REVIEWS

Revisiting Functional Heterogeneity of Microglia and Astroglia

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Abstract—Neuroglia is an important component of the nervous system, whose role in the brain has recently been actively revisited. In addition to maintaining homeostasis of the central nervous system, glial cells are involved in the pathogenesis of multiple brain diseases, which makes their further study highly relevant translationally. With the development of novel research methods, data on a greater heterogeneity of glial cells are becoming available, calling for revision of the existing classifications of microglia and astroglia, as some of them do not fit into the current binary paradigm. Here, we discuss cross-taxon features of microglial and astrocytic cells in mammals and zebrafish, and recent data on glia in normal and pathological conditions, which may form the basis for new systematics of neuroglia and, eventually, can help identify novel therapeutic targets.

INTRODUCTION

Neuroglia are an important constituent of the nervous system, representing a heterogeneous group of cells including astrocytes, microglial cells, oligodendrocytes, ependymocytes, and their progenitors [1]. For a long time, the main function of neuroglia has been considered to provide vital activity, nutrition, and support for neurons. However, the understanding of the biological role of neuroglia has recently significantly expanded [2, 3], encompassing a modulation of neuronal activity [4], neurotransmission [5, 6], as well as pro- and anti-inflammatory processes in the brain [7].

Microglial cells are a population of resident brain macrophages that both perform immune function and modulate synaptic plasticity, neuronal activity [8–12], as well as contribute to the pathogenesis of multiple central nervous system (CNS) diseases. Specifically, microglia express many genes linked to Alzheimer's and Parkinson's diseases, Rett syndrome, schizophrenia, autism, and multiple sclerosis [13–17, 18]. A characteristic feature of microglia is a pronounced transformation in response to CNS pathology, when microglial cells assume an amoeboid/boloid form, migrate to a lesion site, and phagocytize pathogens [19, 20]. Prior to the advent of immunological and molecular research methods,

morphological transformation of microglia was considered a primary sign of its activation in CNS pathology [21, 22], transitioning from the anti-inflammatory (M2) to pro-inflammatory/cytotoxic (M1) phenotype [23]. However, it has now been shown that microglia are also active in the healthy brain, while their morphophysiological features most likely reflect changes in their functions, collectively calling for the revision of the existing, and creation of new, classifications of microglia [24].

The problem of astrocyte classification is also relevant because reactive microglia incite the "activation" of pro-inflammatory A1 astrocytes [25] with the decrease in protective (by analogy to M2 microglia) A2 astrocytes [26]. There is also a problem of cross-taxon homology between micro- and astroglia subtypes, requiring an insight into morphofunctional features of these cells in different vertebrates. Here, we examine the status quo of research on micro- and astroglia in mammals (rodents) and zebrafish (*Danio rerio*), aiming to form the basis for a more comprehensive systematics of neuroglia. This may help obtain a realistic picture of the role of glial cells in normal and pathological CNS processes, eventually identifying novel therapeutic targets.

NEUROGLIA ONTOGENESIS

In mice, microglia as resident brain macrophages [27] arise from three distinct developmental pathways [28] (Fig. 1). In the embryonic period, they derive from erythro-myeloid progenitors (eEMPs) with the c-Kit^{lo}CD41^{lo} phenotype characterized by a low expression of c-Kit receptor tyrosine kinase and integrin alpha-IIb (CD41). In mice, this cell phenotype arises in the yolk sac on embryonic day 8, i.e. before the *anlage* of other glial cell types [29]. Then, these cells give rise to premacrophages (PMPs), which infiltrate the nascent brain through the vasculature [30]. Other macrophages in the adult CNS, shielded by the blood-brain barrier (BBB), also derive from eEMPs, whereas most macrophages outside the CNS are replaced by the first hematopoietic wave from myb-dependent EMPs [31].

In mice, definitive hematopoiesis begins on embryonic day 11 with the generation of hematopoietic stem cells (HSCs), which (like EMPs) first localize in the fetal liver and then in the bone marrow [32]. Microglia originating from EMPs persist throughout adulthood [32], but some of their subpopulations may arise on embryonic day 13 due to the second wave of definitive hematopoiesis [33]. Interestingly, the acquisition of microglia identity in situ is driven by local tissue-specific factors [34], including transforming growth factor beta (TGF-β) [35], whereas the characteristic features of microglia (marker gene expression and epigenetic tags) are rapidly lost when culturing cells ex vivo [36, 37].

A study of microglia origin in the zebrafish demonstrated the contribution of three potential microglia progenitors (eEMP, EMP, HSC) whose spatial and temporal distribution is easier to determine in this model object than in mice because of the transparency of fish embryos [38]. Microglia in zebrafish embryos originate from c-myb-independent eEMPs, but are replaced after birth by c-myb-dependent cells originating from HSCs [38]. Although it is yet to be shown whether fish microglial populations derived from eEMPs and HSCs differ functionally, the ontogenesis of brain macrophages may not only be more intricate than previously thought but also differ significantly across taxa.

Radial glial cells (Fig. 2), which develop from the neuroepithelium and represent the primary neural stem and precursor cells, are considered to be astrocyte precursors in the mammalian nervous system [39]. They localize in the ventricular zone of the brain, and being driven by the regional signals, such as dorsal bone morphogenetic protein (BMP) and ventral sonic hedgehog (SHH) protein, differentiate into different subtypes of progenitors capable of generating neurons, astrocytes, oligodendrocytes, and ependymocytes [40, 41]. This regional diversity of progenitor cells not only underlies the heterogeneity of neurons but also ensures the development of glial subtypes at later stages of ontogenesis [42].

MICROGLIA

Traditional classification

Microglial cell activation can be triggered by exogenous signals, e.g., pathogen-associated molecular patterns (PAMPs, infectious molecular motifs derived from pathogenic bacteria and viruses and including a large variety of such molecules as proteins, lipopolysaccharides, RNA species, etc. [43]), or endogenous signals, e.g., damage-associated molecular patterns (DAMPs, nucleotides, protein
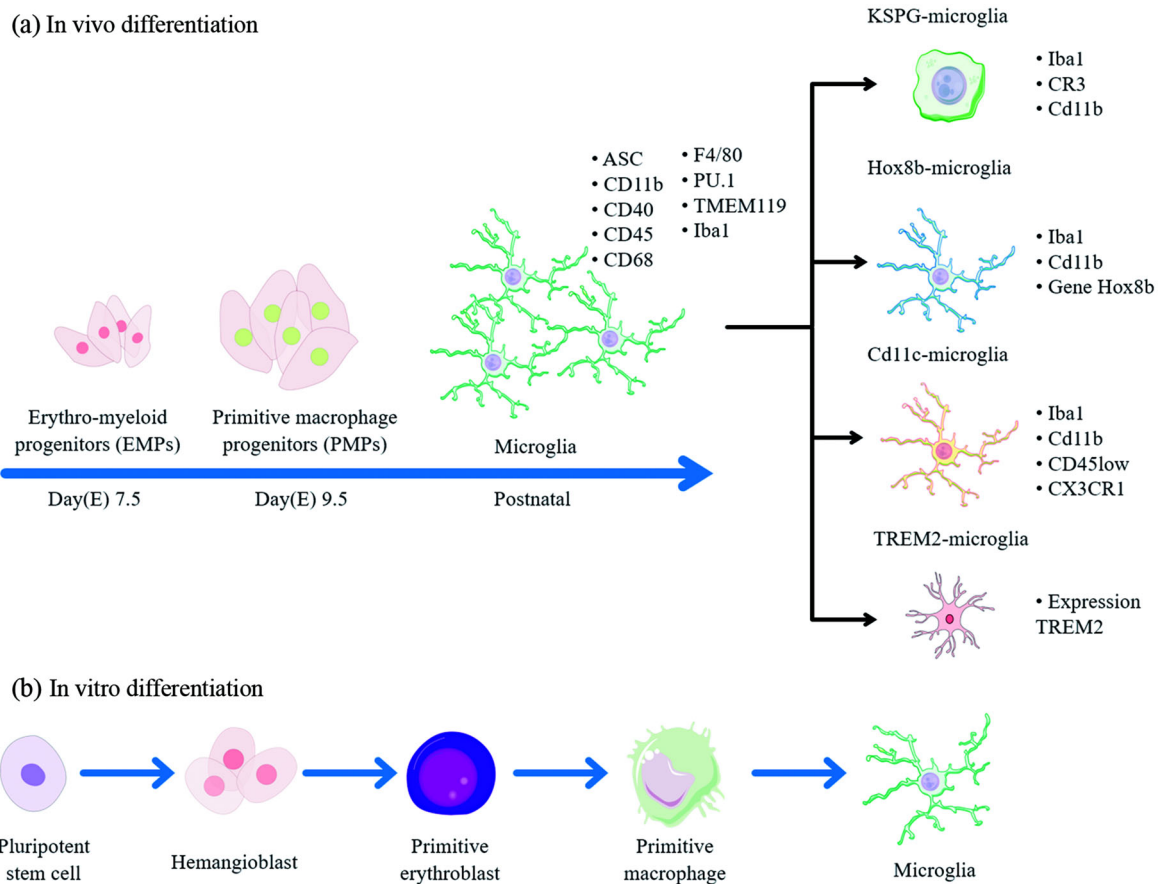
Fig. 1. Putative ontogenesis of microglial cells in rodents (a) and in in vitro models (b). Microglial cells derive from erythro-myeloid progenitors (eEMP) arising on embryonic day 8 in the yolk sac. These cells give rise to the generation of premacrophages (PMF), which infiltrate the primordial brain through the vasculature and subsequently differentiate into different subtypes of microglial cells. Note the microglial cell subtypes identified by the differential expression of the following markers (also see Table 1): Iba1—ionized calcium-binding adapter molecule 1, CR3—complement receptor 3, CD11b—integrin alpha M, Hox8b—homeobox protein, CD45—protein tyrosine phosphatase receptor type C, CX3CR1—CX3C motif chemokine receptor 1 (fractalkine receptor), TREM2—triggering receptor expressed on myeloid cells 2. The protocols for in vitro microglia differentiation (b) begin with reprogramming the induced pluripotent stem cells (iPSCs), originating from the blastocyst inner cell mass, through overexpression of several key transcription factors, followed by differentiation into hemangioblasts, primitive erythroblasts, and primitive macrophages. Some modern protocols enable iPSC transformation into neurons, astrocytes, and oligodendrocytes.

aggregates, including amyloid-beta plaques), as well as cytokines secreted by microglial and astroglial cells [44, 45].

Macrophage polarization to the pro-inflammatory M1 phenotype is activated by the JAK/STAT1 signaling cascade in which STAT1 (signal transducer and activator of transcription 1) [46] activates interferon regulatory factor 5 (IRF5), thus stimulating the production of such pro-inflammatory cytokines as interleukins (IL) IL-6, IL-1β, IL-12, IL-23 and tumor necrosis factor (TNF), as well as chemokines CCL5, CCL20, CXCL1, CXCL9, CXCL10 that recruit immune cells [23]. Activated M1 microglia are characterized by the expression of NADPH oxidase (generating superoxide radical and other reactive oxygen species, ROS), inducible NO synthase (iNOS), and matrix metalloproteinase 12 (MMP-12) [47, 48], as well as membrane IgG receptors CD16 and CD32, T lymphocyte activation antigens CD40 and CD86 [49, 50], and proteins of the major histocompatibility complex class II (MHC II) that mobilizes immune cells for the inflammatory response [50] (Fig. 1).

In turn, IL-4, IL-13 and IL-10, as well as the transcription factor PPARγ, activate the anti-inflammatory M2 phenotype, promoting the restoration of

Table 1. Selected microglia markers differentially expressed in rodents

Microglia sub-types	Additional cell markers	Function	References
KSPG microglia	Iba1, CR3, CD11b	Appear in pathological conditions of the nervous system, e.g., Alzheimer's disease, traumatic brain injury, stroke	[62, 63, 65]
Hox8b microglia	Iba1, CD11b	Involved in the regulation of anxiety levels, grooming and social behavior	[33, 67, 69]
Cd11c microglia	Iba1, CD11b, CD45^{low}, CX3CR1	Involved in neurogenesis and myelination	[71]
TREM2 microglia	—	Regulation of cell proliferation, survival and metabolism. Involved in Alzheimer's disease pathogenesis	[73, 74]
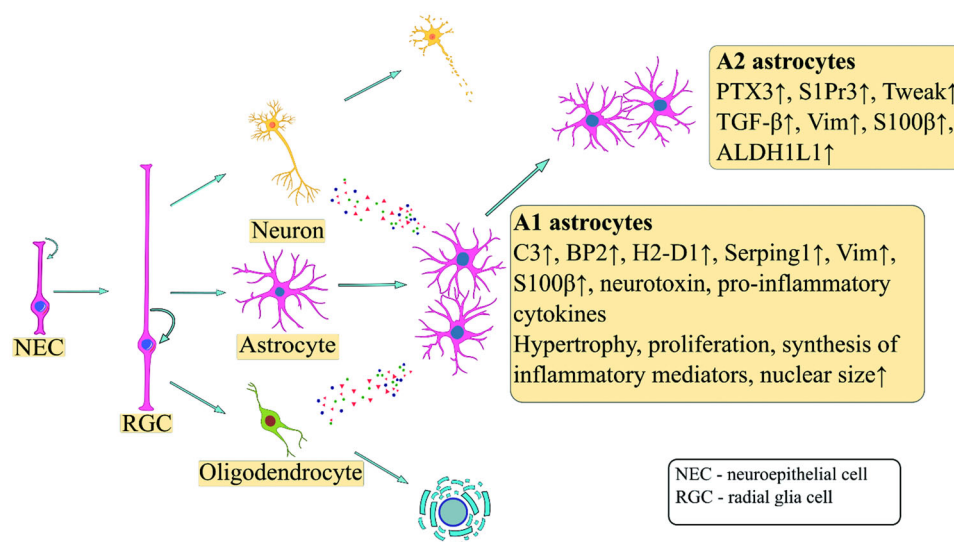
Fig. 2. Putative ontogenesis of mammalian astrocytes. Astrocyte progenitors in the mammalian nervous system are radial glia cells (RGCs) developing from neuroepithelial cells (NECs), which are also the progenitors of neurons and oligodendrocytes. The diagram shows populations according to a classical binary A1/A2 astrocyte classification with unique subtype-specific markers. C3—complement component 3, BP2—insulin-like growth factor binding protein 2, Serping1—serpin family G member 1, Vim—vimentin, S100β—S100 calcium-binding protein B, PTX3—pentraxin 3, S1Pr3—sphingosine-1-phosphate receptor 3, Tweak—cell surface-associated type II transmembrane protein, TGF-β—transforming growth factor beta, ALDHIL1—10- formyltetrahydrofolate dehydrogenase.

nervous system homeostasis [51]. M2 microglia secrete anti-inflammatory cytokines IL- 10, IL-4 and TGFβ, chemokines CCL2, CCL22, CCL17 and CCL24, growth factors IGF-1 (insulin-like growth factor 1), FGF (fibroblast growth factor), CSF-1 (colony-stimulating factor 1), neurotrophic factors NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor), neurotrophins 4/5, GDNF (glial cell line-derived neurotrophic factor) and pro-granulin [50, 52]. M2 cells are characterized by cell surface markers, specifically, CD206 (mannose receptor recognizing glycoprotein-derived glycan chain residues on the surface of microorganisms) [53] and CD163 (macrophage scavenger receptor mediating the internalization of hemoglobin-haptoglobin complexes by macrophages and thus reducing oxidative stress) [54]. Important biomarkers are arginase-1 (ARG1) expression by M2 cells, which cleaves arginine into urea and ornithine to form proline and polyamides required for tissue repair [55, 56], as well as

the ratio of secreted interleukins to cell surface receptors in microglial cells (e.g., IL-12^{high}/IL-10^{low} [57] and CD14^{high}/CD16—characterizing M1, and CD14^{low}/CD16+ characterizing M2 microglia) [58]. Intermediate phenotypes between M1 and M2 are identified by analyzing markers attributable to the two phenotypes simultaneously (e.g., CD86+/CD206+). The presence of M1 markers MHCII and CD86 against the background of high IL-10 and low IL-12 levels, as well as the absence of FIZZ1 and Ym1 (which is characteristic of M2), may also indicate an intermediate microglial phenotype [23].

However, the subdivision of microglia into two polar phenotypes is currently under active revision, since microglial cells perform different functions in the CNS, respond differently to triggers, and are characterized by different molecular markers [24]. Another argument in favor of revisiting the binary classification of microglia is its regional heterogeneity. For example, the microglial self-renewal rate both under normal conditions and when exposed to external stimuli [59, 60], as well as differential gene expression [61], often assessed to identify cell subpopulations of other tissues and only recently applied to microglia, differ depending of the brain structure (Table 1, Fig. 1). Some recently characterized subtypes of neuroglial cells, reflecting their more intricate heterogeneous nature and functional role in the CNS, will be discussed below.

Newly recognized microglia subtypes

KSPG microglia. Rodent microglia are characterized by heterogeneity of expression of keratan sulfate proteoglycan (KSPG), extracellular matrix molecules involved in the regulation of cell adhesion and axonal growth [62]. KSPG-expressing microglia subtype (KSPG microglia) is detected via the 5D4 antibody predominantly in the hippocampus, brainstem and olfactory bulbs, as well as in the cerebral cortex and cerebellum. Morphologically, this subpopulation is represented by ramified microglial cells and is characterized by the markers Iba1 (ionized calcium-binding adapter molecule 1 also known as allograft inflammatory factor 1, AIF-1), CR3, and CD11b [62, 63]. CR3 is a phagocytic complement component C3 receptor involved in the regulation of soluble beta-amyloid clearance and thus indicative of a role for microglia in the pathogenesis of Alzheimer's disease [64]. KSPG microglia were also

detected in the rodent brain during pathological processes, including stroke, neurotrauma and amyotrophic lateral sclerosis models [65, 66].

Hox8b microglia. This subpopulation comprises ramified microglial cells described in the cortex and olfactory bulbs, characterized by the presence of Iba1 and CD11b markers, and expressing the *Hox8b* gene [33, 67]. These cells coexist with a *Hoxb8*-negative subpopulation while being indistinguishable from them by the expression of other microglial gene signatures (*Tmem119*, *Sall1*, *Sall3*, *Gpr56* and *Ms4a7*) and the genes associated with hematopoietic ontogenesis (*Clel12a*, *Klra2* and *Lilra5*) [33, 68]. Interestingly, *Hoxb8* is expressed not in the adult brain, but by microglial progenitors prior to CNS infiltration [33]. To date, there is no consensus on the functions of Hox8b microglia. However, *Hoxb8* knockout mice demonstrate pronounced CNS disorders, such as increased anxiety, pathological self-grooming, and social behavior deficit [69], indicating the great importance of this hematopoietic gene in the CNS.

CD11c microglia. The cells expressing integrin 11c (CD11c) [70], which are found predominantly in the corpus callosum and cerebellar white matter, are distinguished as a separate subpopulation. This type of microglia expresses genes responsible for neurogenesis and myelination and secretes insulin-like growth factor 1 (IGF1), a decrease in the level of which impairs myelination during development. Thus, CD11c microglia in the brain of neonatal mice is likely to be implicated in neuro- and myelinogenesis. Morphologically, these cells are ramified microglia and express Iba1, CD11b, and CX3CR1 as biomarkers [71].

TREM2 microglia. Microglia are also heterogeneous by the expression level of triggering receptor expressed on myeloid cells 2 (TREM2) [72]. Like CR3, TREM2 is involved in the pathogenesis of Alzheimer's disease, coordinating cell clustering around amyloid-beta plaques and regulating proliferation, survival and metabolism of brain cells [73]. The highest density of TREM2-positive microglia occurs in the cingulate gyrus and lateral entorhinal cortex, the lowest in the hypothalamus and frenulum, and these cells are absent in the periventricular regions [74].

Neurogenesis-supporting microglia

Microglia are distinctly heterogeneous by the

presence of fractalkine receptor CX3CR1, as the subventricular zone and olfactory bulb have been found to contain a lesser number of CX3CR1-expressing microglial cells, which are additionally less ramified [75, 76]. In the subventricular zone of adult mice, they are TREM2-negative, while half of them are also Iba1-negative. By contrast, in the olfactory bulb, these cells express TREM2, and one third of them are CD68-positive. This microglial subpopulation is assumed to be essential for neuroblast survival and migration [61, 77].

Satellite microglia

The so-called satellite microglia, unramified glial cells contacting the neuronal soma, can be distinguished separately [78]. They are located predominantly in the cortex, hippocampus, thalamus, and striatum, and are characterized by the classical microglial markers Iba1, CD11b and CX3CR1, while expessing no unique markers of the own [78, 79]. First discovered in mice, this subtype of microglia has also been found in rats and primates [3, 79].

Dark microglia

The so-called "dark" (i.e., more optically dense) microglia interact with blood vessels and occur in the hippocampus, cortex, hypothalamus, and amygdala. In contrast to the other populations, it contains oxidative stress markers (condensed cytoplasm, hypertrophied Golgi apparatus, altered mitochondrial morphology) [80]. Interestingly, the density of dark microglia increases in Alzheimer's disease, suggesting an elevated level of oxidative stress against the background of developing pathology [81]. In addition, dark microglia are characterized by the presence of CD11b, TREM2 and 4d4, weakly express Iba-1 and CX3CR1, and probably participate in vascular remodeling and maintaining the BBB [82, 83].

Other microglia classifications

The expression of unique combinations of biomarkers in different pathological conditions of the nervous system can be used as an additional classification of microglia. Such disease-associated microglia (DAM) are TREM2-positive, with increased *Apoe*, *Axl*, and *Spp1* and decreased *Cx3cr1* and *P2ry12* expression [84, 85]. Ontogenetically, these cells derive from resident microglial cells and inflammatory macrophages. The most striking

example is the MGnD phenotype—Alzheimer's disease- and multiple sclerosis-associated microglia [86], found both in the 5XFAD mouse model of Alzheimer's disease and in brain samples from patients with this pathology. At the same time, a separate tau-associated microglial cluster has been found in humans, but undetected in mice, suggesting the possibility of cross-species heterogeneity of microglial cells [87]. While the issues on the functional role of this subpopulation remain open, the transition to the MGnD phenotype is known to be regulated by TREM2 [84, 88, 89]. Although MGnD has received more attention as the first DAM phenotype studied, it is not the only one. For example, there have been described microglial phenotypes responsive to interferon (IRM) [90], accumulating lipid droplets (LDAM) [91], as well as associated with amyotrophic lateral sclerosis (ALS) [92], glioma (GAM) [93], and Parkinson's disease (PD) [94]. Some of the DAM phenotypes characteristic of pathological conditions in adulthood have also been found in the developing human nervous system, suggesting that developmental transcriptional programs are reactivated in neurodegenerative pathologies [95].

Changes in the phenotype of microglia under chronic stress are also an important issue to consider. In rodents, the development of an inflammatory response in the brain, the key role in which is played by the pro-inflammatory cytokine IL-1β, occurs against this background [96, 97]. Interestingly, data on the development of neuroinflammation differ between in vitro and in vivo models. Specifically, activation of microglia adrenoreceptors exerts pro-inflammatory effects in vivo and anti-inflammatory effects in vitro [98, 99]. Also of interest is chronic stress-induced priming of microglial responses due to which rodents develop stress hypersensitivity that persists even after the cessation of the stressor [100]. Collectively, this raises the question of long-term changes in microglia after stress exposure, including in affective pathologies, as well as their possible correction via modulation of microglia and its priming.

Microglia peculiarities in zebrafish

It is also important to analyze cross-species morphofunctional features of microglia, the understanding of which may have translational significance. In general, expression of microglial biomarker genes (e.g., *irf8*, *spi1*, *csf1ra*, *csf1rb*, *mpeg1.1*, *slc7a7*,

p2ry12, and *p2ry13*) is highly conserved in zebrafish, rodents and humans. For example, the expression of microglial genes in zebrafish is 43−45% overlapping with that in rodents [101]. Meanwhile, genes related to metabolic processes, organism development, and immune responses are the most, and associated with microglial stress responses are the least, conserved [101]. Molecular phenotyping of zebrafish microglia is far less developed than in rodents, as most of differentially expressed markers have not been described in zebrafish (except TREM2, which is involved in the switching of microglial phenotypes in rodents) [102, 103]. Although TREM2 has been shown to play a role in the anti-inflammatory activity of both zebrafish and rodents, it is not completely clear to what extent its expression in fish is differential and suitable for classification [104].

The term DAM is also currently uncommon in zebrafish, but has been described by the zebrafish transcriptome in a model of Alzheimer's disease, which may essentially be fundamental for extrapolating this classification to this model organism as well. Specifically, in the zebrafish model of Alzheimer's disease, the expression of 353 genes in the brain is altered compared to controls, whereas 128 differentially expressed genes are found in humans [105]. However, despite the difference in the number of such genes across species, some of them are involved in similar processes, such as antigen presentation, iron homeostasis, and lysosomal activity [105].

ASTROGLIA

Traditional classification

As with microglia, a binary classification has also been applied to astrocytes (Fig. 2). The A1 phenotype was thought to be pro-inflammatory and induced by M1 microglia-produced cytokines (e.g., Il1α, TNF, and C1q), while its cells undergo morphological and genomic changes, ceasing to perform useful functions (e.g., synapse maintenance) and becoming neurotoxic [25], in contrast to the A2 phenotype (see below). In cell cultures, A1 astrocytes have been shown to secrete a neurotoxin that triggers neuroapoptosis [106, 107]. In addition, they are capable of inducing oligodendrocyte death and slowing down the differentiation of their progenitors, leading to hypomyelination [106], as well as enhanc-

ing synaptic inhibition, leading to cognitive disorders in mice [108]. The pro-inflammatory effect of A1 astrocytes is implemented through the secretion of complement component 3 (C3), a protein of the immune system [106]. A1 astroglia can exacerbate the state of the nervous system in various pathologies. For example, a high level of C3 expression occurs in patients with Alzheimer's disease, while C3a receptor (C3aR) inhibition eliminates cognitive impairments in a mouse model of Alzheimer's disease [109]. C3 is also a participant of the microglia–astrocyte interaction: microglia are the first to be activated by pathological stimuli and further activate astrocytes, which in turn modulate microglia activation, migration, and phagocytosis through cytokine secretion [110].

In the mouse model of Alzheimer's disease, a decrease in C3 leads to suppression of M1 microglia and pro-inflammatory cytokines, thereby attenuating neurodegeneration [111], while an increase in the level of this protein, on the contrary, enhances microglial phagocytosis and thus promotes early synapse destruction [112]. Damaged neurons, in turn, recruit additional reactive astrocytes and microglia. Thus, the interaction between microglial and astroglial pro-inflammatory cells may be synergistic and context-dependent in neurodegenerative diseases. Moreover, molecular characterization of A1 cells via transcriptome analysis indicates differential expression of a number of marker proteins (C3, GBP2, H2-D1, Serping1 [113]), which even within the same astrocyte subtype may have different sensitivity and specificity in different CNS pathologies [114].

The A2 phenotype, likewise A, has traditionally been considered anti-inflammatory and promoting neuronal survival, growth, and repair [115]. A2 astrocytes are characterized by differential expression of marker genes encoding the calcium-binding protein S100 A10 (S100a10), pentraxin-3 (PTX3), S1Pr3, and Tweak [113]. The functional role of A2 astrocytes is mostly opposite to that of A1 cells, suppressing microglial cell activation through secretion of transforming growth factor-beta (TGF-β) [116] and promoting oligodendrocyte differentiation, as well as white matter protection, during brain injury [117]. On the other hand, in a mouse model of neonatal white matter injury, A2 astrocytes impair myelination through prostaglandin E2 secretion,

Table 2. Selected open questions related to studying glial cell heterogeneity

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• How do context-dependent alterations of gene expression in microglia and astrocytes affect their functional properties?

• What approaches can be used to revise the classification of glial cells to take into account the functional role of different populations and context-dependent states?

• Is the approach to systematization of glial cells based on RNA sequencing relevant without considering proteome and metabolome data?

• How to overcome the problem of lack of reliable antibodies for alternative model objects, zebrafish?

• To what extent can the results of studies in model subjects such as zebrafish and rodents be extrapolated to humans, given differences in gene expression and cellular phenotypes?

• What is the main biological reason for the greater heterogeneity of human microglial cells compared to model objects?

• What functions are performed by cell populations unique to humans and what approaches are optimal for their study?

• How can we systematize and compare glial cell data between different model entities, given the differences in cell heterogeneity across species?

• Data on the origin of rodent microglial cells have been obtained predominantly from a single line of mice. How relevant are these results for other rodent lines and species?

• The RNA sequencing data on which reclassification attempts are based can only indirectly indicate cell function. How can we assess the function of phenotypes identified by RNA sequencing?

• What functions are performed by unique microglia populations in different model objects?

• How are glial cells involved in neuroregeneration in zebrafish?

• How are disease-associated glial cell (DAM) phenotypes comparable across different model species?

• How similar are the disease-associated glial cell (DAM) phenotypes in different models of the same pathology, such as in a genetic model of Alzheimer's disease and in the administration of beta-amyloid?

• How does environment affect alteration of microglia phenotype?

• How do local tissue-specific factors affect the acquisition of microglia identity in situ?

• Are the generally accepted glial cell markers (such as GFAP for astrocytes and Iba-1 for microglia) an adequate way to assess the total glial cell pool, given the differential expression in different populations?

• Do differences in KPSG expression levels in microglia in different rat lines reflect functional differences related to the role of this microglia?

• What new discoveries in the neurobiology of microglia and astroglia may change current ideas about the pathogenesis of neurodegenerative diseases and approaches to their treatment?

• What is the potential of A2 astrocyte-based cell therapy for neurodegenerative diseases/neuroinflammation/ischemia/CMD?

• What is the effect of npvp on the neurotoxic functions of astroglia phenotype A1?

• What is the subpopulation composition of astrocytes during zebrafish/mouse/human ontogenesis?

• How do the calcium waves generated by astrocytes of different phenotypes differ? What are the differences between A1 and A2 phenotypes in vitro and in vivo, in in vitro 2D, 3D models, organoids and assembloids?

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thus (like A1 astrocytes) demonstrating the importance of context [118]. Overall, a large number of questions on the role of astrocytes in the CNS and therapy selection for brain injuries remain unanswered (Table 2). Nevertheless, the current binary classification of astrocytes is rather a simplification

that does not reflect the whole set of astrocytic cell phenotypes and requires further reconsideration of the classification of the entire glial cell system [24, 26, 119].

Novel approaches to astrocyte classification

The problem of astrocyte heterogeneity is being also actively discussed in the literature. For example, astrocytes display differential gene expression depending on their location in different cortical layers, forming at least 9 subpopulations [120]. This expression changes upon induction of lipopolysaccharide (LPS)-mediated neuroinflammation, revealing two major populations of the most responsive cells. The first expresses genes specific to the white matter (*Vim* encoding the structural protein vimentin) and deep cortical layers (*Id3* encoding a DNA-binding protein inhibitor), and in neuroinflammation, neuroprotection-associated genes (*Timp1* encoding metalloproteinase inhibitor, *Gpx1* encoding antioxidant glutathione peroxidase, *Hspb1* encoding neuroprotective heat shock protein, and *Gap43* encoding neurotoxicity suppressor protein) [120]. Furthermore, this cluster expresses interferon-induced genes (*Psmb8*, *Ifitm3*), as well as those involved in the process of antigen presentation (*H2-K1*, *H2-T23*, *H2-D1* encoding histocompatibility antigenes) and the marker gene *Timp1* [120]. In contrast, the second population of astrocytes is almost undetectable in the resting state, but characterizes by the expression of genes involved in IFN-dependent transcription regulation (*Stat1* and *Stat2*), as well as in antigen processing (*Tap1* and *Tap2*) and presentation (*H2-Q4*, *H2-K1*, *H2-Ab1*, *H2-D1* and *H2-T23*), during inflammation [120]. These astrocytes may have increased their ability to present antigens due to interferon exposure. This population occurs in the region of the lateral and third ventricles, hippocampus, and cortical layer I, where the cells actively interact with vessels [120].

Other astrocyte subpopulations also respond to inflammation, but to a lesser extent. For example, the subgroup with normally high level of the astrocytic marker *Gfap* and synaptogenesis marker *Thbs4* [120], begins expressing genes that encode C3, CD109 (a protein that inhibits TGF-β signaling) and Igfbp7 (a factor that inhibits VEGF-induced angiogenesis) during inflammation. Among astroglia subpopulations, which are less involved in the inflammatory process, there was found a group of cells expressing the synaptomodeling gene *Sparc26*, the oligodendrocyte-specific gene *Nkx6-2*, and the gene *Gria1* encoding the AMPA glutamate receptor subunit [120], which is highly conserved in zebrafish, rodents, and humans [121]. Overall, there are now prerequisites for the formation of disease-associated astroglial phenotypes similar to the DAM phenotypes, which would allow the identification of pathology markers and potential therapeutic targets. For example, astrocyte phenotypes have been found to be activated and changed during aging, neurodegenerative diseases (including Alzheimer's, Parkinson's, and Huntington's diseases), CNS infections, and acute traumatic brain injury [106, 122−126].

Astrocyte peculiarities in zebrafish

An important cross-taxon difference between the CNS in mammals and zebrafish is the absence of conventional astrocytes in the latter [127]. As initially assumed, the function of astrocytes in zebrafish is taken over by specialized cells of radial glia, which express glial biomarkers (e.g., glial fibrillary acidic protein, GFAP), are neuronal progenitors, and are accordingly involved in neurogenesis [128]. Via confocal microscopy, it has been shown that radial glial cells in fish begin transforming into astrocyte-like cells on day 2 after fertilization and possess additional traits characteristic of mammalian astrocytes, e.g., glutamine synthase (GS) expression. Fibroblast growth factor receptors (fgfr3 and fgfr4) have also been demonstrated to play a critical role in the morphogenesis of zebrafish astrocytes [129]. Nevertheless, there is still no unambiguous evidence that zebrafish astrocytes are similar to their mammalian counterparts.

Astrocytes are also actively involved in fish CNS regeneration, representing one of the pivotal features of this model object. For example, following spinal cord transection in zebrafish, in contrast to mammals, radial glial cells form not a glial scar, but glial bridges that help reunite the transected spinal cord and provide a substrate for subsequent axonal outgrowth [128]. In general, the response of neuroglia to spinal cord transection recapitulates the reaction of neurogenesis, and therefore astrocyte modulation, aimed at the formation or enhancement of the radial glial phenotype, may contribute to a more favorable regenerative response of the mammalian CNS.

CHALLENGES AND PROSPECTS
OF NEUROGLIA RESEARCH

The question of glial cell classification is quite relevant and important. On the one hand, the main problem in the study of microglia is the established application of the binary M1/M2 peripheral macrophage nomenclature, limiting their role in the CNS mainly to immune regulation. The same challenge exists for the binary classification of astrocytes, limiting their phenotypes to being ether 'neurotoxic' or 'neuroprotective', without consideration for the specific state of the nervous system, which is distinct in different pathologies. On the other hand, cancelling the "convenient" polar dichotomy necessitates the construction of novel neuroglia classification systems. This problem is currently being explored by using RNA sequencing data to reveal common cell populations by gene expression patterns. However, changes in gene expression can only indirectly attest to cell functions and do not reflect their anatomical location. Moreover, various biomarker genes may have inconstant (fluctuating) expression levels, which may reflect the intricate dynamics of CNS pathological states, whereas context-dependent, newly characterized microglial subpopulations mainly reflect specific activation states of pre-existing microglia rather than a mix of individual subpopulations.

The problem of data systematization across the model species (due to cell heterogeneity in different species) is also evident, which further complicates the translation of results to humans. For example, human microglia are represented by multiple subtypes, while in other species, including mice and monkeys, such a significant heterogeneity is not observed or has not yet been studied. In addition, a large number of distinct differentially expressed microglial genes, including those associated with neurodegenerative diseases, are found in humans and rodents, which also indicates a possible significant cross-taxon difference in the function of neuroglia [130].

Nevertheless, the study of glial cells on relatively new (for neurobiology) model objects, such as zebrafish, is quite promising from both evolutionarily physiological and practical viewpoints. Specifically, zebrafish are a convenient organism for engineering transgenic constructs because of the peculiarities of their genetics and the transparency of

embryos (that develop rapidly and externally), enabling the imaging and manipulation of specific cell types. For example, using CRISPR-Cas9 techniques, fibroblast growth factor (Fgf) receptors have been shown to play a role in the development of zebrafish astrocytes [129]. Transgenic fish strains can also be used to visualize cell lines, e.g., those co-expressing fluorescent protein in cells expressing the astrocyte marker GFAP [131]. In addition, a large number of genetic models of nervous system disorders have recently been generated in fish, including numerous transgenic models of Alzheimer's and Parkinson's diseases, as well as tauopathies [132–134]. This provides an opportunity to characterize pathogenesis-associated phenotypes of microglial and astroglial cells, which may simplify the typing of highly conserved states of these glial types, which are characteristic of humans as well.

The study of glial cell states, unique to zebrafish, is also an important task, as it may provide an answer to the question of the fish nervous system's high regenerative capacity. For example, regeneration is assumed to be driven by interactions between radial glial cells and macrophages and to be mediated by macrophage-produced TNF [135]. Accordingly, the identified novel aspects of neuroregeneration can be further employed in human therapies. Another question is whether gene expression is enough to determine the state of a cell, as there is no clear understanding of how changes in expression transform the cellular phenotype. While more complete picture of pathogenesis requires proteomic studies [136], however, proteomic analysis is difficult for rarer model objects, since a number of methods are based on the use of antibodies, which are currently developed mainly for rodents and humans. The problem of antibody scarcity for zebrafish and other model objects can be possibly solved by using alternative research methods, e.g., RNA in situ hybridization, which still does not allow proteome assessment, but at least solves the problem of anatomical localization of the genes expressed [137].

In general, the study and systematization of glial cells in various taxa is an urgent task of modern evolutionary physiology and neurobiology. The revision of glial cell classification is actively underway and is likely to remain relevant for a long time to come. It is vigorously stimulated by the development of novel cellular and molecular methods for brain research

and the accumulation of experimental data that do not fit well into the traditional 'binary' glial paradigm. However, at present, there is still insufficient knowledge base to establish a novel optimal and comprehensive classification of neuroglia. Specifically, the results of RNA sequencing need to be supplemented with the assessment of the functional role of cells, their localization, and proteomic profiling.

In turn, untangling the functional roles of different cell populations and identifying their context-dependent states within CNS may shed light on the pathogenetic mechanisms of brain diseases and help determine novel therapeutic strategies. In addition to translational problems, there also remain many open fundamental questions (Table 2) regarding the origin of microglial cells, comparability of zebrafish and mammalian astrocytes, and the peculiarities of microglia–astrocyte interactions in different animal species. Addressing these and other questions will open up new avenues for future studies in the field of neurobiology and pathophysiology of glia.

AUTHORS' CONTRIBUTION

Conceptualization (M.M.K., A.V.K.), literature screening (M.M.K., A.K.V., N.V.S., A.V.K.), data analysis and discussion (M.M.K., A.V.K.), manuscript writing and editing (M.M.K., A.K.V., N.V.S., A.V.K.), final version discussion and approval (M.M.K., A.K.V., N.V.S., A.V.K.).

FUNDING

ETHICS APPROVAL

This work does not contain any experimental animal or human studies.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflict of interest.

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Translated by A. Polyanovsky