



Insights Into Central Nervous System Glial Cell Formation and Function From Zebrafish

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The term glia describes a heterogenous collection of distinct cell types that make up a large proportion of our nervous system. Although once considered the glue of the nervous system, the study of glial cells has evolved significantly in recent years, with a large body of literature now highlighting their complex and diverse roles in development and throughout life. This progress is due, in part, to advances in animal models in which the molecular and cellular mechanisms of glial cell development and function as well as neuron-glia cell interactions can be directly studied *in vivo* in real time, in intact neural circuits. In this review we highlight the instrumental role that zebrafish have played as a vertebrate model system for the study of glial cells, and discuss how the experimental advantages of the zebrafish lend themselves to investigate glial cell interactions and diversity. We focus in particular on recent studies that have provided insight into the formation and function of the major glial cell types in the central nervous system in zebrafish.

Keywords: zebrafish, radial glia, Müller glia, astrocyte, OPC, oligodendrocyte, microglia

INTRODUCTION

The Importance of Glia

Although originally characterised as bystanders to neuronal function by Virchow in 1846, constituting the “glue” of the nervous system (Somjen, 1988), the possible roles of glia in supporting efficient nervous system function have since been vastly explored. It is now well established that glial cells are important throughout life, with recent research highlighting their significant contributions to nearly all aspects of nervous system function (Barres, 2008; Allen and Lyons, 2018). Since their initial description over a century ago, glial cells have been investigated in many diverse model organisms, each providing distinct strengths, with many aspects of glial biology highly conserved across species from invertebrates to humans (Freeman and Doherty, 2006; Lyons and Talbot, 2015; Shaham, 2015; Zuchero and Barres, 2015; Jäkel and Dimou, 2017; Yildirim et al., 2019). Here, we focus on the contribution of the zebrafish as a model organism to investigate the development and function of the major glial cell types in the vertebrate central nervous system (CNS), from radial glial cells, Müller glial cells, astrocytes, oligodendrocyte progenitor cells (OPCs) and oligodendrocytes (OLs) through to microglia.

THE ADVANTAGES OF ZEBRAFISH TO STUDY GLIAL CELLS

The zebrafish is well suited to follow the highly dynamic activities of glial cells, their interactions with other cells, and the molecular mechanisms that control their formation and function throughout life. This is because of a number of distinct advantages of the zebrafish as a vertebrate model system [as reviewed by Lieschke and Currie (2010) and Stewart et al. (2014)]. The small size of adult zebrafish (a few cm long) and their relatively quick life cycle (embryo to sexually mature adult in circa 2–3 months) make zebrafish a cost-effective model organism to maintain in large numbers. Secondly, the external development of zebrafish allows access to embryos from the single-cell stage, including for microinjections, e.g., for early genetic manipulation (Driever and Stemple, 1994; Kawakami et al., 2004). Additionally, the larval zebrafish remains optically transparent for the first weeks of its life, which enables molecules, cells and tissues of interest to be non-invasively imaged within the animal (Swinburne et al., 2015; Bin and Lyons, 2016). Furthermore, despite an analogous generation time when compared to rodents, zebrafish embryos develop very rapidly compared to other models, growing from a fertilised egg to a freely swimming larval fish which displays complex behaviours within a few days of egg fertilisation (Orger and De Polavieja, 2017). This rapid development, coupled with the fact that female zebrafish can produce hundreds of eggs at a time, facilitate large-scale screens on thousands of embryos and larvae over days to weeks. Also, and very importantly, zebrafish have a well conserved genome with other vertebrates (Howe et al., 2013). However, unlike other vertebrates, zebrafish have many duplicated genes following an additional whole genome duplication event in their ancestry (Howe et al., 2013). Despite this, zebrafish exhibit many conserved molecular mechanisms underpinning cellular physiology through to animal behaviour, including regulation of glial cell development (Jadhav et al., 2009; Goldman, 2014; Lyons and Talbot, 2015; Mathews and Appel, 2016b; D’Rozario et al., 2017; Jurisch-Yaksi et al., 2020). Together these properties allow the study of glial cells as they colonise the nervous system of the zebrafish in the first weeks of embryonic-larval life.

Tools to Study Glial Cells and Their Cell-Cell Interactions *in vivo*

A major goal in the field of glial cell research is to understand the cellular behaviour and complex interactions of glia with other cell types, and how these combine to affect overall circuit formation and function. Zebrafish provide the opportunity to directly observe glial cell dynamics and cell-cell interactions *in vivo* over time in the living organism without the need for invasive surgical techniques, which are typically required for analogous analyses in other systems such as murine models (Hill et al., 2018; Hughes et al., 2018). High resolution imaging of fluorescently labelled glial cells of interest can be carried out in the optically transparent zebrafish larvae over the first 1–2 weeks of life, as glial cells populate the CNS (Lyons and Talbot, 2015; Preston et al., 2019; **Figure 1**). At yet later stages, live *in vivo*

imaging can be carried out using zebrafish with mutations in pigment genes such as *golden*, *nacre* and *casper* (Lister et al., 1999; Lamason et al., 2005; White et al., 2008).

Advances in the development of fluorescent reporters means that morphological and functional characteristics of glial cells can be examined within the zebrafish [exemplified throughout and reviewed in Bin and Lyons (2016), Czopka (2016), and D’Rozario et al. (2017)]. These enable intricate processes such as OPC migration and proliferation (Kirby et al., 2006), myelin sheath formation and growth (Klingseisen et al., 2019), and the dynamic behaviour of microglia (Casano et al., 2016), to be investigated at high resolution in real time *in vivo*. In addition, reporters of cell state and function have become available, e.g., those with pH and ion sensitivity, which have enabled investigation of glial cell activity including Ca²⁺ activity in astrocytes (Chen et al., 2020), OPCs (Marisca et al., 2020), and myelinating oligodendrocytes (Baraban et al., 2018; Krasnow et al., 2018), the phagocytic properties of microglia (Peri and Nüsslein-Volhard, 2008; Hughes and Appel, 2020), and even functional activity of entire populations of neurons (Kim et al., 2017; Vanwalleghem et al., 2020) and glia (Baraban et al., 2018; Mu et al., 2019), including as they interact (Diaz Verdugo et al., 2019). In addition, various photo-activatable reagents are available and can allow, for example precisely timed glial cell ablation (Auer et al., 2018), or stimulation of migration (Sieger et al., 2012; Piller et al., 2021). Furthermore, integration of microscopy and behavioural platforms now enables in depth characterisation of glial cells during specific behaviours. For example, arenas wherein zebrafish are presented with virtual reality visual stimuli can now be used to measure neuronal and glial cell activity using optical methods that span from single cell resolution, through to brain-wide analyses of entire cell populations (Engert, 2012; Ahrens et al., 2013; Cong et al., 2017), providing invaluable insight into neural-glia control of circuit function in a live animal (Diaz Verdugo et al., 2019).

Genetic Screening and Investigation

Another major goal in glial cell research is to identify genes required for their development, function and cell-cell interactions. One of the principal ways this has been investigated in the zebrafish is through the use of forward genetic screens, which provides a means for unbiased discovery of gene function. Briefly, adult animals (males in zebrafish) are exposed to mutagens to create random heritable mutations in the genome, with later generation offspring screened for phenotypes of interest, followed by identification of the causative lesion to link phenotype and genotype (Patton and Zon, 2001; Lyons and Kegel, 2019). Although initial forward genetic screens were carried out in invertebrate model organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* (Nüsslein-Volhard and Wieschaus, 1980; Brenner, 2003), innovative work by Streisinger et al. (1981) in the 1980s demonstrated that forward genetic strategies were also feasible in the zebrafish. In the 1990s large-scale mutagenesis-based forward genetic screens were carried out by the Nüsslein-Volhard and Driever laboratories, and highlighted the power of the zebrafish as a model for gene discovery in vertebrates (Driever et al., 1996; Haffter et al., 1996). Since

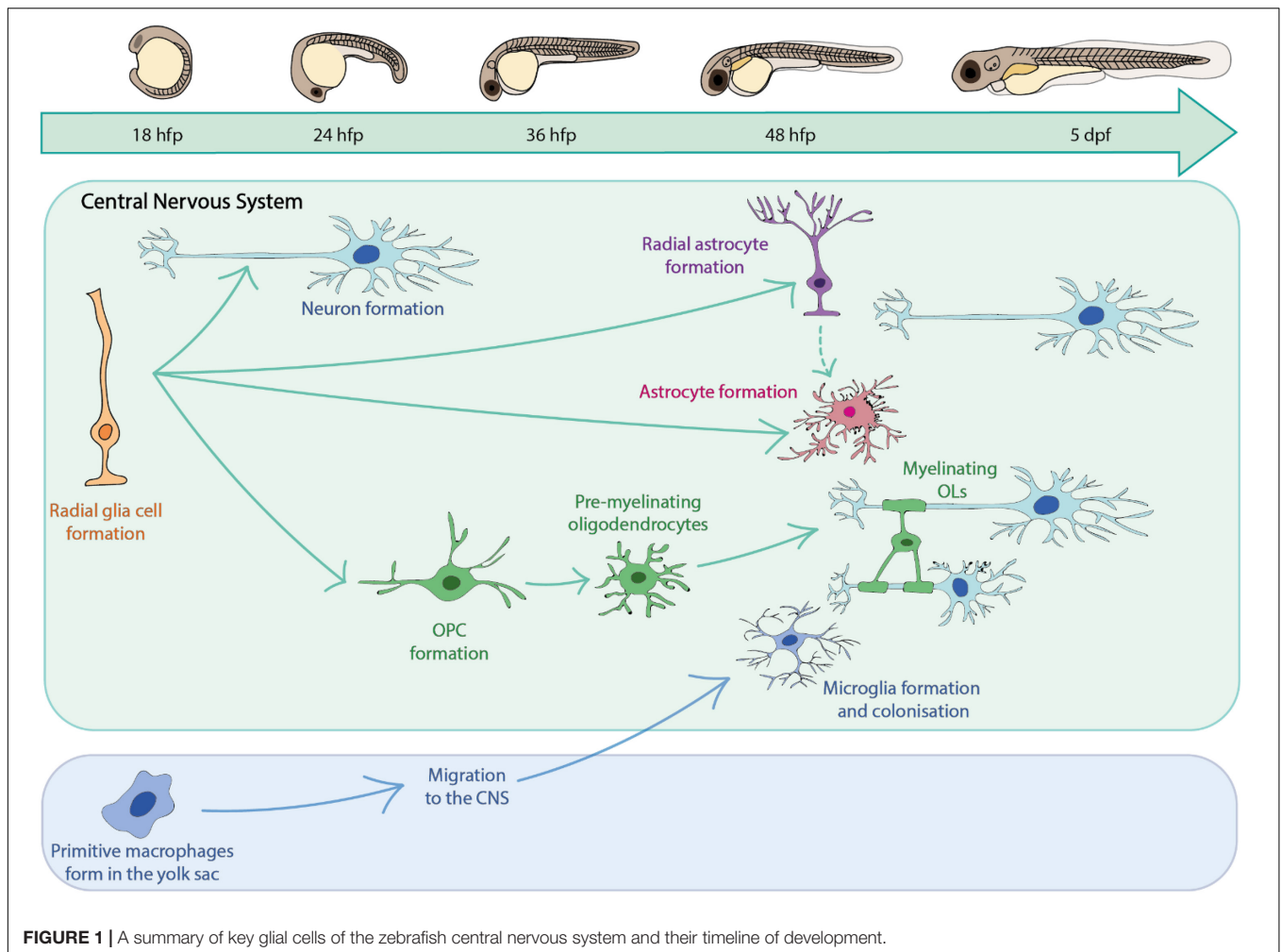


FIGURE 1 | A summary of key glial cells of the zebrafish central nervous system and their timeline of development.

these pioneering screens, which identified hundreds of mutations disrupting numerous biological processes, several dedicated forward genetic screens have been carried out to investigate genes essential for glial cells, including those regulating OPC, oligodendrocyte and microglial cell formation and function (Kazakova et al., 2006; Pogoda et al., 2006; Peri and Nüsslein-Volhard, 2008; Mathews et al., 2014; Mazaheri et al., 2014; Meireles et al., 2014; Shiau et al., 2014; Kearns et al., 2015; Shen et al., 2016; Villani et al., 2019). We will highlight recent insights throughout this piece and, for further information, we direct readers to the following reviews (Lawson and Wolfe, 2011; Kegel et al., 2019). In the now classic three generation forward genetic screen, following mutagenesis of male zebrafish, animals are crossed with female zebrafish to generate a cohort of first generation individuals carrying unique random mutations. These animals are used to seed a second generation, which nowadays often involves combining with transgenic reporters with fluorescently labelled cells or structures of interest to allow for facile screening of third generation offspring for disrupted phenotypes (Lyons and Kegel, 2019). Given the genetic tractability of zebrafish, it is now possible to select from many available stable transgenic lines which specifically label glial

cells [e.g., Kirby et al. (2006), Jung et al. (2010), Preston et al. (2019), Chen et al. (2020), Marisca et al. (2020), Wu et al. (2020)]. Furthermore, mutations in potential glial cell-regulating genes can be investigated in the context of the entire circuit by studying effects on complex forms of neuronal activity or behaviours such as specific swimming patterns, or sensorimotor behaviours including escape responses or prey capture, which are displayed in zebrafish within just a few days of egg fertilisation (Bianco et al., 2011, 2015; Mcclenahan et al., 2012). Developments in genome and RNA sequencing now enable more rapid and accurate identification of the causative lesions that underlie phenotypes than following early screens (Bowen et al., 2012; Henke et al., 2013).

Alongside forward genetic screens, the use of reverse genetic approaches to interrogate specific genes of interest have provided insight into glia in zebrafish, presenting a complement to mutagenesis-based screening approaches. Unlike forward genetic screens, wherein random mutations are induced in the genome, reverse genetic approaches utilise the genetic tractability of the zebrafish to apply targeted approaches to investigate specific gene function. One of the most popular techniques initially used to interrogate gene function in zebrafish utilised synthetic

morpholino antisense oligonucleotides (MOs), which either block mRNA splicing or protein translation (Nasevicius and Ekker, 2000). These tools provided fundamental insights into the development of glial cells in zebrafish, particularly with respect to investigating the conservation in zebrafish of genes known to regulate glial cell development in mammals e.g., Park et al. (2002, 2004). However, although MOs provide a straightforward means to inhibit gene function, they have a number of limitations including off-target effects, variable gene knockdown and depleted efficiency over time, meaning great care must be taken in their use (Stainier et al., 2017). As for essentially all model organisms, the employment of recently discovered gene targeting approaches have revolutionised genetic analyses of zebrafish. For example, genetic knock out or knock in zebrafish can now be efficiently generated using Zinc finger nuclease (ZFN) technology, transcription activator-like effector nucleases (TALENs), and most recently, and most extensively, CRISPR/Cas9-based strategies [for reviews see Huang et al. (2012), Gaj et al. (2013), Ma and Liu (2015), Liu et al. (2017, 2019), Cornet et al. (2018), and Hans et al. (2021)].

The efficiency of new reverse genetic technology is continually improving, enabling more rapid investigation of genes for glial function. One example of this is the use of CRISPR/Cas9 strategies that can now be utilised to reliably induce somatic mutations with high efficiency in F0 embryos, called “crispants.” These significantly increase the speed with which genetic manipulations can be investigated (Keatinge et al., 2021; Klatt Shaw and Mokalled, 2021; Kroll et al., 2021), which can allow the investigation of duplicated genes, those with potentially redundant functions and even multiple pathways in parallel. From a practical view, these technologies reduce the personnel and space requirements of mutagenesis-based forward genetic screens, thereby allowing scalable investigation of gene function by most laboratories. Despite the efficiency of constitutive gene targeting in zebrafish, cell-type specific analyses of gene function is more technically challenging. A long-standing method to assess cell autonomous vs. non-autonomous gene function in zebrafish has been the creation of genetic chimeras by cell transplantation, which has been used to assess the roles of various factors in zebrafish glia, but is a method most suited to the analysis of single cell behaviour, given its chimeric nature (Lyons et al., 2009; Monk et al., 2009; Perlin et al., 2011; Mensch et al., 2015). Another approach widely used in the field is the expression of specific genes under the control of cell type specific drivers, e.g., the expression of wild-type genes on a mutant background, or the expression of dominant negative or constitutively active forms of genetic regulators, which has also provided insight into glial function in zebrafish (**Supplementary Table 1**). More recently, the creation of cell type specific knockout zebrafish using CRISPR-based strategies has also been carried out (Ablain et al., 2015), and strategies to do so are accumulating and show great promise. Although additional work remains to optimise such approaches for widespread analysis of candidate function in a cell type specific manner, they have already been utilised to provide insight into glial cell specific gene function in zebrafish (Chen et al., 2020; Marshall-Phelps et al., 2020). Complementary molecular approaches to profile cells including RNA sequencing,

which is well established in zebrafish (Mazzolini et al., 2019; Wheeler et al., 2019; Farnsworth et al., 2020; Marisca et al., 2020) and, more recently, spatial transcriptomics, which is being optimised for use in zebrafish (Holler et al., 2021; Okochi et al., 2021), provide invaluable platforms for future investigation of the molecular basis of glial cell function in the zebrafish.

In parallel to forward and reverse genetic approaches to assess gene function, the field of optogenetics has revolutionised neuroscience by allowing researchers to take control of the functional firing and signalling properties of neurons and glial cells (Warden et al., 2012; Cho et al., 2016; Kato et al., 2019; Mu et al., 2019; Lee et al., 2020). A principal set of tools in optogenetics are light-sensitive ion channels, which can be activated to control the electrical activity of cells. Given the ease of transgenesis, small size and optical clarity of zebrafish embryos and larvae, it is no surprise that numerous optogenetic actuators have been employed in zebrafish, principally to assess neuronal circuit function (Antinucci et al., 2020). Furthermore, the emergence of various methods to influence signalling pathways in a light-inducible manner (Arrenberg et al., 2009; Buckley et al., 2016; Harris et al., 2020; Mruk et al., 2020) with readouts of many aspects of cellular function, provides exciting opportunities for future study of glial cells, and the importance of neuron-glia interactions in circuit function in zebrafish.

Chemical Screening

As well as their advantages for genetic screening, zebrafish also provide a platform for drug discovery in a vertebrate model. Their rapid external development, small size (head to tail length of larvae with all major glial cell types present circa 5 mm long), and ability to absorb compounds through their skin, allows simple drug administration to live animals, enabling testing of thousands of compounds, even in multi-well plate format (Macrae and Peterson, 2015). Furthermore, the use of zebrafish enables multiple readouts of drug effects. These include simple phenotypic assessment of general health, development, organ growth and cardiovascular function. Additionally, behavioural readouts can be used to determine drug toxicity, alongside specific transgenic reporters for more in-depth cellular analysis. Since their first use in zebrafish, chemical screens have identified many promising compounds for various biological processes, with several taken to clinical trials [reviewed in Patton et al. (2021) and references therein]. In the context of glial biology, chemical screening based approaches have been utilised to identify compounds to enhance oligodendrogenesis and myelination (Buckley et al., 2010; Early et al., 2018), regulators of astrocytic function (Wheeler et al., 2019) and glial regulators of nerve regeneration (Bremer et al., 2017). Indeed, the investigation of glial cells in the context of regeneration and disease is now intensely investigated across models (Hamon et al., 2016; Cayre et al., 2021) and is a burgeoning aspect of research (Franklin et al., 2020; Garcia-Garcia et al., 2020; Eastlake et al., 2021; Hammond et al., 2021; Schirmer et al., 2021) that will form the basis of future reviews. In this piece, however, we will focus on the larger body of work that to date has investigated the development of glial cells in zebrafish, and begun to investigate their fundamental functional roles in the healthy nervous system.

GLIAL CELLS IN THE ZEBRAFISH; ORIGINS, DIFFERENTIATION AND FUNCTION

Studies in zebrafish have provided insights into glial cells of the CNS, peripheral nervous system (PNS) and indeed those that lie at and play essential roles at the boundary of the CNS and PNS. Insights into how zebrafish have informed our understanding of peripheral and boundary glia have been reviewed elsewhere (Kucenas, 2015; Monk et al., 2015; Ackerman and Monk, 2016; D’Rozario et al., 2017; Fontenas and Kucenas, 2018; Muppurala et al., 2020) and so we will focus this review on the major glial cells of the CNS summarised in **Figure 1**.

Glia of the Zebrafish Central Nervous System

Radial Glial Cells

The first cells in the zebrafish to emerge with glial characteristics are radial glial cells which develop with the formation of the neuroepithelium from approximately 10 hours post fertilisation (Kim et al., 2008). Like other vertebrates, radial glial cells in the zebrafish are classified by their characteristic radial processes, which span from the basal to the apical (ventricular) surfaces of the neuroepithelium. Indeed, radial cells with glial characteristics represent the major progenitor cell of the early developing CNS, which can both self-renew and give rise to differentiated cell types, first to neurons, and later to oligodendrocyte progenitor cells, and then to astrocytes (Lyons and Talbot, 2015; Jurisch-Yaksi, 2020). In mammals, following developmental neurogenesis and throughout the majority of the neuraxis, most radial glial cells ultimately detach from the ventricular zone, losing their radial morphology and subsequently contributing to the generation of bona fide astrocytes (Kriegstein and Götz, 2003). In other regions of the mammalian CNS, radial glia adopt heterogeneous states with various potentials to contribute to new cell generation throughout life (Götz et al., 2015; Falk and Götz, 2017). In the zebrafish however, neurogenesis is much more protracted and can, in principle, occur throughout the majority of the neuraxis throughout life (Kroehne et al., 2011; Kyritsis et al., 2012; Jurisch-Yaksi et al., 2020). Mirroring this capacity to serve as progenitor cells throughout life, a large proportion of radial glia in zebrafish retain their radial morphology life-long. In addition some radial glia appear to transition into a state with more specialised functions analogous to mammalian astrocytes. For example, both zebrafish radial glial cells and mammalian astrocytes share expression of the water transport protein aquaporin-4 and glutamate transporter *Eaat2b*, suggesting that they may conduct similar functions including regulating CNS water homeostasis and levels of neurotransmitters in the extracellular space (Grupp et al., 2010; McKeown et al., 2012; Papadopoulos and Verkman, 2013). Indeed, more recent studies have shown that some radial glial-like cells adopt gross morphological and functional features that overlap with those of mammalian astrocytes (Mu et al., 2019), and yet others appear to differentiate into bona fide astrocytes as discussed in the “Radial Astrocytes” and “Astrocytes” sections and summarised in **Figure 2** below.

Müller Glial Cells

In the mammalian CNS, additional glial cells with radial morphologies are known to be present in specific brain areas, e.g., Bergmann glia in the cerebellum and Müller glia in the retina, the latter of which have been extensively investigated in zebrafish. The vertebrate retina consists of six principal neuronal cell types and one primary glial cell, the Müller glial cell. Müller glial cells have a radial morphology (akin to radial glia) and extend over all three retinal layers, giving them the ability to contact neurons, maintain homeostasis and influence retinal structure (Goldman, 2014). Several developmental regulators which control the highly complex radial morphology of Müller glia cells and their even spacing throughout retinal layers have been identified in the zebrafish by utilising CRISPR based screening (Charlton-Perkins et al., 2019). Interestingly, a number of these appear to be highly conserved across species (Fairchild et al., 2018), suggesting that Müller glia morphogenesis is regulated by highly conserved differentiation programmes. Genes including *pax2a*, *itga5*, *itga6* and *itgb1a*, as well as those in the *nephrins* and *cadm* gene families have been prioritised as interesting candidates for further future investigation (Charlton-Perkins et al., 2019).

Under normal conditions Müller glial cells have several conserved functions across model organisms, including recycling neurotransmitters, maintaining ionic balance and interacting with retinal microglial cells. For example, mammalian Müller glia, like their radial glial counterparts, express glutamate transporter *EAAT2*, and expression of *Eaat2* orthologs is also observed in zebrafish Müller glia (Niklaus et al., 2017), which mediates removal of excess glutamate released from photoreceptor synapses, implying conservation in the roles of these cells across vertebrates. Indeed, when *Eaat2a* was depleted in zebrafish larvae, reduced electroretinographic responses were recorded (Niklaus et al., 2017), supporting the conclusion that Müller glia can influence synaptic function in the zebrafish, as in mammals. Furthermore, Müller glia have been shown to play an essential role in maintaining the tissue integrity of the retina, as retinas in zebrafish lacking Müller glia cells tear apart due to reduced tensile strength (MacDonald et al., 2015).

Similar to radial glial cells, Müller glia have been shown to be highly regenerative in response to injury in zebrafish as Müller glia cells can regenerate neurons, as well as all major retinal cell types (Powell et al., 2016; Sifuentes et al., 2016; Wan and Goldman, 2016). In contrast, Müller-glia derived progenitors in mammalian models have a more limited capacity to regenerate different cell types (Goldman, 2014). Therefore, future studies investigating the differences between mammalian and zebrafish Müller glia cells will be essential to determine the signalling pathways that are essential for efficient retinal repair.

The range of roles carried out by Müller glia cells in the retina highlights the functional diversity of glial cells with radial morphologies in different regions of the CNS. In the zebrafish, many distinct markers have been used to identify different radial glial cell populations (Bernardos and Raymond, 2006; Cuoghi and Mola, 2009; Farnsworth et al., 2020), with some cell morphologies and functions displaying characteristic biology

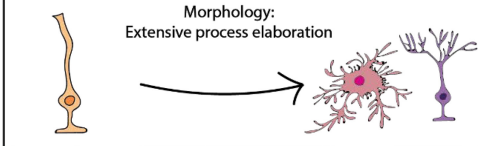
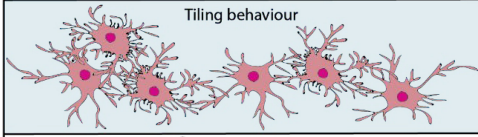
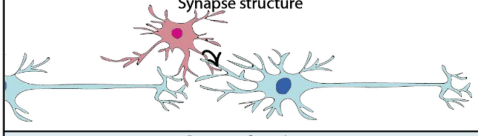
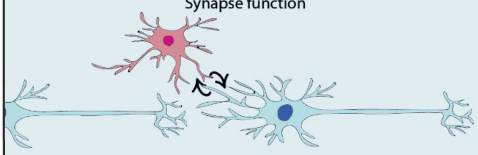
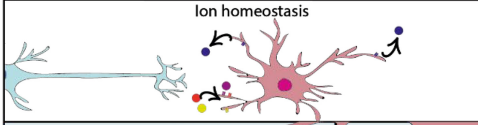
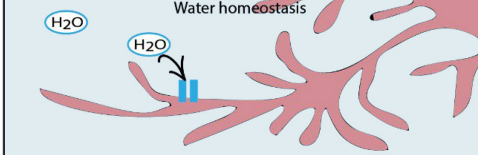
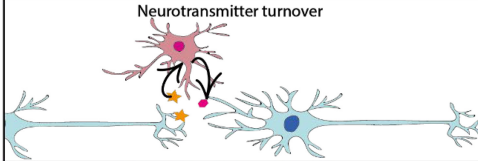
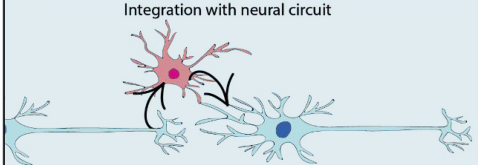
| Function / Feature / Characteristic | Zebrafish | Mammals |
|--|-----------|-----------|
| | Astrocyte | Astrocyte |
|  <p>Morphology: Extensive process elaboration</p> | ✓ | ✓ |
|  <p>Tiling behaviour</p> | ✓ | ✓ |
|  <p>Synapse structure</p> | ... | ✓ |
|  <p>Synapse function</p> | ... | ✓ |
|  <p>Ion homeostasis</p> | ... | ✓ |
|  <p>Water homeostasis</p> | ... | ✓ |
|  <p>Neurotransmitter turnover</p> | ... | ✓ |
|  <p>Integration with neural circuit</p> | ✓ | ✓ |

FIGURE 2 | Key characteristics and functional properties of zebrafish and mammalian astrocytes. For simplicity we refer to astrocytes as cells with a radial morphology, or those which have adapted a morphology akin to radial astrocytes. The use of “...” refers to areas of astrocyte biology which require further investigation.

assigned to radial glial cells whilst others are more reminiscent of astrocyte-like cells.

Radial Astrocytes

Studies in mammals have made it entirely clear that astrocytes are potent regulators of various aspects of neural circuit

formation and function (Freeman, 2010; Clarke and Barres, 2013; Stogsdill and Eroglu, 2017; Dallérac et al., 2018; Farhy-Tselnickner and Allen, 2018; Perez-Catalan et al., 2021; Sancho et al., 2021). Astrocytes play a number of essential roles in the mammalian nervous system including modulating synapse formation, pruning, and physiology (Chung et al., 2015;

Farhy-Tselnicker and Allen, 2018). Additionally, astrocytes can metabolically support the CNS, with extensive interactions with the vasculature and most major cell types of the CNS, including neurons, OPCs, oligodendrocytes and microglia (Clemente et al., 2013; Chung et al., 2015; Allen and Eroglu, 2017; Nutma et al., 2020). Reflecting their multiple roles, mammalian astrocytes have a complex morphology with an array of cellular processes that make connections with a host of these cellular targets (Allen and Lyons, 2018). However, the extent to which astrocyte-like cells with such complex morphology exist in zebrafish, or contribute to neural circuit structure and function was, until recently, quite unclear. In a tour de force study, Mu et al. (2019) showed that radial glial/astrocyte-like cells with complex morphologies do indeed exist in zebrafish and play an important role in neural circuit computations. In this study cells with both a radial process and an endfoot at the ventricular surface also had a huge network of cellular processes in synaptic regions. By whole-brain *in vivo* imaging of calcium transients of these cells as well as neurons while zebrafish were executing a specific behaviour, the authors were able to ascribe a role for these radial astrocytes in circuit computations (Mu et al., 2019). In this study, Mu and colleagues investigated the mechanisms of futility-induced passivity, or giving-up behaviour, which is a behaviour employed by many animals to conserve energy in-between high-activity phases (Warden et al., 2012; Andalman et al., 2019). Zebrafish elicit a visuomotor behaviour called the optomotor response to stabilise their position in the moving currents of river water, e.g., to not be swept downstream in a river upon an increasing current (Orger et al., 2000; Vladimirov et al., 2014). This response can be recapitulated experimentally in a virtual reality system by projecting moving bar patterns to the fish to simulate water flow and, in response, zebrafish elicit forward swims to stabilise their position relative to the moving bars. In one iteration of this experimental paradigm, animals are actually head-restrained using agarose while the bar patterns are projected and fictive swimming is induced, which can be assessed by electrophysiological recording of motor output. Sensory feedback corresponding to this measured fictive motor output is then provided back to the animal by way of adjusting the moving bar pattern. However, the experimenter can change the sensory feedback by altering the moving bar patterns. For example, if one simply keeps the bars moving at the same speed, i.e., not adjusted in line with the fish's predicted motor output, the fish will have the "experience" of being swept along by the virtual current, despite its attempts to stabilise its position. After a certain period (tens of seconds) with such feedback, animals "give-up" on this apparently futile behaviour, hence the term futility-induced passivity.

By carrying out whole-brain light-sheet imaging of calcium activity in effectively all brain neurons, together with that of radial astrocytes during futility-induced passivity, the authors showed that while the activity of certain specific neurons decreased upon the switch to giving up, radial astrocyte activity in specific brain regions increased, and in fact proceeded, both the decrease in neuronal activity and the switch in behaviour. This implicated radial astrocytes in driving the giving up behaviour. The causal role of radial astrocytes in regulating

both neuronal activity and the switch to the passive state was investigated in numerous complementary manners, using optogenetic, chemogenetic, pharmacological and cell-ablation based manipulations of radial astrocytes (Mu et al., 2019). Although the precise circuit mechanisms remain to be fully defined, this study provides direct evidence that radial astrocytes can integrate and in turn regulate neuronal activity and behaviour in a living vertebrate. Even though the cells whose activity was imaged in this study were referred to as radial astrocytes it remained unclear what proportion of these astrocyte-like cells actually retained a radial-like morphology. Given the historical lack of documentation of cells with a truly astrocytic (star-like) non-radial morphology in amniotes, it was assumed that essentially all such cells may retain a radial morphology and thus a hybrid radial glial/astrocytic state. However, a very recent study has shown that this is not necessarily the case and that cells with definitive astrocyte-properties exist in fish (Chen et al., 2020) (Figure 2).

Astrocytes

It is important to note that the study of glial cells in zebrafish remains of relatively modest volume compared with that of mammalian models, and until the advent of transgenic reporter tools, approaches to visualise glial cell morphology in zebrafish typically relied on a restricted number of antibody-based labelling approaches (Grupp et al., 2010). Therefore, the fact that cells with a bona fide astrocyte nature had not been identified reflected more absence of evidence than evidence of absence. Indeed, it was a breakthrough in transgenic reporter technology that unlocked the evidence for astrocytes in zebrafish. Starting with an *in situ* hybridisation screen of candidate astrocyte markers from the mammalian literature, Chen et al. (2020) selected the glutamate aspartate transporter (*Glast*) as a strong candidate marker of astrocytes in zebrafish. Upon making transgenic constructs and stable reporter lines using *glast* regulatory sequence, *Glast* expressing cells were shown to have a number of similarities to mammalian astrocytes (Figure 2). Using live *in vivo* imaging Chen and colleagues first demonstrated that zebrafish *Glast*-expressing cells transform in morphology from radial glial cells into cells that display complex morphologies akin to mammalian astrocytes (Figure 2). Furthermore, *Glast*-expressing cells display tiling behaviours, in order to maximise CNS coverage, similar to mammalian astrocytes (Bushong et al., 2002) (Figure 2). Using the *glast* regulatory sequence to drive expression of a genetically encoded indicator defined patterns of Ca²⁺ activity highly characteristic of those seen in mammals (Nett et al., 2002; Wang et al., 2019) were observed (Figure 2), which importantly were shown to be sensitive to regulation by norepinephrine, as in mammals (Ding et al., 2013; Paukert et al., 2014). Additionally, *Glast*-expressing cells in the zebrafish expressed glutamine synthetase in their somata and processes, which is also enriched in mammalian astrocytes and is known to be essential for neuronal interactions (Norenberg and Martinez-Hernandez, 1979). Interestingly, astrocyte-like *Glast*-expressing cells in the zebrafish spinal cord were also found to closely associate with synapses, identified utilising the presynaptic vesicle glycoprotein 2A, suggesting that zebrafish astrocytes may play an important

role in mediating synaptic formation and function (**Figure 2**). Given the large body of literature indicating that astrocytes regulate effectively all stages of synapse formation and function in mammals (Chung et al., 2015; Farhy-Tselnicker and Allen, 2018), it will be important to determine to what extent this is true in zebrafish (**Figure 2**). For example, given their close proximity to synapses, similar to astrocytes in mammalian models (Stogsdill et al., 2017), it is possible that zebrafish astrocytes may play a role in the clearance of ions and neurotransmitters from the extracellular space, and potentially also in gliotransmission. Given the power of zebrafish for investigation of cell structure, function and interactions at sub-cellular resolution, one can envision future studies that combine high resolution imaging of glial cells with functional imaging of neuronal and synaptic activity during the execution of specific behaviours, to explore the regulation of synaptic formation and function by astrocytes in zebrafish. One area of increasingly obvious importance from studies in mammals is that of heterogeneity with respect to subtypes and states of astrocytes (Oberheim et al., 2012; Poskanzer and Molofsky, 2018; Bellini et al., 2019), which will also be important to investigate in zebrafish by combining molecular profiling studies with the advantages of visualising cell structure and function *in vivo* using zebrafish in health, and ultimately also in disease-relevant experimental paradigms.

Oligodendrocyte Lineage Cells

Oligodendrocyte Progenitor Cells

In addition to astrocytes and neurons, radial glial cell progenitors can also produce OPCs. OPCs (also known as NG2 cells) are an abundant group of progenitor cells in the CNS throughout life, which are principally known for their ability to generate myelinating oligodendrocytes (Mitew et al., 2014) (**Figure 3**). OPCs and oligodendrocytes are, to date, the most extensively studied glial cell lineage in zebrafish, with the focus having been on their formation and functions in the embryonic hindbrain and spinal cord. The majority of such early born OPCs in the zebrafish initially derive from the progenitor of motor neuron (pMN) domains of the ventral CNS, as in mammals (Lu et al., 2002; Park et al., 2002; Zhou and Anderson, 2002; Shin et al., 2003; Lamason et al., 2005; Li et al., 2007; Kucenas et al., 2008). Later in development, OPCs and oligodendrocytes in the zebrafish and other vertebrates colonise the entire brain (Richardson et al., 2006; Bergles and Richardson, 2016), but because these have been much less extensively studied in zebrafish we will focus on studies which have investigated the development of OPCs from the pMN domain.

Oligodendrocyte Progenitor Cell Specification. As in mammals, pMN progenitors in zebrafish initially generate motor neurons, then switch to producing OPCs in a process tightly regulated by a collection of transcription factors, signalling molecules and extracellular cues. For example, various transcription factors including Olig2 and Nkx2.2, and signalling pathways including Hedgehog have all been shown to have important conserved roles in OPC specification in zebrafish and mammals (Lu et al., 2000, 2002; Park et al., 2002; Kirby et al., 2006; Sun et al., 2006; Li et al., 2007; Kucenas et al., 2008; Lyons and Talbot,

2015) (**Supplementary Table 1**). More recently, mechanisms of Sonic hedgehog signalling regulation were investigated by Scott et al. (2020) who explored the effects of transcriptional repressor *prdm8* on OPC specification. By live *in vivo* imaging they show that pMN cells in *prdm8* mutant embryos have elevated Sonic hedgehog signalling and prematurely switch from motor neuron to OPC production (Scott et al., 2020). Intriguingly, *Prdm8* was also shown to influence the fate of oligodendrocyte lineage cells, as *prdm8* mutant embryos have increased numbers of oligodendrocytes but lack sufficient OPCs. Together, this suggests that the morphogen Sonic hedgehog plays an important role in controlling the timing of OPC specification in zebrafish (Ravanelli and Appel, 2015; Scott et al., 2020). Similarly, the Notch canonical signalling pathway also influences the timing of the pMN domain progenitor differentiation switch from motor neurons to OPCs. This has been shown in zebrafish where reduced Notch activity induced excess formation of primary motor neurons and reduced numbers of OPCs (Appel et al., 2001; **Supplementary Table 1**). In contrast, constitutive Notch activity promoted OPC production (Appel et al., 2001; **Supplementary Table 1**). Complementing this study, Snyder et al. (2012) found that disrupting *fbxw7*, a ubiquitin ligase that targets Notch for degradation, results in excess OPC production (**Supplementary Table 1**). Taken together, these data suggest that Notch signalling tightly regulates the timing of the pMN progenitor differentiation switch, and therefore plays a central role in OPC specification.

Concomitant with their specification, OPCs in zebrafish and mammals delaminate from the neuroepithelium and migrate towards their axonal targets. One interesting candidate which has been identified in zebrafish to affect the timing of neuroepithelial delamination to OPCs in the zebrafish is the microRNA *mir-29*, which can regulate the expression of the polarity genes *pard3* and *prkci* (Hudish et al., 2013; **Supplementary Table 1**). How OPCs are specified in other regions of the neuraxis and at later stages, will require ongoing analyses, and in particular the way in which radial-glial-like progenitors present in zebrafish can give rise to OPCs following injury or demyelination awaits in-depth analyses ahead of comparison with mammals.

Oligodendrocyte Progenitor Cell Proliferation and Migration. Following delamination from the neuroepithelium OPCs in mammals proliferate extensively and migrate widely to take up positions throughout the parenchyma in which they both continue to proliferate and also adopt specialised functions, including the generation of myelinating oligodendrocytes (**Figure 3**). In contrast, at the stages and regions principally studied to date in zebrafish, OPCs have a relatively small volume to colonise. Indeed, the total distance that OPCs migrate from the pMN domain in the ventral spinal cord to nearby ventral axonal tracts where oligodendrocytes first differentiate is on the order tens of microns with dorsal regions of the hindbrain or spinal cord remaining only 100–200 μm from the pMN domain. These small distances mean that the OPC population doesn't undergo a huge degree of expansion through proliferation and migration at early embryonic stages. To what extent principles of OPC population expansion and migration observed in mammals, e.g., whereby OPCs follow the brain vasculature to migrate

| Function / Feature / Characteristic | Zebrafish | Mammals |
|-------------------------------------|-----------------------|-----------------------|
| | OPC / Oligodendrocyte | OPC / Oligodendrocyte |
| OPCs generate oligodendrocytes | | |
| Non-canonical functions of OPCs | | |
| Myelination by oligodendrocytes | | |
| Metabolic support to the axon | | |
| Activity regulated myelination | | |

FIGURE 3 | Key characteristics and functional properties of zebrafish and mammalian OPCs and oligodendrocytes. The use of “...” refers to areas of OPC/oligodendrocyte biology which require further investigation.

over long distances (Tsai et al., 2016), are conserved in fish will require analyses at later stages and in other regions of the CNS when and where migratory routes are longer and, likely more complex. Nonetheless, several cell-cell interactions, as well as specific molecular factors have been identified that influence both OPC proliferation and migration in the developing zebrafish (Supplementary Table 1).

For example, OPC proliferation appears to be responsive to the density of axons fated for myelination in the embryonic zebrafish CNS, with OPC proliferation reduced in a mutant that lacked axons fated for myelination in the posterior spinal cord, Almeida and Lyons (2016). One set of axonal signals known to regulate many aspects of the oligodendrocyte lineage in mammals, including OPC proliferation, are those associated with neuronal activity (Geraghty et al., 2019). Indeed, a recent

study has indicated that neuronal activity also influences OPC proliferation in the zebrafish where a pharmacologically-induced increase in neural activity resulted in increased OPC proliferation (Marisca et al., 2020). Although the axonal signals (activity-related or not) that regulate OPC proliferation in zebrafish await identification, receptors on oligodendrocytes that influence proliferation have been uncovered (Supplementary Table 1). In addition to proliferation, neuronal activity has also been implicated in regulating OPC migration in mammals (Harlow et al., 2015) and recently it was found that disruption to the AMPA receptor subunit GluR4A (encoded by *gria4a*) expressed on OPCs impaired migration in zebrafish (Piller et al., 2021) (Supplementary Table 1).

Following migration to their target regions, OPCs undergo a set of cell-cell interactions to demarcate the space in which

they will ultimately reside. During this tiling behaviour OPCs continuously extend and retract processes to sample their environment and appear to be highly influenced by contact with surrounding OPCs, whereby contact between distinct OPCs appears to induce repulsion of contacting processes (Kirby et al., 2006). Indeed, when a single or small group of OPCs are ablated, nearby OPCs increase cell division to replenish numbers, fill the space, and reinitiate tiling behaviours (Kirby et al., 2006). This appears to be a largely conserved mechanism between species, given that similar observations were made in the cortex of mature mice, where neighbouring OPC processes exhibited contact-mediated withdrawal, and increased proliferation following damage to neighbouring OPCs (Hughes et al., 2013). Recently, in a mouse model, the molecular mechanism for this OPC-OPC interaction was proposed (Chavali et al., 2020), conservation of which remains to be tested in zebrafish, but appears likely, given the remarkable similarity in associated cell behaviour across species.

Oligodendrocyte Progenitor Cell Diversity. The broadly accepted view of OPCs is that they are principally sources of myelinating oligodendrocytes. However, it is now becoming clear that OPCs can exhibit a diverse array of functions beyond their roles in making myelinating oligodendrocytes (Harrington et al., 2020; Akay et al., 2021; Clayton and Tesar, 2021) (**Figure 3**).

The existence of OPCs with potentially distinct fates was first investigated in zebrafish by Kucenas et al. (2008), who showed that OPCs that express the transcription factor *Nkx2.2a*, an ortholog of rodent *Nkx2.2*, typically differentiate into myelinating oligodendrocytes, whereas OPCs that do not express *nkx2.2a* mostly remain as non-myelinating OPCs. The fate of distinct zebrafish OPCs was recently investigated further in a study that combined fate mapping of individual OPC clones, morphological characterisation of their dynamic behaviour by time-lapse microscopy, and associated single cell RNA sequencing (Marisca et al., 2020). This study confirmed the existence of two discrete groups of OPCs in the zebrafish spinal cord, those with cell bodies located within axonal tracts and those with cell bodies surrounded by the cell bodies of other cells, principally neurons (Marisca et al., 2020). Interestingly, OPCs with cell bodies located in the axon-dense areas tended to directly generate myelinating oligodendrocytes, whereas those with cell bodies in neuron/soma-rich areas tended to self-renew and remain relatively stable, although these cells could also generate the OPCs that moved to axon-rich areas. These two OPC types displayed both unique branching morphologies and very different patterns of calcium activity and interactions with axons. Interestingly, and perhaps surprisingly, OPCs with their cell bodies within the neuron-rich areas had higher levels of calcium activity, but yet infrequently differentiated, despite having extensive processes that made very stable contact with axons. Interestingly and correspondingly, these OPCs had a molecular signature of being responsive to neuronal activity, and it was these cells whose proliferation was responsive to changes in activity noted earlier (Marisca et al., 2020). This suggests that although neuronal activity can regulate OPC proliferation, it may not, directly at least, influence oligodendrocyte differentiation.

The function of OPCs beyond their generation of oligodendrocytes has been hard to experimentally disentangle, but has recently been investigated in zebrafish, by taking advantage of the presence of abundant OPCs in a brain area, the optic tectum, that does not become myelinated in the developing larva. The optic tectum is the largest retino-recipient brain structure in zebrafish, receiving input from a large proportion of retinal ganglion cell axons and is analogous to the superior colliculus in mammals. Upon arrival at the tectum, distinct retinal ganglion cell axons enter specific tectal layers where they make precise connections with defined tectal neuron dendrites (Erskine and Herrera, 2007). To investigate a potential role for OPCs in connectivity with target neurons, Xiao et al. (2021) ablated tectal OPCs and found that this resulted in enlarged axonal arbors in the tectum and branching of retinal ganglion cell axons beyond the tectal neuropil where target dendrites are located. Indeed, live imaging implicated OPCs in regulating axonal pruning/remodelling *in vivo*, which had previously been predicted by *in vitro* studies in mammals (Goldberg et al., 2004). Very interestingly, ablation of tectal OPCs in zebrafish also resulted in impairments to visual function. Recent investigation in rodent models suggest that OPCs can engulf axons in mammals (Buchanan et al., 2021), and even directly modulate neuronal activity (Sakry et al., 2014). Given the availability of reporters to label OPCs as well as synaptic and neuronal activity in zebrafish, it will be possible to directly and more deeply interrogate how OPCs affect both neuronal structure and function *in vivo* using zebrafish. As well as determining the currently unrecognised roles of OPCs in larval zebrafish, another exciting area of future study will be determining the functions of OPCs in the adult zebrafish brain (Tsata et al., 2020), both in health and disease.

Oligodendrocyte Differentiation. The process of oligodendrocyte differentiation from OPCs is one that can occur by default in the absence of axonal signals (Zeller et al., 1985; Lee et al., 2012; Bechler et al., 2018), upon the activation of gene expression programmes that ultimately converge on building the myelin sheath [reviewed in Czopka and Lyons (2011), Bergles and Richardson (2016), and D'Rozario et al. (2017)]. This default differentiation programme appears to be conserved *in vivo* in zebrafish, as evidence by relatively unaltered oligodendrocyte differentiation observed in mutants with a large reduction in axons fated for myelination (Almeida and Lyons, 2016). As observed in mammals, there appears to be a critical period following differentiation in which oligodendrocytes either commit to myelination, or undergo cell death (Takada and Appel, 2010; Almeida and Lyons, 2016). In general, the molecular regulation of oligodendrocyte differentiation and initiation of myelin gene expression appears well conserved with mammals (**Supplementary Table 1**). For example, the lysosomal transcription factor *Tfeb* was recently identified through a forward genetic screen in zebrafish as a negative regulator of myelination, whereby its abrogation leads to premature and excess myelination (Meireles et al., 2018). In parallel, mice lacking *TFEB* in oligodendrocytes were also found to exhibit precocious and excessive myelination (Sun et al., 2018). In the mouse this

was found to be mediated, at least in part, by reduced cell death of newly generated oligodendrocytes. It remains to be determined precisely to what extent the molecular control of oligodendrocyte survival and myelination are distinct or overlap downstream of Tfeb, and how Tfeb and other major transcription factors co-operate to control oligodendrocyte differentiation.

Myelination by Oligodendrocytes. Similar to other vertebrates, zebrafish oligodendrocytes make multiple myelin sheaths on many axons upon differentiation (**Figure 3**), and although some differences in components have been noted the overall molecular composition and ultrastructural nature of myelin in zebrafish and mammals appears broadly similar (Jahn et al., 2009, 2020; Czopka and Lyons, 2011; Preston and Macklin, 2015; Czopka, 2016; Siems et al., 2021) (**Figure 3**). By live imaging of oligodendrocytes in zebrafish over time using transgenic reporters, Czopka et al. (2013) found that individual cells initiate formation of essentially all of their sheaths within a matter of hours following the initiation of the first sheath, pointing to a restricted period in which oligodendrocytes have to select axons for myelination. Following the selection of axons, the process of myelination continues *via* wrapping of the innermost layer of the myelinating process around and along the axon in a spiralling manner (Snaidero et al., 2014; Nawaz et al., 2015). Following the initial dynamic period of their formation, most sheaths are maintained over time, except for occasional sheath retractions (Czopka et al., 2013; Auer et al., 2018). Whereas initial myelin sheath formation occurs over a matter of hours, imaging studies indicated that subsequent wrapping-based sheath growth occurs over several days, before slowing down and growing in step with the overall growth of the tissue (Auer et al., 2018).

But how are specific axons selected for myelination and why do oligodendrocytes avoid other axons, and indeed other cellular targets for myelination? Almeida et al. (2018) recently found that the relative abundance of oligodendrocytes and axons fated for myelination influences targeting, given that in mutants with fewer axons fated for myelination or in animals with excess oligodendrocytes, oligodendrocytes made myelin around inappropriate targets including neuronal cell bodies. In contrast to myelinating neuronal cell bodies, oligodendrocytes in animals with fewer axons fated for myelination do not myelinate incorrect axons, suggesting more stringent mechanisms preventing their myelination, likely a combination of typically being of smaller axon calibre and expressing inhibitory signals (Klingseisen and Lyons, 2018). Following a forward genetic screen in zebrafish, it was subsequently found that the cell adhesion molecule Neurofascin functions in oligodendrocytes to prevent inappropriate myelination of cell bodies, a mechanism conserved in rodents (Klingseisen et al., 2019). This finding was also made in a parallel study, which implicated the neuronal partner of oligodendrocyte Neurofascin, Contactin1, in mediating correct myelin targeting (Djannatian et al., 2019). Studies in zebrafish have also indicated that certain axons can positively influence myelination (Almeida et al., 2011; Nelson et al., 2020), with many studies now demonstrating that neuronal activity can influence myelination (Nave and Werner, 2014; Baraban et al., 2016; Swire and French-Constant, 2018; Williamson and Lyons,

2018). Studies in zebrafish have contributed to this, with evidence that blocking synaptic vesicle release from all neurons reduces the number of myelin sheaths made by oligodendrocytes and increasing activity increases sheath number (Mensch et al., 2015). Correspondingly, experiments in which the synaptic vesicle release of only some axons was affected indicated that myelination is biased towards higher activity axons (Hines et al., 2015; Koudelka et al., 2016), but interestingly that this only remains true of certain neuronal subtypes (Koudelka et al., 2016), a feature that has more recently also been shown to be true in rodents (Yang et al., 2020).

The mechanisms by which vesicle release regulates myelination was recently investigated by live-cell imaging a transgenic reporter (SypHy) that allows assessment of vesicular release along axons together with a reporter that allows assessment of myelination along the same axons. Somewhat surprisingly, it was found that axonal synaptic vesicle fusion increases upon myelination, and in fact requires myelination. Upon myelination, axonal vesicular fusion becomes enriched adjacent to sites of myelin sheath formation, where it becomes increased upon increasing neuronal activity, which in turn promotes myelination. This led to a feedforward model of activity-regulated myelination, whereby myelination stimulates the localised axonal vesicular release that in turn consolidates myelin sheath growth along axons (Almeida et al., 2021). Although the various molecular mechanisms that regulate the axonal vesicular release remain to be determined, the cell adhesion molecule *N*-cadherin has been proposed to mediate the effects of neuronal activity on myelination in zebrafish (Chen et al., 2017), and may do so by influencing vesicular recycling, as has been shown in mammals (Van Stegen et al., 2017). Recent data has also indicated that axons employ mechanisms that consolidate synapse formation in myelination. This was evidenced by observations that oligodendrocytes in zebrafish can localise the postsynaptic scaffold protein Psd95 to myelin sheaths, and that myelination can be dysregulated by interfering with molecules involved in synapse organisation (Hughes and Appel, 2019; **Supplementary Table 1**), also seen in mammals (Elazar et al., 2019). In addition to direct signalling between axons and myelinating processes, recent studies have also shown that neuronal activity can influence myelination indirectly, including through regulation of endothelin signalling from the vasculature, which influences myelin sheath production by oligodendrocytes in an Endothelin receptor B dependent manner in both fish and mice (Swire et al., 2019). Future studies will be required to elucidate the potentially multiple activity related axonal signals that influence myelination and the receptors on myelinating processes or on intermediate cells that mediate these effects.

Although the mechanisms of activity-regulated myelination remain to be fully disentangled, two parallel studies in zebrafish indicated that they may influence specific codes of Ca²⁺ activity in myelin sheaths, that in turn affect myelination. By live imaging individual myelin sheaths, distinct modes of Ca²⁺ activity were found to prefigure either myelin sheath retractions or myelin sheath elongation (Baraban et al., 2018; Krasnow et al., 2018). Global inhibition of neuronal activity indicated that roughly half

of the calcium transients in myelin sheaths were regulated by neuronal firing (Krasnow et al., 2018). A causal role for localised myelin Ca²⁺ in mediating myelination was evidenced by the finding that localised Ca²⁺ dependent Calpain protease activity was responsible for driving myelin sheath retractions, Baraban et al. (2018) and that manipulating free Ca²⁺ levels influenced sheath elongation (Krasnow et al., 2018). How the firing patterns of individual neurons influences distinct Ca²⁺ activities in myelin sheaths remains to be determined, but is likely to be experimentally tractable in zebrafish by combining optogenetic control of neuronal activity with analyses of corresponding myelin responses. Another mechanism that has been proposed to influence myelination downstream of neuronal activity is that of the local translation of proteins, including myelin structural proteins such as myelin basic protein at the axon-myelin interface (Wake et al., 2011). It has been known for some time that oligodendrocytes transport mRNAs encoding myelin proteins to their distal myelinating processes (Colman et al., 1982), and a forward genetic screen in zebrafish revealed that such mRNA transport in oligodendrocytes is dependent on the kinesin motor protein Kif1b (Lyons et al., 2009). A more recent study has shown that neuronal activity can affect the regulation of mRNAs within myelin sheaths in zebrafish, and that specific 3'UTR elements in *mbp* mRNA regulate its localisation and local translation (Torvund-Jensen et al., 2018). To further investigate the mechanisms that regulate mRNA localisation to myelin sheaths, Yergert et al. (2019) tracked mRNAs as they were transported *in vivo* in the zebrafish. In doing so, distinct 3'UTR elements were identified which direct mRNA to areas of active sheath growth. This study also showed that additional mRNAs have conserved 3'UTR sequences, which appear to drive their localisation to myelin sheaths, including the mRNA encoding the RNA binding fragile X mental retardation protein FMRP, *fmr1* (Yergert et al., 2019). The importance of *fmr1* in directing sheath growth has also been demonstrated in zebrafish, whereby oligodendrocytes lacking *fmr1* have shorter myelin sheaths (Doll et al., 2020), a phenotype that will be important to pursue in the context of understanding circuit function and relevance to developmental disorders.

In addition to mechanisms potentially related to activity-regulated myelination, studies in zebrafish have also provided insight into fundamental cell biology of myelination by oligodendrocyte in the CNS. Live imaging studies in zebrafish placed actin as a major regulator of sheath wrapping, as in mammals (Nawaz et al., 2015; Zuchero et al., 2015), and more recently this has been shown to be influenced by the p21-activated kinase 1 (Pak1) in zebrafish (Brown et al., 2021). In parallel to its role in influencing myelin targeting, oligodendrocyte Neurofascin has been shown to support the growth of myelin sheaths along axons, likely through its interaction with axonal Caspr (Klingseisen et al., 2019). In addition to the regulation of the growth of individual myelin sheaths, the total amount of myelin made by oligodendrocytes is an important factor, and one that has been shown to be regulated by the akt-mTOR pathway (Mathews and Appel, 2016a), under the influence of the ubiquitin ligase Fbxw7 (Kearns et al., 2015), a factor that when absent can lead to Schwann cells in the peripheral nervous

system myelinating multiple axons like oligodendrocyte through an unknown mechanism independent of its control of Mtor (Harty et al., 2019). Perhaps surprisingly, it remains unclear how the overall production of myelin is regulated over time, an area important for investigation in the future.

How Does Myelin Affect Circuit Function?

Myelin sheaths are well known to facilitate rapid action potential propagation along axons, through their lipid-rich, multilamellar composition (Nave and Werner, 2014; Stadelmann et al., 2019), their physiology (Suminaite et al., 2019), and their role in organising key ion channels along axonal subdomains, such as nodes of Ranvier (Sherman and Brophy, 2005; Stassart et al., 2018). In addition, myelin sheaths are thought to represent a conduit for the metabolic support of axons by oligodendrocytes, which has also been proposed to regulate axonal function and integrity (Nave, 2010; Nave and Ehrenreich, 2018) (Figure 3). Furthermore, the fact that myelination can be modified by neuronal activity (Figure 3), and that changes in myelination can affect conduction, has led to the hypothesis that activity regulated myelination may contribute to nervous system plasticity (Fields, 2015; Chang et al., 2016; Almeida and Lyons, 2017; Monje, 2018; Suminaite et al., 2019). The zebrafish offers a unique opportunity to investigate these questions. The first steps towards investigating how CNS myelin in zebrafish affected neural circuit function were however only recently investigated by Madden et al. (2021) in a mutant with disrupted *myrf*, myelin regulatory factor, which affected axonal conduction and zebrafish behaviour. They found that *Myrf* was required for normal myelination in the CNS (Supplementary Table 1), as in rodents (Bujalka et al., 2013), and that zebrafish *myrf* mutants displayed reduced action potential conduction velocity and have a decreased ability to sustain high frequency action potential firing. To assess the effects of these changes in circuit function on behaviour zebrafish startle responses were studied, which displayed an increased latency to perform startle responses and an aberrant behavioural choice upon sensory stimulation (Madden et al., 2021). Although much remains to be discovered, this study highlights the potential of the zebrafish to integrate electrophysiological protocols with live *in vivo* imaging and behavioural assays, which could also be combined with functional imaging of neuronal and circuit activity to fully interrogate how myelination affects neuronal function and circuit plasticity.

Microglia

Microglia Formation

Microglia are the resident immune cells of the central nervous system (Ransohoff and Cardona, 2010) (Figure 4). Recent genetic tracing, live cell imaging and transcriptomic sequencing data has revealed two waves of microglial development within the zebrafish (Xu et al., 2016; Ferrero et al., 2018; Wu et al., 2020) (Figure 1). The first wave originates from a population of primitive macrophages in the yolk sac (Herbomel et al., 2001; Ginhoux et al., 2010; Schulz et al., 2012). These migrate to the hematopoietic tissue [the rostral blood island (RBI)], and then from peripheral tissues to the optic tectum during early embryonic development (Herbomel, 1999). A second wave,

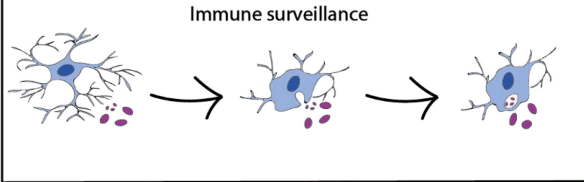
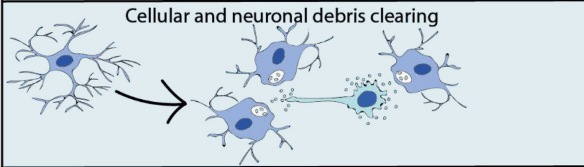
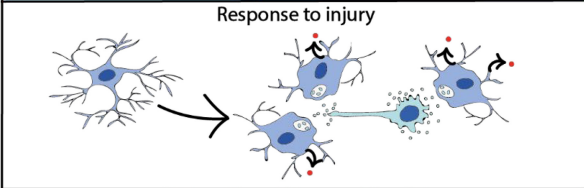
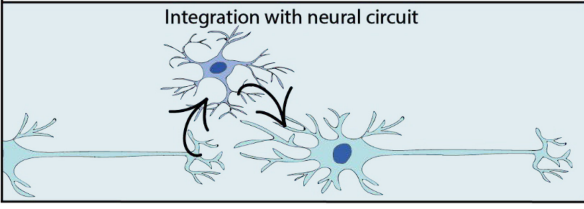
| Function / Feature / Characteristic | Zebrafish | Mammals |
|--|-----------|-----------|
| | Microglia | Microglia |
|  <p>Immune surveillance</p> | ✓ | ✓ |
|  <p>Cellular and neuronal debris clearing</p> | ✓ | ✓ |
|  <p>Response to injury</p> | ✓ | ✓ |
|  <p>Integration with neural circuit</p> | ✓ | ✓ |

FIGURE 4 | Key characteristics and functional properties of zebrafish and mammalian microglia. The use of “...” refers to areas of microglial biology which require further investigation.

which derives from hematopoietic stem cells from the ventral wall of the dorsal aorta replaces the original population later in development (Ferrero et al., 2018; Wu et al., 2020; Silva et al., 2021). A number of conserved transcription factors have been shown to be required for microglia development in zebrafish and mammalian models including Pu.1 and Irf8 (Rhodes et al., 2005; Li et al., 2011; Shiao et al., 2015) (**Supplementary Table 1**). Additionally, studies in the zebrafish have identified factors essential for the infiltration of yolk sac derived macrophages into the brain. For example, the receptor for macrophage-colony-stimulating factor (Csf1r), has been shown to be essential for microglia migration, as loss of function mutations in both paralogs which encode this receptor induce a complete loss of microglia in the developing zebrafish brain (Herbomel et al., 2001; Wu et al., 2018) (**Supplementary Table 1**), similar to findings in mammals (Ginhoux et al., 2010). Further dissection of the role of Csf1r using fate-mapping have identified distinct roles for the distinct paralogs of the *csf1r* gene in the zebrafish (Braasch et al., 2006); *csf1ra* and *csf1rb*. Whereas *csf1ra* is important for the initial developmental wave, and later microglia maintenance in the adult zebrafish brain, *csf1rb* has been shown to be essential

for colonisation of the CNS by the second microglial wave (Ferrero et al., 2021).

Further studies have also highlighted an essential role for Xpr1, a phosphate exporter, in microglial colonisation of the brain (Meireles et al., 2014), where disruption of both paralogs reduces microglial number in the CNS (**Supplementary Table 1**). However, unlike the *csf1r* paralogs which independently impair microglial number, *xpr1b* appears to play a much more fundamental role in microglial function than *xpr1a* which was not required for microglial formation. Like the *csf1ra* mutants, *xpr1b* mutants do have a primitive macrophage population but, unlike the *csf1ra* mutation, even after migration into the brain these cells fail to differentiate into microglia, even in later larval developmental stages. Also deriving from a forward genetic screen for genes that regulate microglial formation was the identification of the Nod-like receptor Nlrc3-like gene as being essential for microglial infiltration to the brain. Interestingly, Nlrc3l appears to function to downregulate excessive inflammation, whereby mutants exhibited hugely increased inflammation and aggregation of primitive macrophages in the yolk and vasculature preventing their migration to the brain (Shiao et al., 2014)

(**Supplementary Table 1**), bringing up important questions on how peripheral inflammation can regulate CNS microglia, a topic of increasing broad interest.

Upon entry into the CNS, differentiation of microglia is classified based on up-regulation of *apoE*, downregulation of *I-plastin* and a ramified cell morphology (Herbomel et al., 2001), which can be regulated *via* a number of signalling pathways (Yang et al., 2021). Recent work indicates that the process of microglial precursor colonisation of the zebrafish CNS is promoted by programmed neuronal cell death, a normal aspect of early nervous system development in vertebrates (Nijhawan et al., 2000; Dekkers et al., 2013). Interestingly, recent studies in the zebrafish have shown a direct link between the numbers of neurons undergoing apoptosis in different CNS regions and the level of microglial infiltration into them. This was demonstrated by Xu et al. (2016) who showed that inhibition of neuronal cell death reduces the number of microglial precursors which enter the optic tectum in the developing zebrafish. To investigate the signals which may mediate this process, Casano et al. (2016) utilised a photoconvertible protein expressed in microglia to track the progress of microglia in a system with inducible neuronal cell death. Enhanced infiltration of microglia into specific regions with significant neuronal apoptosis was shown to be mediated by nucleotide-mediated chemotaxis, which has also been reported in murine studies (Haynes et al., 2006), where nucleotide release is associated with cell or tissue damage. Taken together these data demonstrate that neuronal apoptosis mediates microglial CNS infiltration into specific regions, which is used during zebrafish development to colonise the CNS and influence microglia number.

Functional Roles of Microglia

The process by which microglia engulf dying neurons has been investigated in the zebrafish by time-lapse imaging (Peri and Nüsslein-Volhard, 2008). Neuronal engulfment is reliant on combined interactions between Tim-4 and Bai1, two phosphatidylserine receptors on the microglial surface (**Supplementary Table 1**). Specifically, Bai1 is involved in phagosome formation and transport whilst Tim-4 is necessary for phagosome stabilisation *via* actin polymerisation (Mazaheri et al., 2014). Once phagocytosed, the Rag-Regulator complex is essential in the formation of lysosomes and efficient digestion of neuronal debris (Shen et al., 2016). Further investigations have added to the list of phagocytic signals that could prompt engulfment of dying neurons by microglial cells in the zebrafish including lysophosphatidylcholine, a phospholipid known to be released from apoptotic neurons (Xu et al., 2016) which is also a potent chemoattractant for microglia in rodents.

But how can microglia coordinate their migration towards phagocytic targets, and how are microglia recruited long distances throughout the CNS? One candidate signalling pathway, which has been shown to facilitate long range migration of microglia to damaged neurons is calcium signalling. Utilising a targeted laser neuronal ablation approach to damage neurons in the brains of larval fish expressing calcium reporters in microglia Sieger et al. (2012) observed rapid calcium waves which determined the direction and magnitude of the microglial

response, as killing fewer neurons resulted in shorter range Ca²⁺ waves and migration of local microglia only. Furthermore, preventing the formation of such Ca²⁺ waves or chelating extracellular Ca²⁺ reduced microglia migration to sites of damage, suggesting that Ca²⁺ signalling plays an essential role in neuronal-microglial interactions. But how could neuronal signalling influence the generation of Ca²⁺ waves within microglia? Interestingly, the generation of Ca²⁺ waves was found to be dependent on glutamate and NMDA-receptor signalling, which may facilitate communication between neuronal and microglial cells in a number of physiological as well as disease/injury situations, given that microglial contacts with neurons have been shown to be affected by neuronal activity (Hughes and Appel, 2020). Microglia have also been implicated in synapse formation and function including synaptic pruning and removal of inappropriate synaptic contacts in mammals, which is a normal aspect of vertebrate development. In the zebrafish the contribution of glia to synapse formation, strengthening and pruning is far less extensively studied than in mammals, and remains an exciting area for future research. That being said, there is evidence to suggest that microglia in the zebrafish may play important roles in synaptogenesis. For example, a recent study from Silva et al. (2021) describe a novel subpopulation of synapse-associated microglia present in the midbrain and hindbrain. Synapse-associated microglia express the complement gene *C1qc*, a known protein which also regulates synaptic engulfment by microglia in rodents (Stevens et al., 2007; Hong et al., 2016), which could suggest that it may mediate similar functions in the zebrafish.

Microglia can also regulate homeostatic neuronal activity in zebrafish. This was explored in a study by Li et al. (2012) who integrated *in vivo* imaging of microglial cells, whole-cell electrophysiology recording, and glutamate uncaging. By monitoring microglial morphology, process dynamics and neuronal activity they showed that local increases in neuronal activity attracted microglial processes, which contacted the somas of highly active neuronal cells. Once microglia-neuron contacts were established after about 5 min this contact downregulated spontaneous activity in the neuron, which remained at a low level for at least 7 min even after the interaction. Further mechanistic investigation showed that this process was dependent on pannexin-1 hemichannels on the neuronal soma, triggering the release of signals which attract microglia *via* activation of the small Rho GTPase Rac in resting microglia (Li et al., 2012). Intriguingly, a recent study utilising a mouse model has provided further evidence that microglia may mediate negative feedback mechanisms to protect the brain from excessive activity, highlighting a conserved feature of microglia biology across species (Badimon et al., 2021). Together these results raise fascinating questions about the roles of microglia in regulating neural excitation in the healthy CNS, the potential roles of microglia at synapses, and the effects these may have on neuronal function if such interactions become perturbed in disease.

Interestingly, neuronal regulation of microglial function has recently been demonstrated to include microglial phagocytosis of non-neuronal cells. For example, microglia have recently been proposed to be capable of phagocytosing myelin sheaths during

development. Similar to synapses and neurons, more myelin sheaths are initially produced in development than are eventually maintained over time. Whilst much of this was demonstrated to be driven by oligodendrocytes retracting myelin sheaths, as visualised *in vivo* in the zebrafish, Hughes and colleagues have recently described a contribution of microglia to developmental myelin sheath elimination. By studying the interactions of microglia with myelinated axons, they observed close association of microglia with both neuronal somas and myelinated regions of the axon (Hughes and Appel, 2020). Very interestingly, in animals with fewer microglia, individual oligodendrocytes had a greater number of myelin sheaths, without any significant effect on oligodendrocyte number, directly implicating microglia in the control of myelination by single oligodendrocytes (Hughes and Appel, 2020). This data raises a number of questions regarding the potential impacts of microglia in life long myelin plasticity and refinement of neural circuit structure and function, which await investigation. Microglia remain in the CNS throughout life with the ability to quickly transition into distinct functional states. Given that recent gene expression profiling studies have shown larval zebrafish microglia to have a conserved microglia signature *in vivo* compared to other model organisms, this places the zebrafish in a central position for future study of these cells (Mazzolini et al., 2019), in the healthy nervous system, but also in various disease modelling contexts, which are becoming established in zebrafish.

DISCUSSION

The current landscape of zebrafish glial research is an exciting one given the rapid advances in genetic manipulation strategies, imaging techniques, probes for labelling, fluorescent reporters, ablation techniques, optogenetics, drug screening and the ability to integrate many of these whilst also studying behavioural responses. Additionally, advances in cell type specific investigation of gene function, and platforms to simultaneously investigate glial and neuronal function within the context of behavioural outputs will, no doubt, place the zebrafish in a central position for future investigation of glial cells in health and disease.

Recent research has highlighted the diversity of functions played by glia in the zebrafish, and how their similarities with other model organisms can be used to identify conserved biological pathways. Moreover, differences between the highly regenerative zebrafish and other organisms provide a useful comparison where novel pathways with potential implications

in repair can be investigated. Given the range of essential roles that glia perform in development, circuit function and lifelong maintenance of the healthy nervous system, it follows that disruption to glia, or changes to the functional roles which they play can characterise disease. The zebrafish, as a model organism, is emerging as a popular choice to study glial cells in disease and regeneration. For example, the roles of glial cells in epileptic seizures, brain tumours, degenerative disease, remyelination, peripheral nerve regeneration, retinal regeneration, and spinal cord repair are now being studied in zebrafish models of disease (Mokalled et al., 2016; Sifuentes et al., 2016; Karttunen et al., 2017; Chia et al., 2018, 2019; Frøyset et al., 2018; Saleem and Kannan, 2018; Diaz Verdugo et al., 2019; Neely et al., 2020). Given the applications of zebrafish to investigate glial cells throughout life, in disease and for screening applications we believe that this makes for exciting future research into glial cell function to complement that in other models.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2021.754606/full#supplementary-material>

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