Glial Cell Development and Function in the Zebrafish Central Nervous System

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ABSTRACT

Over the past decades the zebrafish has emerged as an excellent model organism with which to study the biology of all glial cell types in nervous system development, plasticity, and regeneration. In this review, which builds on the first version of this book chapter, we will summarize how the relative ease to manipulate the zebrafish genome and its suitability for intravital imaging have helped understand principles of glial cell biology with a focus on oligodendrocytes, microglia, and astrocytes. We will highlight recent findings on the diverse properties and functions of these glial cell types in the central nervous system, and discuss open questions and future directions of the field.

INTRODUCTION

All animals with a central nervous system (CNS) have glia, but only the vertebrate CNS contains three glial cell types: oligodendrocytes, microglia, and astrocytes. The zebrafish is amongst the simplest vertebrate model organisms used in biosciences and its popularity has increased steadily since its introduction in the 1980s by George Streisinger (Streisinger et al. 1981). Several properties make zebrafish a superb model for experimental research. They are highly fecund with a single pair giving rise to hundreds of offspring in each mating. Embryos develop externally making them easily accessible to the experimenter. As they develop from a fertilized egg to a freely swimming animal in less than five days, they are an ideal model for developmental studies. From five days post-fertilization (dpf) onwards, young zebrafish start hunting for prey, meaning that they have formed functional neural circuits and are able to carry out complex sensory-motor transformations. During all these early stages, zebrafish remain relatively small (under 1 cm in length) and optically transparent, which allows one to study glial development and function at unprecedented detail and without the need for surgical intervention. These combined features have made the zebrafish an exquisite model for genetic, pharmacological, cellular, physiological, and behavioral analyses in the intact living animal.

Although zebrafish represent evolutionary distant relatives to mammals with a CNS of lower complexity (about 100,000 neurons in a larval fish brain) and a different neuroanatomy, it is important to emphasize that principles of nervous system formation and function are highly conserved across species. A comparative study has revealed that at least 70% of human genes have at least one ortholog in zebrafish (Howe et al. 2013). Likewise, the past two decades have shown that development and function of zebrafish glia are highly conserved compared to

mammals, from key transcription factors that regulate development to molecular signals and cellular dynamics that regulate the interaction of glia with other CNS cell types that surround them.

In this chapter, we aim to provide an update of the excellent contribution by David Lyons and William Talbot in the first edition of this book (Lyons and Talbot 2015). Since then, major progress has been made to understand properties and functions of glia due to the possibility to live image all glial cell types at single cell resolution in the entire animal (Figures 1 and 2). Here, we will summarize our current understanding of zebrafish glial biology, and discuss open questions and future directions.

OLIGODENDROCYTES

As in mammals, zebrafish oligodendrocyte lineage cells form an abundant population throughout the CNS where they co-exist in different states from undifferentiated precursors to myelinating oligodendrocytes throughout development, adulthood, and aging. How individual oligodendrocytes progress through their lineage, how they communicate with surrounding neurons (and glia), when to differentiate, and which axons to select for myelination are fundamental questions that had remained unanswered for a long time. In addition to what we have learned from zebrafish about the genetic control of oligodendrocyte development (comprehensively reviewed by(Lyons and Talbot 2015; Preston and Macklin 2015; Ackerman and Monk 2016; Czopka 2016), the suitability of young zebrafish for non-invasive live cell microscopy along with the development of reagents and technologies to visualize and manipulate oligodendrocytes, has allowed the study of oligodendrocyte biology in real time in the intact

living animal. Indeed, zebrafish remains the only system in which one can live image oligodendrocyte-neuron interactions from the moment cells are specified to the point where they have formed mature myelin sheaths *in vivo*, and perform sophisticated genetic manipulations to understand mechanisms. The past 10 years have provided a substantial collection of studies in which *in vivo* imaging and cellular genetic manipulations have revealed fundamental properties OPCs, oligodendrocytes, and myelin. In the following sections we will focus on summarizing these studies.

Formation of oligodendrocyte precursor cells and regulation of their lineage progression

Oligodendrocyte precursors (OPCs) are specified in defined CNS regions from where they migrate and disperse throughout the CNS. In the spinal cord of zebrafish, like in all vertebrates, the first OPCs arise from the pMN domain defined by the *olig2* transcription factor, which initially gives rise to motor neurons followed by the generation of OPCs through the recruitment of new wave of neural progenitors to the pMN domain (Park et al. 2002; Rowitch 2004; Ravanelli and Appel 2015). Throughout the CNS, OPCs display diverse properties with regard to gene expression profile, physiological properties, and ability to differentiate (Viganò et al. 2013; Marques et al. 2016; Spitzer et al. 2019). Consequently, it has been a long-standing question in the field whether the observed diversity of OPCs reflects intrinsically different types of OPCs, or rather different states of the same cell (reviewed by (Dimou and Simons 2017; Foerster et al. 2019; Kamen et al. 2021). Marisca and colleagues addressed this question using an integrated approach in zebrafish to identify molecular, anatomical, and physiological differences between OPCs whilst monitoring their lineage formation and probing their function over time (Marisca et al. 2020). They found that the zebrafish spinal cord contains a network of OPCs with different

morphological complexities and process remodelling dynamics, depending on their local microenvironment. Although all these OPCs contact the same cohorts of myelination-competent axons, they have targets available that they can myelinate, only some OPCs differentiate readily while others do not. To test how these different groups of OPCs relate to another, *i.e.*, if different OPCs seen at any point in time simply represent different states of the same cell, Marisca et al. generated clonal trees of OPC fates and interrelationships, which revealed a functional segregation between OPCs; some remain undifferentiated in either quiescent or proliferative states to regulate their overall numbers, while other still proliferative OPCs become primed for differentiation and subsequent myelination. Interestingly, OPCs that persist as quiescent cells rarely differentiated to myelinating oligodendrocytes. Instead, quiescent OPCs could re-enter cell cycle and divide in a calcium-dependent manner to give rise to a daughter cell, which then frequently proceeded to myelination. These results show that, although all OPCs represent different states of their lineage, lineage progression is not linear for each individual OPC and that a hierarchy exists within their overall population.

What makes myelinating and non-myelinating OPCs different? Marisca and colleagues found that a combination of intrinsic and extrinsic factors regulate the likelihood of an OPC to differentiate. Extrinsic, because OPCs did not differentiate when the OPC cell body was surrounded by neuron cell bodies (regardless of the OPC processes contacting myelination competent axons). Intrinsic, because all myelinating oligodendrocytes that Marisca *et al*. identified in their clonal analyses were formed from a cell that had undergone a recent cell division. The finding that recently divided OPCs differentiate with a higher frequency is consistent with reports on oligodendrocyte generation from OPCs in the developing mouse

cortex (Hill et al. 2014), as well as remyelination (Foerster et al. 2020), but it differs from the adult mouse cortex where direct differentiation of OPCs has been reported that have been persisting for long periods of time (Bacmeister et al. 2020). Future work will be needed to dissect if readily differentiating OPCs in the adult animal are already somewhat primed and just need to have a break released to proceed to myelination, or if fundamentally different mechanisms exist between developmental and adult oligodendrogenesis.

Choosing axons for myelination and making the right number of sheaths with the right length

Once an OPC has entered its terminal differentiation program, each individual cell appears to have only a narrow time window to establish its maximum number of myelin sheaths. Live cell imaging studies in zebrafish showed that processes of differentiating oligodendrocytes either form nascent axon ensheathments, or alternatively retract back to the cell body within just a few hours after forming its first myelin sheath (Czopka et al. 2013; Almeida and Macklin 2023). After this time oligodendrocytes do generally not form any new sheaths, although sheaths can still be eliminated by either retraction (Czopka et al. 2013; Liu et al. 2013), as well as microglia-mediated phagocytosis (Hughes and Appel 2020; Djannatian et al. 2023).

One important implication of this stereotyped behavior of myelin sheath formation by individual oligodendrocytes is that each oligodendrocyte must carefully choose which axons to myelinate as they rapidly lose the competency to do so. How oligodendrocytes select their axons is not fully understood. Axon caliber is a major determinant of ensheathment fate in the CNS. In zebrafish, the largest caliber axon (the Mauthner axon) is also the first one myelinated, followed by other axons of smaller yet still relatively large caliber (Almeida et al. 2011; Koudelka et al. 2016).

However, CNS axons of a very large range of calibers are ultimately myelinated, meaning that additional regulatory factors must be present.

Currently, the prevailing view is that there is no single determinant of myelination fate in the CNS, but that this process is under the influence of several factors that may be employed in a context-dependent manner. Over the past 10 years, the concept of 'adaptive' myelination has emerged and is understood as the regulation of myelination in response to changes in nervous system activity, based on observations that white matter content, oligodendrogenesis, and myelination increase in response to changes to experience and learning (Scholz et al. 2009; Makinodan et al. 2012; McKenzie et al. 2014). Oligodendrocytes in mammals and zebrafish are perfectly equipped to sense neural activity using a wide range of neurotransmitter receptors and voltage-gated ion channels (Maldonado and Angulo 2015; Marisca et al. 2020). Several studies in zebrafish revealed that neuronal activity directly tunes myelin sheath formation at the level of individual cells down to single sheaths. Systemic blockade of axonal vesicle release reduces the overall number of myelin sheaths formed per oligodendrocyte, and vice versa, an increase of neural activity increases the number of sheaths per cell (Mensch et al. 2015). Furthermore, blocking vesicle release in single axons biases towards axon ensheathment of non-silenced axons in choice situations (Hines et al. 2015). How these effects are mediated at the molecular level? They may involve direct neurotransmitter- and/or depolarization-induced signalling in oligodendrocytes as it was recently described that axon-OPC synaptic contacts can predict regions of sheath formation (Li et al. 2022), but also involve more indirect cascades. For example, one study using zebrafish and mice showed that myelin sheath numbers formed by individual oligodendrocytes involved signalling via endothelins released from the vasculature,

possibly linking neurovascular communication to the regulation of oligodendrocyte behavior (Swire et al. 2019).

After differentiating oligodendrocytes have selected target axons for myelination, sheaths need to grow to the right length. Again, long-term *in vivo* imaging in zebrafish has revealed principles of myelin sheath dynamics by showing that nascent sheaths grow dynamically and at highly variable rates for the first three days after their respective initiation (Auer et al. 2018). After this phase, sheaths continued to extend at slow rates that are similar to the overall body growth of the larval zebrafish. Therefore, differences in the length between individual myelin sheaths are established during the first days after their respective formation (Auer et al. 2018). A series of related studies revealed that this early phase of variable sheath growth is regulated by dynamic neuron to oligodendrocyte communication. Newly formed sheaths exhibit intracellular calcium transients which can be raised by neuronal activity, and which can regulate their stabilization, extension or shrinking (Baraban et al. 2017; Krasnow et al. 2017). Indeed, post-synaptic proteins have been detected in paranodal regions, which are sites of axonal vesicle fusion (Hughes and Appel 2019; Almeida et al. 2021), and the disruption of both axonal vesicle release as well as synaptic and non-synaptic adhesion molecules in paranodal regions impair myelin sheath extension (Hughes and Appel 2019; Djannatian et al. 2019; Klingseisen et al. 2019; Almeida et al. 2021). Together, these studies suggest that neuronal activity during the early phases of sheath growth may ultimately determine whether a long or a short myelin sheath will be formed.

Although axonal activity can directly regulate ensheathment fate and sheath growth, it should be noted that axonal activity is not an absolute requirement for myelination. Oligodendrocytes still

myelinate when all action potentials are blocked by tetrodotoxin in zebrafish (Mensch et al. 2015). Furthermore, an activity-dependent control of sheath growth alone may not be sufficient to regulate how entire axons get myelinated along their length. Axon myelination patterns can be highly specific and form over long periods of time, which frequently leads to the formation of intermittent 'patchy' myelination with sheaths that have no direct neighbors in zebrafish and mice (Tomassy et al. 2014; Auer et al. 2018). Therefore, growing myelin sheaths need to know (or be told) when and where to stop extending to form a heminode (and ultimately a node) in a desired place. Contact-mediated repulsion by neighboring sheaths is one mechanism to stop them growing (Auer et al. 2018). This process requires internodal and paranodal adhesions as their disruption results in myelin sheaths that overgrow each other (Djannatian et al. 2019). However, how do sheaths stop growing when they don't meet another sheath? One simple explanation would be that the axon itself provides stop signals. Evidence for such cues comes from two zebrafish imaging studies which showed that growing myelin sheaths frequently extend asymmetrically from the feeding cytoplasmic process (Auer et al. 2018). In some cases, this was due to the presence of axon collateral branches, which provide a physical barrier that stop sheaths extending further (Auer et al. 2018). In other cases, however, sheaths stopped growing in one but not the other direction even though no obvious physical barrier was present. Here, a later study revealed that the presence of pre-nodal clusters along unmyelinated axon stretches can serve as stop signal for growing myelin and therefore prefigure node of Ranvier position (Vagionitis et al. 2022). Another possibility to form myelin sheaths of a desired length with nodes in a specific position comes from very recent observations using zebrafish, mice, and human organoids where cytoplasmic bridges connecting adjacent myelin sheaths across a node of Ranvier have been observed (Call et al. 2022). Although the meaning of these paranodal

bridges is presently unclear, it is tempting to speculate whether they represent a secondary constriction to split an existing myelin sheath into two, and thus an entirely new mechanism to regulate sheath length and node position. Together, these collective studies using zebrafish have revealed different ways of ongoing axon-oligodendrocyte crosstalk to dynamically regulate if and how axons get myelinated over time. Many of these processes are modulated by neuronal activity and thus adaptive, which opens new avenues to investigate how such adaptive myelination in turn changes axon and consequently circuit function.

Repairing a demyelinated axon

Damage to myelin and disease-mediated loss of myelin are hallmarks of CNS injury and demyelinating diseases like multiple sclerosis (MS), which have lasting and irreversible consequences for axonal health and function (Franklin and ffrench-Constant 2017). Although zebrafish do not get MS, just like any other non-human species, they are a valuable model to understand principles of regenerative oligodendrogenesis. Various models to demyelinate axons have been established and range from focal single cell demyelination using photosensitizers (Auer et al. 2018), toxin-induced demyelination using cuprizone (Jaronen et al. 2022) and lysolecithin (Münzel et al. 2014; Cunha et al. 2020; Morris and Kucenas 2021), as well as chemogenetic models to induce oligodendrocyte death using targeted expression of nitroreductase (Karttunen et al. 2017) and TRPV1 channels (Neely et al. 2022). *In vivo* imaging of oligodendrocyte dynamics in these models has, for example, revealed that myelinating oligodendrocytes that survive experimental demyelination can sometimes form new myelin sheaths, but that these sheaths are frequently mistargeted to non-axonal compartments (Neely et al. 2022). Inspired from these observations in zebrafish, the same study confirmed that such

mistargeting can also be found human MS lesions and may in fact impair neuronal function and hinder efficient myelin repair. This work showcases how discoveries from zebrafish can help understand aspects of human disease without attempting to directly model the disease.

What do OPCs do in the CNS beyond making myelin?

Owing to the fact that OPCs always form a constant number of resident CNS cells, it has been a long-standing question about the role OPCs in the CNS besides being the cellular source of myelinating oligodendrocytes. However, answers have remained largely elusive, primarily due to the circumstance that it is technically difficult to manipulate OPC function without indirectly affecting myelination. Several regions of the mammalian CNS contain OPCs but remain largely devoid of myelin, and would thus be suitable to specifically test OPC-specific functions without indirectly interfering with myelin formation (*e.g.*, superficial layers of the cerebellar cortex and olfactory bulb glomeruli). However, reagents and assays to specifically target OPCs in these regions have remained sparse. Recently, Xiao et al. identified the optic tectum of larval zebrafish as a CNS region that allows the precise study OPC functions without indirectly interfering with myelination (Xiao et al. 2022). The zebrafish optic tectum is the region where retinal ganglion cell axons synapse to tectal neurons. This region is easily accessible to the experimenter, densely interspersed with OPCs, but it contains hardly any myelin (Figure 2). Importantly, during these stages larval zebrafish have a functional visual system, thus allowing one to directly probe the roles of OPCs in a functional neural circuit. Using this model, Xiao *et al.* found through different perturbation methods that the absence of OPCs from the tectum impaired the precise formation and remodeling of retinal ganglion cell axon arbors, which consequently degraded the acuity of

visual processing, thus providing a direct role for OPCs in sculpting neural circuits (Xiao et al. 2022).

The finding that tissue resident OPCs have mature roles over and above their canonical roles in myelin formation raises a vast range of open questions. Firstly, how do OPCs exert their effects to fine-tuning circuit connectivity? They could either guide axons, as has been shown in the context of glial scar formation and CNS damage where OPCs inhibit axon growth (Tan et al. 2005). Alternatively, they could prune axons by phagocytosis. Indeed, it was recently shown in the mammalian visual system that OPCs can ingest axonal presynaptic compartments (Auguste et al. 2022; Buchanan et al. 2022). Regardless of the mechanism of action, by being an active player in neural circuit development, dysfunctional OPCs may likely contribute to a vast range neurodevelopmental and neuropsychiatric disorders where the fine-tuning of circuit connectivity are dysregulated. For example, in a recent sequencing study of patients who suffered from major depressive disorders, about 50% of dysregulated genes were in fact encoded by OPCs (Nagy et al. 2020). In the light of the findings from zebrafish where OPCs directly regulate circuit connectivity (Xiao et al. 2022) it may thus be that OPCs themselves directly contribute to mental illness, which will be interesting research directions to address in the future.

MICROGLIA

Although microglia are immune cells that originate outside the brain parenchyma, many studies have demonstrated that they play essential roles in the development and homeostasis of the brain. Indeed, today we know that microglia have many functions besides fighting pathogens, ranging from synaptic patterning, neurogenesis, neuronal removal, survival, and axon guidance.

Moreover, the notion that microglia participate in many, if not all, neurodegenerative disorders affecting the CNS has generated a great deal of interest in these cells, pushing scientists to investigate how microglia respond to neuronal changes, with the zebrafish serving as an ideal model.

Intrinsic and extrinsic processes contribute to microglial brain colonization

Microglia come from yolk sac primitive macrophages that colonize the embryonic brain as highly migrating cells (for review, see (Prinz et al. 2017)). In mice and fish, this process relies on the tyrosine kinase colony-stimulating factor 1 receptor (Csf1r). In mammals, this receptor is responsible for both brain colonization and microglial survival (Erblich et al. 2011; Pridans et al. 2018; Rojo et al. 2019), and pharmacological inhibition of CSF1R can be used to deplete the microglial population (Elmore et al. 2014; Squarzoni et al. 2014). In mouse, this receptor has two ligands, Csf1 and Interleukin 34 (Il34) (Lin et al. 2008), with distinct expression patterns and non-redundant functions (Zeisel et al. 2015; Cahoy et al. 2008; Greter et al. 2012; Wang et al. 2012; Easley-Neal et al. 2019; Kana et al. 2019). In contrast, zebrafish have two Csf receptor paralogs, Csf1ra and Csf1rb, resulting from genome duplication in teleosts (Braasch et al. 2006). There are no microglia in the absence of both paralogs (Oosterhof et al. 2018); however, less severe phenotypes are observed when only one of the two genes is mutated (Ferrero et al. 2020). Fish microglia colonize the brain in two waves; the first wave occurs during embryogenesis to establish primitive microglia, and the second occurs later to set up the adult population (Xu et al. 2015; Ferrero et al. 2018). Within this framework, Csf1ra and Csf1rb play distinct functions; Csf1ra is responsible for establishing primitive microglia, while Csf1rb is a regulator of microglial development in adults (Ferrero et al. 2020). Interestingly, in these mutants, when one

population is absent, the other one is smaller, pointing to the fact that the primitive and adult microglial populations might be interdependent (Ferrero et al. 2020). There is also evidence that microglial progenitors infiltrate the mammalian cortex in multiple waves and via different routes (Swinnen et al. 2013; Smolders et al. 2019), and in humans, microglia appear to colonize the brain in a stepwise manner during gestation (Menassa and Gomez-Nicola 2018). It is intriguing to speculate that, like in fish, these microglial colonization waves might be interdependent and account for the regional heterogeneity observed in mammalian microglia (see below).

Another interesting aspect of brain colonization is understanding how microglial precursors find their way to the brain. Studies in fish have shown that these cells are attracted by neuronal cell death, a key feature of brain development. Indeed, long-range signals from dying neurons attract microglial precursors into the CNS, highlighting the importance of neuronal cell death in shaping the brain's immune system (Casano et al. 2016; Xu et al. 2016). Reducing the rate of neuronal cell death leads to fewer microglia while an increase in apoptosis results in more microglia colonizing this organ (Casano et al. 2016). Research using zebrafish has also shown that lysosomes and their regulation in microglia influence brain colonization. Indeed, zebrafish microglia lacking components of the Rag regulatory complex -GTPases that function as heterodimers on lysosomes- have enlarged lysosomes and undigested apoptotic material (Shen et al. 2016). Moreover, these mutants have fewer microglia in the brain, suggesting that defects in lysosomes and cargo processing can affect essential microglial functions like migration and differentiation. In *raga* mutants, lysosomal genes are upregulated, and microglial brain colonization defects can be rescued by ablating *tfeb* and *tfe3*, transcription factors required for activating lysosomal pathways (Iyer et al. 2022). Recent research on microglia that colonize the

developing retina has shown that blood vessels provide ways for these cells to enter neurogenic eye regions, suggesting that guidance factors may be present on the surface of these blood vessels to facilitate microglial migration (Ranawat and Masai 2021).

The interaction between microglia and other glia in the central nervous system

Interactions between microglia and the local environment are of significant interest not only for understanding how microglia influence brain development and functionality but also for uncovering how changes in brain physiology affect key microglial behaviors. A recent study investigated how microglia engulf developing myelin sheaths, a function that could impact higher brain functions such as memory and learning (Hughes and Appel 2020; Djannatian et al. 2023). Fluorescent labeling of microglia and oligodendrocytes allowed visualization of cellular interactions and revealed the presence of engulfed myelin in microglia. Importantly, this study uncovered a functional link between the level of neuronal activity and the removal of myelin by microglia as optogenetic manipulations that make neurons less active led to more myelin in microglia (Hughes and Appel 2020). Depleting microglia did not affect the number or distribution of oligodendrocytes; however, it altered the morphology of myelin sheaths, which appeared shorter and often misshaped, suggesting a link between these two cell types and a role for microglia in myelination by oligodendrocytes (Hughes and Appel 2020). In line with this, a recent study using electron microscopy in mouse and in vivo confocal light microscopy in zebrafish has shown that during early development microglia engulf myelin fragments (Djannatian et al. 2023). This process depends on the presence of phosphatidylserine lipids on myelin, a signal that also mediates the engulfment of apoptotic cells and synaptic pruning (Mazaheri et al. 2014; Scott-Hewitt et al. 2020). Another study in this direction has shown that in

humans, some *CSF1R* variants cause ALSP (adult-onset leukoencephalopathy with axonal spheroids and pigmented glia), a leukodystrophy characterized by fewer microglia and a cognitive decline (Ranawat and Masai 2021). Introducing these human ALSP-causing *CSF1R* variants in the fish genome recapitulates the microglial reduction seen in patients (Ranawat and Masai 2021). Interestingly, transcriptomics and proteomics approaches revealed upregulation of genes in astrocytes associated with enhanced endocytosis, indicating that astrocytes might try to compensate for the loss of microglia in these mutants. This points to the existence of critical feedback compensatory mechanisms within the glial populations of the CNS.

Microglial transcriptional and functional heterogeneity

Single-cell transcriptomics approaches have demonstrated that microglia display a high degree of transcriptional heterogeneity (for review see (Masuda et al. 2020). A big question in the field is how differences in gene expression translate into functional diversity. Understanding this will provide important insights into how the microglial population differs in its responses to challenges and changes in brain physiology. In the zebrafish, we can distinguish two adjacent brain regions, the synaptic-rich hindbrain (HD) and the neurogenic optic tectum (OT). Interestingly, in these two areas, microglia display different morphologies; hindbrain microglia are ramified, while optic tectum microglia are more ameboid (Wu et al. 2020; Silva et al. 2021). In addition, these cells exhibit regionally specific gene signatures; HD microglia are enriched for complement cascade components, whereas OT microglia are enriched for cathepsins and lysosomal enzymes. Interestingly, these cells also appear to perform different functions, and cathepsin-enriched microglia in the OT engulf apoptotic neurons, while complement-expressing microglia in the HB are likely to interact more with synapses (Wu et al. 2020; Silva et al. 2021).

The direct comparison of these regional microglial populations represents a first step toward linking gene expression to function, an important goal in the field. While microglia have been seen to populate and adapt to specific areas within the CNS, time-lapse imaging in zebrafish has shown that these cells can also leave the CNS, for example after spinal root injury (Green et al. 2019). Indeed, in response to damage in the periphery, microglia migrate out of the spinal root in a glutamatergic signaling-dependent manner to phagocytose debris. Green and colleagues discovered that once these microglia return to the CNS, they respond faster to a second injury and are more phagocytic than cells that remain in the spinal cord. Thus, live imaging of the fish illustrates the remarkable plasticity of these cells and the importance of investigating the spatial-temporal dynamics of microglial state transitions and adaptations.

Role for microglia in removing neurons and modulating their activity

Several studies have examined one of the main functions of microglia, which is the engulfment of neurons during brain development (Peri and Nüsslein-Volhard 2008). Engulfing an entire neuron can be challenging, as microglia must also sort and recycle the products that derive from the degradation of this cell. These late steps in phagocytosis remain poorly understood, mainly due to the difficulty of studying these processes *in vivo*. However, understanding the mechanism by which microglia process engulfed neurons is a fundamental goal, as many well-known Alzheimer's disease risk factors are genes that are required in microglia to degrade and transport lipids that derive from neuronal degradation (Thorlakur et al. 2013; Keren-Shaul et al. 2017; Nugent et al. 2020). Moreover, diseased microglia are often characterized by the presence of lipid aggregates (Marschallinger et al. 2020). A study in zebrafish tracked phagosomes inside microglia to follow the fate of the neuronal cargo in these cells (Villani et al. 2019). Live

386 imaging showed that phagosomes containing dead neurons shrink progressively and fuse with
387 the gastrosome, a previously undescribed cellular compartment that allows efficient processing
388 of the apoptotic cargo (Villani et al. 2019). The gastrosome, also found in mammalian
389 macrophages, contains membrane fragments and expands dramatically when phagocytosis
390 increases, indicating that cells such as microglia must also limit neuronal uptake to allow
391 digestion and maintain their shape. Indeed, a hallmark of microglia is their highly ramified
392 morphology, characterized by the presence of multiple dynamic protrusions that these cells use
393 to scan the brain parenchyma and engulf several neurons per hour (Villani et al. 2019). The
394 mechanisms that allow microglia to use their branches to identify and engulf apoptotic neurons
395 successfully remain unclear. Zebrafish live imaging approaches have shown that microglia,
396 despite having many branches, always select one branch and engulf one neuron at a time (Möller
397 et al. 2022). This branch selection process strongly correlates with the movement of the
398 microglial centrosome that translocates rapidly into one branch towards the forming phagosome.
399 Microglia with two centrosomes -a condition obtained by overexpressing core centrosomal
400 components- engulf more neurons and even remove two neurons simultaneously, indicating that
401 centrosomal migration is a rate-limiting step in microglial neuronal engulfment (Möller et al.
402 2022). The targeted movement of the microglial centrosome has been shown to involve the
403 PLC/DAG signaling cascade, which also operates in T-cells at the immunological synapse,
404 reinforcing the idea of a possible evolutionary link between these two critical cellular interphases
405 (Möller et al. 2022). Besides looking at neuronal microglial interactions during brain
406 development, several studies have also focused on how microglia respond to tumours induced by
407 AKT1oncogene overexpression in neural cells (Chia et al. 2018, 2019). Interestingly, dynamic
408 interactions between microglia and these $AKT1^{+}$ neuronal cells are mediated by ATP signaling

409 that attracts microglia in a *p2ry12*-dependent manner, similar to microglial attraction towards
410 neuronal injuries (Chia et al. 2019). These interactions are not phagocytic but might promote
411 tumour growth as microglial depletion reduces $AKT1^{+}$ neuronal cell proliferation (Chia et al.
412 2019). There has also been considerable interest in the role of microglia in synaptic elimination,
413 a process also known as pruning and first described in mice (Paolicelli et al. 2011; Schafer et al.
414 2012). Interestingly, although it is established that microglia participate in synaptic pruning, it is
415 an ongoing debate of whether microglia do so by actively removing synapses through
416 engulfment (Eyo and Molofsky 2023). Here, the fantastic properties of zebrafish for in vivo live
417 cell imaging of how microglia engage with synapses during circuit remodelling could be used to
418 help resolve these open questions. Furthermore, studies in zebrafish and mice have demonstrated
419 a non-phagocytic role for microglia in the modulation of neuronal activity (Li et al. 2012;
420 Merlini et al. 2021). Indeed, live imaging in zebrafish has revealed that microglial processes
421 contact highly active neurons and that in turn these interactions lead to the downregulation of
422 both spontaneous and induced neuronal activity (Li et al. 2012), suggesting an important role for
423 microglia in neuronal modulation.
424
425 In conclusion, today we now know that microglia are an integral part of the CNS glial pool and
426 that these cells perform a variety of important functions. As we continue to study microglia, their
427 phenotypes, and dynamic state transitions, one clear goal is the development of novel strategies
428 for modulating microglial activities in vivo. The zebrafish model system will remain an
429 invaluable and indispensable resource in this pursuit.
430
431 **ASTROCYTES**

Astrocytes are morphologically complex glial cells that extend dense cellular processes to interact closely with neuronal synapses, brain vasculature, and other glial cells in the CNS. The most numerous cells in the mammalian brain, astrocytes support neuronal activity, maintain homeostasis of the CNS, and are implicated in the control of neural circuit development and function (Clarke and Barres 2013; Nagai et al. 2021; Perez-Catalan et al. 2021). Moreover, many studies suggest astrocytes play key roles in neurological diseases (Molofsky et al. 2012; Burda and Sofroniew 2014). Despite their importance, compared to our understanding of neuronal development and function, we know very little about how astrocytes develop, what the diverse function of astrocytes might be in different brain regions, and how these properties are regulated.

During development, immature astrocytes derive from radial glial cells. Astrocytes elaborate their cellular processes during postnatal development, coincident with the period of active CNS synaptogenesis, and ultimately form intimate associations with neuronal synapses that are crucial for both cell types (Bushong et al. 2002). How astrocytes establish and maintain their remarkable morphologies is not known. Astrocytes also powerfully control neuronal development. For instance, astrocyte-secreted Thrombospodin promotes synapse formation via its neuronally-expressed receptor (Christopherson et al. 2005; Eroglu et al. 2009), and additional astrocyte-derived factors are also required for synapse formation and maturation (Kucukdereli et al. 2011; Allen et al. 2012). Based on efforts from several labs, it seems highly likely that additional molecules regulating astrocytic process growth, plasticity, and sculpting of neural circuitry await discovery. Finally, astrocytes respond to neurotransmitter release by increasing intracellular calcium levels (Cornell-Bell et al. 1990; Dani et al. 1992), which has been proposed to participate in neural circuit control. For example, norepinephrine powerfully controls astrocyte

calcium signaling in mammals (Shigetomi et al. 2016), and a conserved neuromodulatory event (via invertebrate analogs of norepinephrine) regulates neurotransmission changes and behaviors in *Drosophila* (Ma et al. 2016).

Although most of our understanding of astrocyte biology derives from investigation of mouse models, numerous studies suggest striking conservation of astrocyte biology across species (Oikonomou and Shaham 2011; Stork et al. 2014). Curiously, zebrafish had long been proposed to not possess stellate astrocytes until recently, and radial glial cells had been historically proposed to functionally substitute for astrocytes (Grupp et al. 2010; Lyons and Talbot 2015). Recent work, however, has identified astrocytes in zebrafish (Chen et al. 2020), thus positioning zebrafish as a new model to study astrocyte biology *in vivo*.

Discovery and characterization of zebrafish astrocytes

In mammals, radial glia serve as neural progenitors during early development; by late neurogenesis, most radial glia regress their radial processes from the ventricles and become stellate-like astrocytes (Rowitch and Kriegstein 2010). Similarly, in zebrafish, radial glia have been characterized in various CNS regions during development (Lyons et al. 2003; Johnson et al. 2016). However, in contrast to mammals, zebrafish radial glia persist in most regions of the adult CNS and are thought to be at least in part responsible for the impressive CNS regenerative capacity observed in this species (Kroehne et al. 2011; Than-Trong and Bally-Cuif 2015). Radial glia-like cells in zebrafish are present in the brain and in some regions have elaborated processes near synapses (Lyons and Talbot 2015; Mu et al. 2019), suggesting these cells could perform key functions of astrocytes.

Chen et al. recently sought to test if zebrafish radial glia perform necessary astrocytic functions or if a subset of zebrafish radial glia transform into stellate astrocytes that morphologically and functionally resemble mammalian astrocytes (Chen et al. 2020). Previous studies in zebrafish relied on Gfap (glial fibrillary acidic protein) as a marker and which is also expressed in zebrafish radial glia. Instead, Chen et al. focused on Glast (glutamate aspartate transporter or EAAT1), which is encoded by two orthologs in zebrafish, *slc1a3a* and *slc1a3b*. Transgenic lines and expression constructs were created in which membrane and nuclear markers were expressed under the *slc1a3b* promoter, thus enabling global and single-cell resolution analysis of Glast^{+} cells. Using these tools, Chen et al. observed radial astrocytes ((Mu et al. 2019); see next section) in the hindbrain, Bergmann glia-like cells in the cerebellum, and cells with the appearance of stellate astrocytes in the spinal cord (Figure 2).

Focusing on these stellate cells, which we hereafter refer to as zebrafish astrocytes, Chen and colleagues demonstrated their genesis from radial glia precursors by time-lapse imaging, showed that these cells express additional astrocyte markers, elaborate fine processes during synapse formation, tile with other astrocytes, exhibit spontaneous microdomain calcium transients with similar kinetics as mouse and *Drosophila* astrocytes, and that these microdomain calcium transients are sensitive to norepinephrine. In all, this work demonstrated that the zebrafish CNS houses a population of astrocytes very similar to those in mammals and *Drosophila*, providing further support for the notion that astrocytes are an ancient, well-conserved CNS cell type. Going forward, zebrafish will represent a powerful tool to study astrocyte development and function *in vivo*.

Roles for astrocytes in neural circuitry

It is critical to study astrocyte biology *in vivo* to understand their role in the control of neural circuits. Zebrafish represent an ideal system to do so, as demonstrated in a recent elegant study by Mu and colleagues who investigated how astrocytes regulate circuit function using whole brain imaging as animals executed simple behaviors (Mu et al. 2019). The authors examined the optomotor response in zebrafish larvae, which is a robust reflex enabling animals to maintain position in response to current. In these experiments, head-fixed larvae respond to moving gratings with swim bouts that attempt to match the presented optical flow (Orger et al. 2008). If the visual feedback following fictive swimming bouts is withheld, zebrafish eventually stop responding to the moving grating and become passive, a behavior that has been compared to learned helplessness in mammals (Nagai et al. 2021). Using this behavioral test coupled with whole-brain calcium imaging and cell specific perturbations, Mu et al. found that a zebrafish radial astrocytes are causal in regulating passivity generated by "futile" swim attempts such that radial astrocyte activation increased passivity, while silencing decreased passivity. After accumulated unsuccessful attempts, noradrenergic neurons in the medulla oblongata become active, and the released norepinephrine activates the α1-adenoceptor on radial astrocytes, which then activate GABAergic neurons in the brain stem to trigger behavioral passivity. In all, this work established zebrafish radial astrocytes as an essential player in a circuit that mediates an adaptive behavioral response.

It is interesting to note that spinal cord astrocytes (Chen et al. 2020) and hindbrain radial astrocytes (Mu et al. 2019) exhibit morphological differences, with hindbrain radial astrocytes

maintaining a long primary process between the cell body and the dense branches. However, given similarities in molecular markers (both express *glast* and *gfap*) and responses to norepinephrine signaling, it seems likely that spinal cord astrocytes and hindbrain radial astrocytes represent the same cell type or closely related cell types in different CNS areas, whereby surrounding cells or structural constraints might play a role in regulating morphogenesis.

Roles for astrocytes and radial glia in injury and disease models

The injury response of zebrafish radial glia and bridging glia, particularly in the adult CNS, has been extensively discussed (e.g., (Lyons and Talbot 2015; Jurisch-Yaksi et al. 2020; Becker and Becker 2022)) and we direct the reader to these resources for more information. In the future, it will be important to test whether stellate astrocytes, which are abundant in the larval CNS (Chen et al. 2020) persist in the adulthood and how these cells respond to injury and repair. Beyond injury, the study of zebrafish astrocytes can be a powerful contribution to our understanding of disease models. For example, zebrafish have been used for decades in numerous models of epilepsy (Yaksi et al. 2021), and recent work has uncovered key roles for radial glia in pentylenetetrazole (PTZ)-induced epilepsy. Diaz-Verdugo and colleagues demonstrated that Ca^{2+} signaling in radial glia is highly active and strongly synchronized compared to neurons before seizures began. During seizures, synchronization of radial glia and neural activity increased, and activation of radial glia using optogenetic approaches could strongly modulate neural activity by glutamate and gap junctions (Verdugo et al. 2019).

CONCLUSIONS AND FUTURE DIRECTIONS

The use of zebrafish began as a discovery model to identify genes important for different aspects of development and has since then transitioned towards a highly versatile model organism with which to study glial cell biology by combining genetics, imaging, and physiology of intercellular communication in an intact living animal. With an ever-increasing set of reagents and assays to visualize and manipulate cells of interest, and the continued advancement of microscopy approaches available, we anticipate that intravital imaging will continue to be one of the main strengths that this model provides to longitudinally study glial cell function and their complex interactions in the same animal over time. With the advent of CRISPR/Cas9-mediated genome editing, the direct targeting of specific genes is now highly efficient and allows for the rapid generation of knock-ins, for example to insert floxed alleles to enable cell type specific gene disruption, which has historically been unavailable to the community (Liu et al. 2022). We anticipate that such approaches will also become standard when studying glial cell biology in zebrafish in the next few years. Regardless of these technological considerations, moving the field forward will also necessitate looking beyond understanding the biology of glial cells themselves. It will be very interesting to dissect how glial cells integrate into the multicellular CNS, and how they help regulate formation, function, and dysfunction of the CNS. Possessing all the major classes of glial cells as well as a true vasculature, zebrafish represent a powerful tool with which to study glial development, neuron-glial interactions, glial-glial interactions, and glial-vascular interactions, all in intact circuits in a living, behaving vertebrate. Some recent studies have already integrated glial biology into systems neuroscience questions (Mu et al. 2019), and vice versa integrated circuit approaches into questions that relate to glial biology (Xiao et al. 2022), showing that zebrafish research is excellently suited to move forward towards an integrated understanding of glial for nervous system formation, function, and dysfunction.

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FIGURE LEGENDS

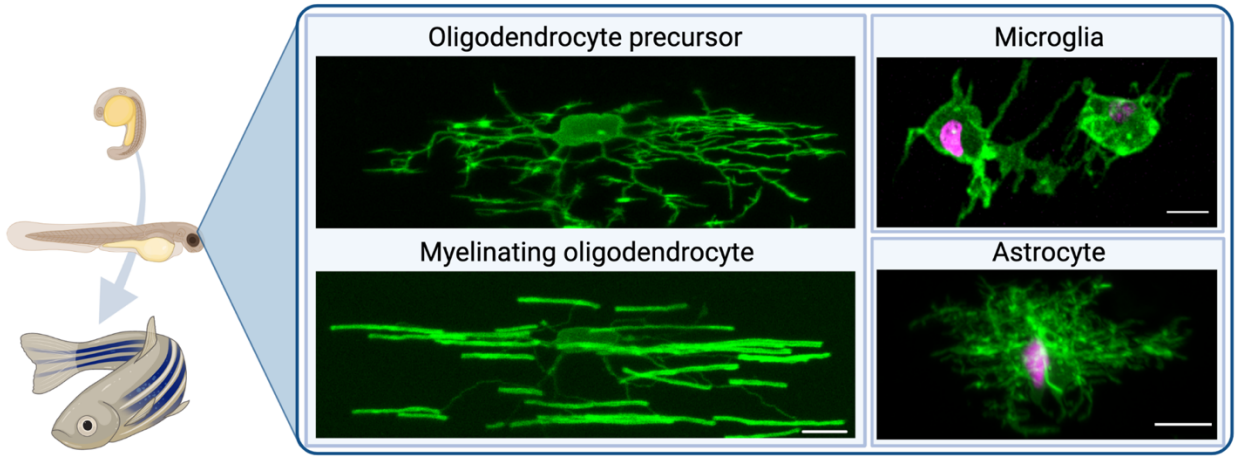

Figure 1: Visualising different glial cell types in living zebrafish.

Left: Schematic of different stages of zebrafish development from embryo through adulthood. Right: *In vivo* microscopy of different glial cell types in young zebrafish. The same oligodendrocyte at different stages of development labelled with an olig1:memEYFP transgenic reporter (image reproduced from Auer et al., 2018). Microglia are labelled in a double transgenic line with membrane-targeted tagRFP (green) and nuclear nls-Crimson (magenta) (Tg(mpeg1:Gal4; UAS:lyn- tagRFPT); Tg(spi1b:Gal4-UAS:NLS-Crimson)). A single astrocyte is labelled with membrane myrGFP (green) and nuclear H2A-mCherry (magenta) driven by the *glast* promoter. Cell was imaged from the larval spinal cord at 6 dpf (image credit, Jiakun Chen). Scale bars 10 µm.

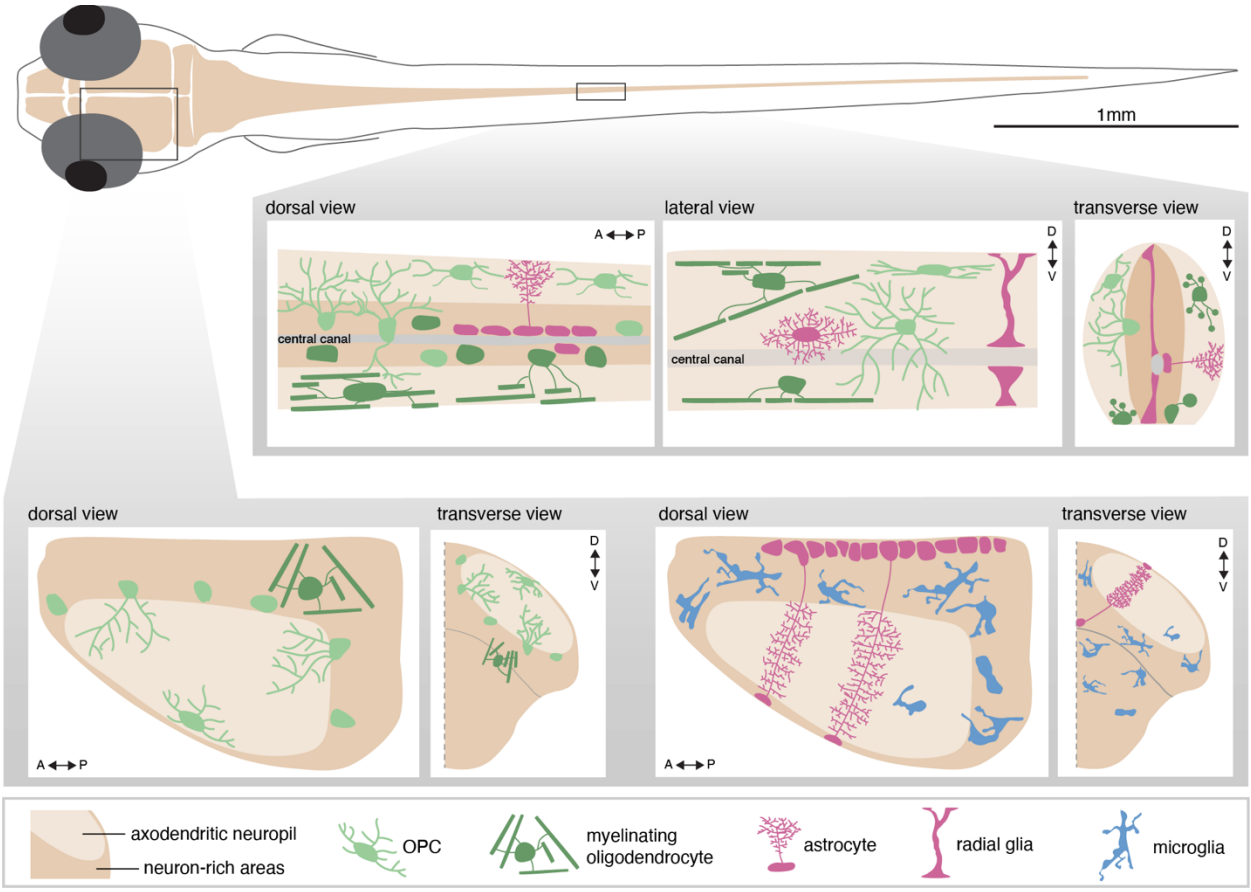

Figure 2: Distribution of morphologies of different glial cell types in zebrafish.

Top: Schematic dorsal view of a larval zebrafish and its central nervous system. Boxes indicate the positions of detailed zoom-ins underneath to outline positioning and morphologies of different glial cell types at the level of the optic tectum (left box with zoom-in at bottom) and the spinal cord (right box with zoom-in in the middle). A=anterior, P=posterior, D=dorsal, V=ventral.