared.

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