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# Glial Cell Development and Function in Zebrafish

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The zebrafish is a premier vertebrate model system that offers many experimental advantages for in vivo imaging and genetic studies. This review provides an overview of glial cell types in the central and peripheral nervous system of zebrafish. We highlight some recent work that exploited the strengths of the zebrafish system to increase the understanding of the role of Gpr126 in Schwann cell myelination and illuminate the mechanisms controlling oligodendrocyte development and myelination. We also summarize similarities and differences between zebrafish radial glia and mammalian astrocytes and consider the possibility that their distinct characteristics may represent extremes in a continuum of cell identity. Finally, we focus on the emergence of zebrafish as a model for elucidating the development and function of microglia. These recent studies have highlighted the power of the zebrafish system for analyzing important aspects of glial development and function.

Following the pioneering work of George Streisinger in the early 1980s, the zebrafish has emerged as a premier vertebrate model system (Streisinger et al. 1981). A key strength of the zebrafish is that the embryos and early larvae are transparent, allowing exquisite cellular analysis of many dynamic processes, including cell migration, axonal pathfinding, and myelination, among many others (e.g., Gilmour et al. 2002; Lyons et al. 2005; Czopka et al. 2013). The zebrafish also has many advantages for large-scale genetic studies, including relatively small size and rapid development, high fecundity, and the ability to manipulate the ploidy of gametes and early embryos (Kimmel 1989). Through the 1980s and early 1990s, insightful studies of several interesting mutations elegantly exploited these experimental advantages (e.g., Kimmel et al. 1989; Ho and Kane 1990; Hatta et al. 1991; Grunwald and Eisen 2002), attracting many researchers from other fields to the zebrafish system. Following the explosion of interest in the zebrafish in the 1990s, advances in many areas have added to the strengths of the system, including large-scale screens that identified thousands of new mutations (Driever et al. 1996; Haffter et al. 1996), rapid transgenesis (Kawakami et al. 2004), new methods for imaging and tracking all cells during development (Huisken 2012), genetic mapping and sequencing to identify genes and mutated loci (Postlethwait et al. 1994; Howe et al. 2013), optogenetic methods to control neural activity (Portugues et al. 2013), the advent of targeted nucleases to create mutations in genes of interest (Huang et al. 2011; Sander et al. 2011; Bedell et al. 2012; Chang et al. 2013; Hwang et al.

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2013), and small molecule screening approaches to isolate compounds with novel biological activities in vivo (Peterson and Fishman 2011).

Many fundamental similarities in physiology and body plan unite the zebrafish and other vertebrates (Kimmel 1989). In addition, analysis of genes and genomes has revealed that sequence, expression, and function of many genes are conserved among zebrafish and other vertebrates (Postlethwait and Talbot 1997; Howe et al. 2013). Thus, insights from studies in zebrafish will apply broadly to other vertebrates, including humans. On the other hand, there are important genetic, genomic, and physiological differences among vertebrates. It is, therefore, important to keep possible differences in mind and to recognize that analyzing the diversity among different species may enhance overall understanding of important processes. For example, zebrafish and other teleosts have a much more extensive regenerative ability than mammals, so that studies of fin, heart, and spinal cord regeneration in zebrafish may suggest avenues toward new therapeutic approaches in humans (Gemberling et al. 2013; Becker and Becker 2014).

In this review, we provide an overview of different types of glia in the zebrafish, with a focus on some recent studies that highlight the power of the zebrafish system to analyze different aspects of glial development and function.

## SCHWANN CELLS

Schwann cells are the myelinating glia of peripheral nerves (Jessen and Mirsky 2005). Schwann cell precursors, which derive from the neural crest, migrate with growing axons as nerves are developing. After migration is complete, the process of radial sorting begins, during which Schwann cells associate with individual axons in the bundle of the developing nerve (Jessen and Mirsky 2005; Raphael and Talbot 2011). A promyelinating Schwann cell associates with a large-diameter axon and begins the process of myelination, which culminates with the formation of a compact myelin sheath. Many studies in mammals have defined important regulators of Schwann cell development and myelination, including axonal neuregulin signals and their glial ErbB receptors and key Schwann cell transcription factors including Sox10, Oct6, Brn2, and Krox20 (Jessen and Mirsky 2005; Nave and Salzer 2006; Pereira et al. 2012). Analysis of expression patterns, cell behavior, and mutant phenotypes in zebrafish indicates that the functions of these genes are broadly conserved among vertebrates (e.g., Kegel et al. 2014; reviewed in Raphael and Talbot 2011).

A number of approaches have identified zebrafish mutations disrupting Schwann cell development and differentiation, including different genetic screens, as well as reverse genetic approaches to explore candidate genes, such as *krox20* (Dutton et al. 2001; Grant et al. 2005; Pogoda et al. 2006; Monk et al. 2009). In addition to showing that many genes previously known to have important roles in mammals have conserved functions in zebrafish, these studies highlighted some new functions of these genes, including, for example, the role of axonal Nrg1-TypeIII as a guidance cue that directs the migration of Schwann cells in growing nerves (Lyons et al. 2005; Perlin et al. 2011). Moreover, analysis of mutants lacking Schwann cells has defined some interesting functions of the cells themselves, including controlling the timing of sensory organ differentiation, preventing accumulation of voltage-gated sodium channels at inappropriate locations along axons, and remodeling the basement membrane of the skin (Grant et al. 2005; Voas et al. 2009; Raphael et al. 2010; Lush and Piotrowski 2014). These topics have been reviewed extensively (Raphael and Talbot 2011; Pereira et al. 2012; Glenn and Talbot 2013b). Therefore, we focus here on recent characterization of Gpr126, a gene with essential function in myelination that was defined initially using genetic approaches in zebrafish.

### Gpr126

A screen for disruptions in myelinating glia identified a number of mutants lacking myelin basic protein (*mbp*) expression in Schwann cells (Pogoda et al. 2006). Initial phenotypic studies showed that most of these mutations disrupted early stages of Schwann cell develop-

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ment. In contrast, two allelic mutations had normal expression of early Schwann cell and axonal markers, despite the absence of *mbp* expression, suggesting that the mutated gene was essential for the initiation of myelination (Pogoda et al. 2006).

Genetic mapping and positional cloning showed that these mutations disrupt Gpr126, an orphan member of the adhesion class of G protein–coupled receptors (Monk et al. 2009). Ultrastructural analysis of the mutants confirmed that Schwann cells are present and associated with apparently normal axons in *gpr126* mutants (Fig. 1A). The Schwann cells in *gpr126* mutants did not progress beyond the promyelinating stage (Fig. 1A), indicating that Gpr126 is essential to initiate myelination (Monk et al. 2009). Analysis of a Gpr126 knockout mouse (Fig. 1A) showed that its function in the initiation of myelination is conserved in mammals

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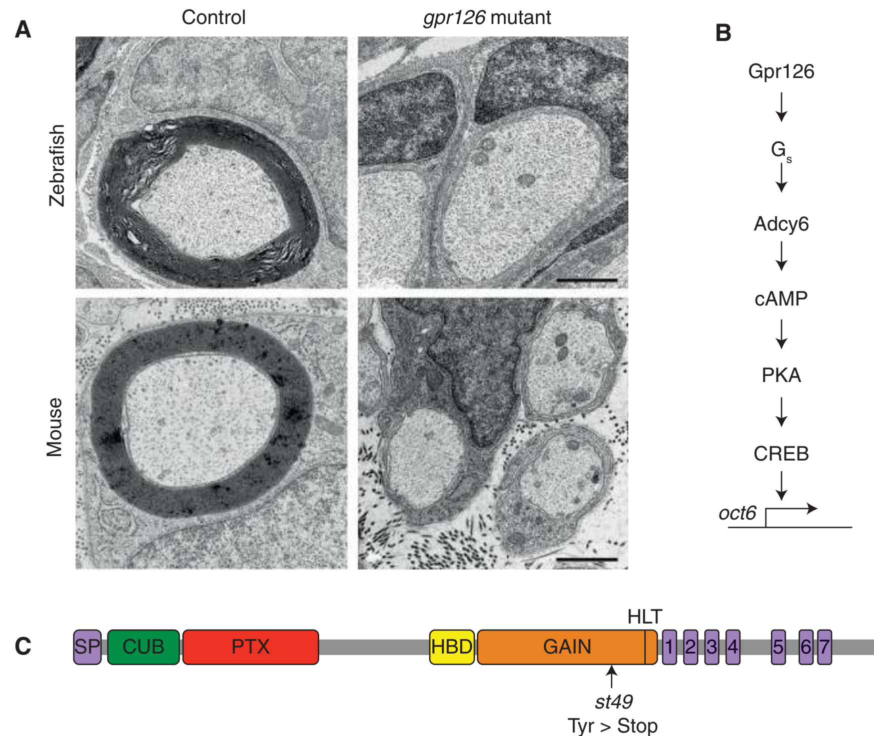


**Figure 1.** The role of Gpr126 in Schwann cell myelination. (*A*) In zebrafish and mouse, *gpr126* mutants lack myelin in peripheral nerves. In control animals, compact myelin is evident in transmission electron micrographs of the posterior lateral line nerve from adult zebrafish (*top*) and the sciatic nerve of P12 mouse (*bottom*). In corresponding images from *gpr126* mutants, Schwann cells associate with axons but do not form myelin. Scale bars, 0.5 µm. (From Monk et al. 2009; reprinted, with permission, from The American Association for the Advancement of Science 2009; and from Monk et al. 2011 and Monk and Talbot, unpubl.; reprinted, with permission, from the author.) (*B*) Simplified model of some components of Gpr126 signaling pathway, beginning with activation of the receptor by ligands that may include type IV collagen (Paavola et al. 2014) and others, and culminating with the transcriptional activation of the *oct6* gene. For more details, see text and recent reviews (Pereira et al. 2012; Glenn and Talbot 2013b). (*C*) Schematic representation of domains in Gpr126, showing the signal peptide (SP), CUB domain, pentraxin domain (PTX), hormone binding domain (HBD), GAIN domain with the amino acids (His-Leu-Thr) of the catalytic triad mediating autoproteolyic cleavage (black line), and the seven transmembrane helices (1–7). In addition to its function in activating heterotrimeric G proteins, Patra et al. (2013) have proposed that the region of the protein amino terminal to the GAIN cleavage site and nonsense lesion in the *st49* zebrafish mutant allele has a distinct function that is required for heart trabeculation in zebrafish.

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(Monk et al. 2011), highlighting the power of genetic screens in zebrafish to identify new regulators of mammalian myelination. Cellular transplantation studies in zebrafish further showed that wild-type Schwann cells were able to differentiate and express Mbp when associated with mutant axons, indicating that Gpr126 acts autonomously in Schwann cells (Monk et al. 2009). Similarly, analysis of a conditional mouse mutation showed that loss of Gpr126 function in Schwann cells caused defects in the radial sorting process , which pairs promyelinating Schwann cells with individual axonal segments and the initiation of myelination (Mogha et al. 2013).

Although much remains to be determined about the signaling pathway, there is increasing evidence that Gpr126 triggers myelination by elevating cAMP levels in Schwann cells (Fig. 1B). Incubating *gpr126* mutant zebrafish with the adenylyl cyclase agonist forskolin restored myelin in the nerves of the mutant, providing the initial evidence that Gpr126 is coupled to $G_s$ (Monk et al. 2009). Recent work in the mouse showed that cAMP levels are reduced in Schwann cells lacking Gpr126 and also provided biochemical evidence that Gpr126 is coupled to $G_s$ and $G_i$ (Mogha et al. 2013). A recent report identified adenylate cyclase 6 (ADCY6) as a candidate effector of Gpr126 signaling in Schwann cells (Laquérriere et al. 2013). In a consanguineous human family, lack of myelin in peripheral nerves was associated with a homozygous missense mutation in ADCY6. Furthermore, knockdown of duplicate *adcy6* genes in zebrafish produced a phenotype similar to *gpr126* mutants (Laquérriere et al. 2013).

Recent studies indicate that protein kinase A (PKA), which is activated by cAMP, is an important effector of Gpr126 signaling (Fig. 1B). Expression of a constitutively active PKA in Schwann cells rescued *mbp* expression in *gpr126* mutant zebrafish (Glenn and Talbot 2013a), and 8-CPT-cAMP, a selective agonist of PKA, rescued myelination in cultures of murine Schwann cells lacking Gpr126 (Mogha et al. 2013). The key substrates of PKA in myelinating Schwann cells are not yet known, but cAMP-response element-binding protein (CREB) is a strong candidate (Mandemakers et al. 2000; Jagalur et al. 2011; Glenn and Talbot 2013b). This transcription factor is a well-known PKA substrate, and it has been implicated in transcriptional activation of *oct6* (Mandemakers et al. 2000). Elevation of cAMP activates Oct6 expression in cultured Schwann cells (Lemke and Chao 1988; Monuki et al. 1989), and Oct6 and its close relative Brn2 are essential for Schwann cells to express *krox20* and initiate myelination at the appropriate stage (Jaegle et al. 2003). The absence of *oct6* expression in Schwann cells is the earliest phenotype evident in nerves of *gpr126* mutant zebrafish (Monk et al. 2009), suggesting that Gpr126 signaling elevates cAMP to activate *oct6* in Schwann cells in vivo.

Interestingly, Gpr126 is not required after myelination initiates, because a short pulse of forskolin not only promotes the onset of myelination in *gpr126* mutants, but also allows the mutants to form compact myelin that matures and persists for months after the treatment (Glenn and Talbot 2013a). Thus, the current data indicate that Gpr126 activates a transiently required signaling pathway that includes $G_s$, adenylyl cyclase 6, and PKA, culminating with the transcriptional activation of *oct6* and perhaps other key downstream regulators of myelination (Fig. 1B) (Glenn and Talbot 2013b). A recent paper reported that type IV collagen, a component of the Schwann cell extracellular matrix, binds to the amino-terminal region of Gpr126 and increases cAMP levels in heterologous cells expressing Gpr126 (Paavola et al. 2014). Therefore, it is possible that interactions between Gpr126 and the Schwann cell extracellular matrix activate signaling and trigger myelination in peripheral nerves.

Gpr126 and other adhesion G protein–coupled receptors (GPCRs) undergo autoproteolytic cleavage at the GPS motif, which is part of the larger GAIN domain (Fig. 1C) (Moriguchi et al. 2004; Araç et al. 2012). The role of this cleavage in receptor function and signaling is not entirely clear (Prömel et al. 2013), but a recent report proposed that the amino-terminal fragment of Gpr126 has a function that is independent of the $G_s$/adenylate cyclase/PKA pathway described above (Patra et al. 2013). This

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hypothesis was put forward to explain phenotypic differences between zebrafish and mouse *gpr126* mutants. Most *Gpr126* mutant mice die before birth with defects in trabeculation of the developing heart, whereas the available zebrafish nonsense mutants are viable with no apparent heart defects (Monk et al. 2009, 2011; Waller-Evans et al. 2010; Geng et al. 2013; Patra et al. 2013; Paavola et al. 2014). A possible explanation is that some functions of Gpr126 are species-specific. Patra et al. (2013), however, proposed that the extracellular region of Gpr126 that is amino terminal to the stop codon in zebrafish mutants homozygous for the *st49* allele (Fig. 1C) could fulfill the function of Gpr126 in heart development. In their analysis of morpholinos targeting different regions of *gpr126*, Patra et al. (2013) report that a translation-blocking morpholino causes a heart defect not seen in available *gpr126* nonsense mutants or embryos injected with splice-blocking morpholinos against downstream exons. These morpholino studies are intriguing, but analysis of targeted mutations in the corresponding regions of *gpr126* is needed to definitively address the role of the amino-terminal region of the receptor and its function in zebrafish heart development.

## OLIGODENDROCYTE LINEAGE

### Specification of Oligodendrocyte Progenitors

The myelinating glial cell of the central nervous system (CNS), the oligodendrocyte, derives from a specific progenitor domain (pMN domain) in the ventral spinal cord of both zebrafish and mammals (Lu et al. 2000, 2002; Novitch et al. 2001; Park et al. 2002; Zhou and Anderson 2002; Shin et al. 2003; Norton et al. 2005). Here, Hedgehog signaling regulates the expression of essential transcription factors, including *olig2*, which are required for motor neuron and oligodendrocyte formation (Novitch et al. 2001; Lu et al. 2002; Park et al. 2002; Zhou and Anderson 2002; Li et al. 2011a; Sun et al. 2011). The pMN domain population of progenitors first generate motor neurons and then oligodendrocytes, and work in zebrafish has indicated that specification of these distinct cell types may depend on the identity of distinct hedgehog ligands (Park et al. 2004; Chung et al. 2013b). Roles for additional factors in regulating the differentiation of oligodendrocyte progenitors have also derived from work in zebrafish. For example, partial loss-of-function of autotaxin, a secreted enzyme that generates lysophosphatidic acid, reduces oligodendrocyte progenitor differentiation (Yuelling et al. 2012). Following a microarray screen (Takada and Appel 2010), that revealed high expression of *swap70* in the oligodendrocyte lineage, studies abrogating *swap70* function revealed a role for this factor in regulating the transition to oligodendrocyte progenitor fate in the pMN domain (Takada and Appel 2011).

Analyses in zebrafish have also provided key insights into how Notch signaling regulates whether pMN domain progenitors differentiate into motor neurons or remain proliferative and acquire oligodendrocyte progenitor identity at a later stage. Disruption of Notch signaling causes premature generation of neurons at the expense oligodendrocyte progenitors, and conversely, activation of Notch1a maintains pMN domain cells in a proliferative state, leading to excess oligodendrocyte progenitors (Park and Appel 2003; Park et al. 2005). Disruption of the F-box and WD repeat domain containing 7 protein, Fbxw7, causes excess oligodendrocyte progenitors to be specified from the pMN domain. Interestingly, *fbxw7* encodes the substrate recognition component of a ubiquitin ligase that targets Notch and other proteins for degradation, and suppression of Notch signaling rescued the excess oligodendrocyte progenitor cell phenotype in *fxbw7* mutants (Snyder et al. 2012), further implicating Notch in regulating progenitors in this domain. The role of Notch signaling in regulating cell fate in vivo is complex, and there are four Notch receptors in zebrafish, Notch1a, Notch1b, Notch2, and Notch3. *notch3* mutant zebrafish have a reduction in early oligodendrocyte progenitor number (Zaucker et al. 2013), but also have disruptions in vascular and pericyte formation (Zaucker et al. 2013; Wang et al. 2014), and another study implicates Notch3 in regulating adult neural stem cell behavior (Alunni et al. 2013). Dissection of *notch3* related phenotypes

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is also of clinical relevance because disruption of human Notch3 causes CADASIL (Joutel et al. 1996), a complex autosomal dominant condition characterized in part by degeneration of small blood vessels and damage to white matter (i.e., myelinated axons) (Chabriat et al. 2009). The analyses of cell behavior and cell–cell interactions that are possible in zebrafish may help elucidate the relationships between vascular and glial phenotypes in *notch3* mutants that result in disease-like pathologies.

## Oligodendrocyte Precursor Cells (OPCs)

Following the specification of oligodendrocyte progenitor cells in the pMN domain, their progeny delaminate from the neuroepithelium to become oligodendrocyte precursor cells (OPCs) that migrate toward axonal tracts, before differentiation into oligodendrocytes. The mechanisms that control delamination are largely unknown, but a recent study in zebrafish provided some intriguing hints. The microRNA miR-219 is specific to the oligodendrocyte lineage, its overexpression promotes oligodendrocyte (OL) differentiation, and its down-regulation prevents OL differentiation (Dugas et al. 2010; Zhao et al. 2010) in both rodents and zebrafish (Zhao et al. 2010). More recently, Hudish et al. showed that miR-219 regulates expression of the polarity genes, *pard3* and *prkci*, in neuroepithelial progenitors, such that in the absence of normal miR-219 function, neural progenitors retain expression and localization of polarity proteins at their apical endfeet. miR-219 deficient progenitor cells also remain proliferative for an inappropriately long time (Hudish et al. 2013), suggesting that miR-219 dependent regulation of polarity proteins might determine whether OL progenitors remain neuroepithelial in morphology (and thus in the cell cycle) or delaminate to become OPCs.

OPCs (also known as Ng2 cells in rodents) have also been considered by some to be a separate differentiated class of glial cell (for discussion, see Mangin and Gallo 2011; Almeida and Lyons 2013). However, it is clear that their general dynamic behavior and ability to generate OLs is conserved not only between zebrafish and rodents, but also between embryonic spinal cord and adult cortex. Kirby et al. (2006) first characterized the behavior of OPCs in the developing zebrafish spinal cord. They showed that when the exploratory processes of different OPCs come into contact, OPCs withdraw from one another. Following cell ablation, neighboring OPCs divide to replenish cell number before showing the same exploratory process behaviors, suggesting that repulsive interactions help establish OPC number and position in the early spinal cord (Kirby et al. 2006). The majority of early spinal cord OPCs then go on to generate mature myelinating OLs (Czopka et al. 2013). Subsequent multipohoton imaging of adult mammalian OPCs revealed that they behaved in an almost identical manner. They also extended processes that withdrew on contacting neighbors, divided slowly over time, increased proliferation on damage to neighboring cells, and differentiated exclusively into mature OLs (Hughes et al. 2013).

Studies in zebrafish have also contributed to our understanding of the molecular control of OPC migration and number. Disruption to the zebrafish neurofibromin1 genes causes OPCs to proliferate excessively (Lee et al. 2010; Shin et al. 2012) and to reduce their pause times during migration (Lee et al. 2010). However, this does not lead to increased myelination in the CNS, showing that the presence of more OPCs does not necessarily result in precocious or ectopic CNS myelination (Shin et al. 2012). *olig2*-expressing progenitor cells exist in the adult zebrafish brain (März et al. 2010b), but their (potentially specialized) function(s) have not been fully explored. Future experiments using zebrafish will continue to illuminate the function of this intriguing cell type(s) throughout life.

## Oligodendrocyte Differentiation

Studies in rodents have identified several key transcription factors, such as Sox10, Olig1, and Myrf, which control the differentiation of OPCs into OLs (see review by Mitew et al. 2013). Mutation of Sox10 prevents terminal differentiation of OLs in mammals (Stolt et al. 2002), and causes OL apoptosis in zebrafish.

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Time-lapse analyses in zebrafish showed that *sox10* mutant OLs initiate axonal wrapping before undergoing cell death (Takada et al. 2010), suggesting that *sox10* is specifically required for survival of cells committed to myelination. Studies in mammals have shown that Sox10 acts together with Olig1 and Myrf to control myelin gene expression (Park et al. 2002; Li et al. 2007, 2011a; Bujalka et al. 2013; Hornig et al. 2013). The expression of *olig1* (Schebesta and Serluca 2009) and its interaction with *sox10* in promoting myelin gene expression is conserved in zebrafish (Li et al. 2007) but the role of Myrf remains to be analyzed.

Transcription factors under the control of the canonical Wnt signaling pathway are also implicated in OL differentiation. Several studies in rodents have indicated that activation of β-catenin negatively regulates OL differentiation (Fancy et al. 2009, 2011, 2014; Ye et al. 2009). Treatment of zebrafish with compounds that stabilize β-catenin (mimicking activation of the Wnt pathway) also disrupted OL differentiation (Kim et al. 2008). In contrast, Tawk et al. showed that reduction of β-catenin signaling (following heat shock induction of a truncated dominant repressor form of the Wnt target tcf3) repressed myelin gene transcription in zebrafish (Tawk et al. 2011). Interestingly, in the same study, activating the Wnt pathway promoted expression of the myelin protein PLP in both fish and rodent cells, suggesting that Wnt can also promote OL differentiation and myelination, and that apparent discrepancies may reflect the timing of Wnt pathway activation in OL lineage cells. Indeed, Wnt signaling also acts at earlier stages of OL development in zebrafish (Kim et al. 2008; 2011). Further complementary studies in fish and rodent models will help disentangle the complex roles of Wnt in the OL lineage.

## Cellular Regulation of CNS Myelination

The mechanisms by which axons and OLs interact to coordinate CNS myelination in vivo remain largely unknown. In both fish and mammals, individual OLs typically myelinate either a small number of relatively large caliber axons or a larger number of smaller caliber axons (Bunge 1968; Butt and Berry 2000; Almeida et al. 2011). To test whether such distinct morphologies reflect the existence of subtypes of OLs or the axonal environment, Almeida et al. (2011) used two independent genetic manipulations to increase the number of large-caliber Mauthner axons in zebrafish and assess how this affected OL behavior. They observed that OLs that typically myelinated only smaller caliber axons in wild type readily myelinated both large and small caliber axons in the presence of extra Mauthner axons (Almeida et al. 2011). This result showed that the mature morphology of myelinating OLs is plastic and determined by the axonal environment. There is increasing evidence that new myelin sheaths can be made on previously unmyelinated axons well into adult life and that there may be some degree myelin sheath turnover along previously myelinated axons through life (for review, see Wang and Young 2013). However, until recently it was not clear when during the life of any single oligodendrocyte it could generate new myelin sheaths. To address this question in vivo, Czopka et al. (2013) performed time-lapse and time-course microscopy of individual oligodendrocytes in zebrafish. They observed that individual OLs always initiated formation of their final new myelin sheath within 5–6 h of the first (Czopka et al. 2013). This remarkably short period of new myelin sheath generation relative to the length of the cell's life appears to be conserved across species, because previous in vitro coculture studies showed that mammalian oligodendrocytes also generate new myelin sheaths for a period of only 12 h (Watkins et al. 2008). Together these data indicate that new myelin sheaths generated at distinct times in life must be made by newly differentiating oligodendrocytes.

Although textbooks indicate that the myelin sheath is wrapped around the axon by growth of the oligodendrocyte's myelinating process at the interface with the axon, this has evaded direct observation. Very recently, Snaidero et al. performed high-resolution imaging of individual myelin sheath "wraps" in vivo using zebrafish (Snaidero et al. 2014). Quantification of fluorescence intensity of membrane-tethered green fluorescent protein (GFP) along the length of

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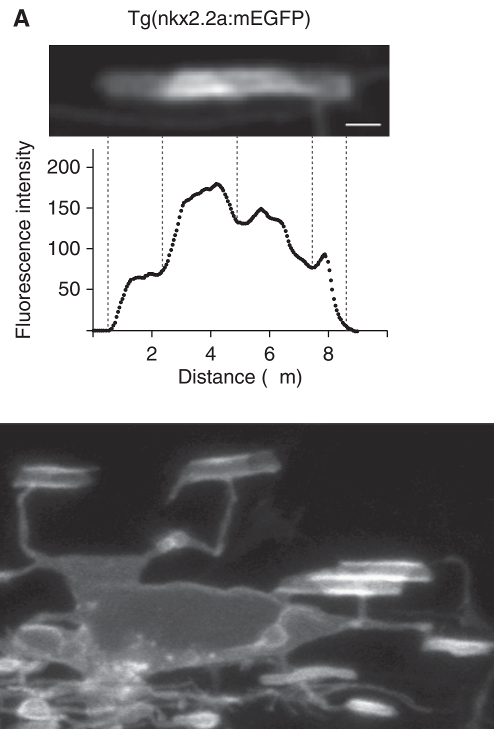


Figure 2. Live imaging of myelinating oligodendrocytes in vivo. (*A*) *Top* panel shows a single nkx2.2a:mEGFP expressing myelin sheath imaged in the living zebrafish spinal cord at 2 days postfertilization (dpf). Note the step-like changes in fluorescence intensity along the length of the myelin sheath, which are quantified in the *bottom* panel, indicating the quantal nature of the step pattern. (*B*) Individual nkx2.2a:mEGFP expressing oligodendrocyte with numerous nascent myelin sheaths, many of which show the characteristic step change pattern of fluorescence intensity along the length of the sheath, with highest intensity always toward the middle relative to the ends. Images were taken on a confocal microscope and subsequently deconvolved. Scale bar, 1 um.

individual myelin sheaths revealed a distinct quantal step pattern, with areas of high intensity in the middle of the sheath and lower intensity at laterally growing distal ends (Fig. 2). These intensity profiles were taken as evidence of the discrete wraps of myelin and time-lapse analyses suggested that the addition of new wraps of myelin first occurs at the center of the sheath and that the individual wraps subsequently extend along the length of the axon. These predictions from in vivo imaging in zebrafish were validated by ultrastructural reconstruction and viral labeling studies in rodents (Snaidero et al. 2014).

## Molecular Regulation of CNS Myelination

Studies in zebrafish have also provided insight into the molecular regulation of myelination. For example, a combination of mutant analyses and chemical studies has shown that cholesterol biosynthesis is required for normal CNS myelin sheath formation in vivo (Matthews et al. 2014). Zebrafish mutant analyses also revealed that the kinesin motor protein Kif1b (Lyons et al. 2009) and the cytoskeletal component α-tubulin 8-like 3a (Larson et al. 2010) are essential for the subcellular localization of myelin basic protein mRNA to myelinating processes, a feature of myelination previously shown to be conserved in fish and mammals (Colman et al. 1982; Brösamle and Halpern 2002). In *kif1b* mutants, ectopic, myelin-like membranes were observed in inappropriate regions, suggesting that the ectopic translation of MBP might contribute to ectopic membrane compaction (Lyons et al. 2009). Additional functions for the

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local translation of *mbp* mRNA during myelination are also likely. A recent in vitro study suggested that neuronal activity might stimulate the local translation of MBP to regulate myelination via phosphorylation-dependent activation of the nonreceptor tyrosine kinase Fyn (Wake et al. 2011). Although Fyn was previously shown essential for normal myelination in vivo, its role was unclear (Umemori et al. 1994). Czopka et al. (2013) used transgenes to drive a constitutively active version of Fyn kinase (ca-Fyn) specifically in zebrafish myelinating OLs. Ca-Fyn OLs generated one-third more myelin sheaths than wild type, and knockdown of Fyn function by morpholino antisense oligonucleotides caused individual OLs to generate fewer sheaths. The sheaths that formed in these animals were of normal length and thickness. Thus these results indicated that Fyn regulates the number of sheaths that an individual OL makes, but not the properties of individual sheaths (Czopka et al. 2013). The signals and mechanisms upstream of and downstream from Fyn in regulating myelination in vivo await elucidation.

Neuregulin ligands and their ErbB receptors have central roles in regulating many aspects of Schwann cell development and myelination (Nave and Salzer 2006; Pereira et al. 2012; Glenn and Talbot 2013b), but mouse and zebrafish mutants in Nrg1, Erbb2, and Erbb3 have no obvious CNS myelin defects at early stages (Lyons et al. 2005; Brinkmann et al. 2008). Despite these genetic analyses, a morpholino study suggested that knockdown of either *erbb2* or *erbb3* interfered with an active process of myelin sheath pruning by OLs, so that individual OLs have more sheaths per cell on average (Liu et al. 2013). However, the morpholino-based knockdown of *erbb* caused animals to have fewer OLs, which was not observed in previous analyses of fish or mice *erbb* mutants (Lyons et al. 2005; Brinkmann et al. 2008; Liu et al. 2013). Interestingly, in vitro studies have shown that, under normal conditions, the density of OLs inversely correlates with myelin sheath number per cell (Rosenberg et al. 2008), suggesting that the "pruning" phenotype may be a secondary consequence of a delay in development and reduced OL number in morpholino-treated animals. Future generation of targeted germline mutants and unbiased genetic screens will help to further elucidate the molecular basis of CNS myelination in vivo.

## Oligodendrocytes during Injury and Disease

Promoting OL differentiation and (re)myelination has been proposed as a therapeutic strategy for the treatment of demyelinating diseases, such as multiple sclerosis (Compston and Coles 2008). Given their suitability for chemical screening and the fact that such screens have lead to clinical trials for other conditions (North et al. 2007; Yuelling et al. 2012; Cutler et al. 2013), zebrafish larvae have been used to identify compounds that promote OL differentiation (Buckley et al. 2010). Combining new live reporters of myelin (Almeida et al. 2011) and transgenic systems to ablate OLs (Chung et al. 2013a), with large-scale chemical screens, will help identify compounds that modify the response to glial damage and the consequent repair process, and thus be of potential therapeutic importance in the future.

## CNS–PNS Boundary

The axons of peripheral nerves have regions in the CNS (where they are myelinated by OLs) and the peripheral nervous system (PNS) (where Schwann cells fulfill the myelinating function). Therefore, mechanisms must exist to keep OLs and Schwann cells at the respective sides of the CNS–PNS boundary. The CNS–PNS barrier, however, can be breached. Following damage to CNS, Schwann cells can invade the CNS and myelinate CNS axons (Blakemore 1977). In addition, overexpression of human Neuregulin 1 type III in the zebrafish CNS can divert Schwann cells into CNS (Perlin et al. 2011). Conversely, in the absence of Schwann cells, OLs can enter the PNS in both zebrafish (Kucenas et al. 2009) and rodents (Coulpier et al. 2010). In rodents, a specialized set of neural-crest derived cells, called boundary cap cells, take up positions at the CNS–PNS boundary. In addition to their role in preventing the exit of

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motor neurons into the PNS in mammals (Vermeren et al. 2003), boundary cap cells may also restrict astrocytes and OLs to the CNS (Coulpier et al. 2010). In addition to this possible role, boundary cap cells can also function as stem cells, and may even be capable of generating CNS cell types including OLs (Zujovic et al. 2011), although this has to be confirmed in vivo. Boundary cap cells have not yet been studied in detail in zebrafish but given the suitability of zebrafish for live-cell imaging and lineage-based studies, this is likely to be a very fruitful area of future research. Another article in this collection discusses the evidence that perineurial cells covering peripheral nerves originate in the CNS (Kucenas et al. 2008; Kucenas 2015).

## Fish Radial Glia and Their Relationship to Mammalian Astrocytes

The fundamental radial morphology of neuroepithelial progenitor cells of the CNS of a variety of vertebrates was described almost 80 yr ago in the seminal work of F. C. Sauer (1934, 1935, 1936, 1937). Neuronal progenitors in the zebrafish neuroepithelium also have a radial morphology, and express markers of radial glia, such as the glial acidic fibrillary protein (GFAP) (Lyons et al. 2003). Extensive work in rodents starting in the early 2000s indicated that these cells are indeed neuronal progenitors (Malatesta et al. 2000; Noctor et al. 2001, 2002). The diversity of neuronal progenitors and stem cells of radial glial-type identity has been discussed extensively elsewhere for both rodents (e.g., Kriegstein and Alvarez-Buylla 2009) and zebrafish (e.g., Grandel and Brand 2012). However, it is clear that there is considerable heterogeneity among neural progenitors between species and brain areas and between embryonic and adult stages, and heterogeneity even among cells that have a very similar radial morphology. In zebrafish, numerous studies indicate that cells expressing radial glial morphology and markers function as progenitor cells throughout life (e.g., Lyons et al. 2003; Lam et al. 2009; Ito et al. 2010; Kroehne et al. 2011; Dong et al. 2012). However, it is also clear that many radial progenitor cells that remain proliferative throughout life do not have radial glial characteristics (März et al. 2010a), but rather have characteristics of simple neuroepithelial cells, for example, expression of the intermediate filament nestin and not GFAP (Mahler and Driever 2007). Conversely, it is also clear that some radial glial cells have more specialized functions beyond their role as progenitor cells.

One striking difference between the zebrafish and mammalian CNS, or more specifically between anamniotes (including fish) and amniotes (including mammals), is the absence of stellate astrocytes in anamniotes. Although a very small number of studies have suggested the existence of stellate or rounded astrocytes in fish (Kawai et al. 2001; Alunni et al. 2005), these observations have not been repeated to date, nor have stellate astrocytes been observed in nonfish anamniotes, such as *Xenopus laevis*. This raises the possibility that some radial glia (which can directly transform into astrocytes at late stages of mammalian nervous system development [Voigt 1989; Barry and McDermott 2005]) become specialized in anamniotes to subserve many of the functions of differentiated astrocytes. Over the past decade, the number of functions associated with mammalian astrocytes has increased greatly, with evidence of their involvement in processes as diverse as responding to neuronal injury (Cregg et al. 2014), providing metabolic support to neurons (Bouzier-Sore and Pellerin 2013), maintaining ionic and osmotic balance and integrity of the blood brain barrier, regulating blood flow (Attwell et al. 2010), regulating the formation (Eroglu and Barres 2010; Allen et al. 2012), pruning (Chung et al. 2013c), and function of synapses (Dallérac et al. 2013), and even regulating learning and memory, where transplantation of human astrocytes into rodents enhanced synaptic plasticity (Han et al. 2013). Detailed discussion of the many roles of astrocytes is beyond the scope of this review, but we briefly consider the evidence that radial glia in zebrafish may serve some specialized roles of astrocytes in mammals.

The "reactive astrocyte" response to nervous system damage is characterized by changes in the morphology of astrocytes (Cregg et al.

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2014). One notable example of the reactive astrocyte response is its contribution to formation of the glial scar, which correlates with the failure of mammalian CNS axons to regenerate (Cregg et al. 2014). Some aspects of the reactive astrocyte response are conserved between mammalian astrocytes and fish radial glia. Reactive glia are present following injury to the adult zebrafish optic nerve (Neve et al. 2012) and spinal cord (Hui et al. 2010), and overexpression of rat Lipocalin-2, an autocrine mediator of reactive astrocytosis in rodents, induces changes in GFAP expressing radial glia in zebrafish (Lee et al. 2009). However, one well-known difference between fish and mammals is their regenerative capacity, particularly that of CNS axons, and one suggestion has been that this, at least in part, reflects differences in the astrocytic versus radial glial response to injury (see review by Becker and Becker 2014). One striking response of radial glia to spinal cord transection in zebrafish is the formation of "glial bridges" that help rejoin the transected spinal cord and provide a substrate for subsequent axonal regrowth (Goldshmit et al. 2012). Intriguingly, this function of zebrafish radial glia depends on fibroblast growth factor (FGF) signaling, and treatment of primate astrocytes with FGF-2 induced them to adopt a similar bipolar morphology in vitro to the zebrafish radial glia morphology observed in vivo. Furthermore, mouse gray matter astrocytes express the FGF pathway target Sprouty4 after spinal cord injury (Goldshmit et al. 2012), suggesting further similarities between these cell types.

In zebrafish, radial glial-like progenitors and the analogous Müller glia in the retina proliferate in response to injury and regenerate neurons and other cell types (Kroehne et al. 2011; Kizil et al. 2012; Gorsuch and Hyde 2013). Following spinal cord transection, the proliferative and neurogenic response of radial glia recapitulates many aspects of developmental neurogenesis (Reimer et al. 2008, 2009), and it has been suggested that this response of radial glia may partly occur in place of the contribution of astrocytes to the glial scar seen in mammals (Becker and Becker 2014). This suggests the hypothesis that manipulation of astrocytes toward a more radial glial-like phenotype could promote a more favorable regenerative response in mammals, which is appealing because cells of the radial glia/astrocytic lineage can function as bona fide neural stem cells in the mammalian adult CNS (Kriegstein and Alvarez-Buylla 2009). However, it is clear from studies of astrocytes in rodents and radial glia in zebrafish that there is an enormous diversity in the types and states of these cell types. Whether this diversity represents the existence of distinct committed cell types or polarized phenotypes of a single cell type remains to be fully elucidated.

The injury response of zebrafish radial glia has been studied more extensively than other astrocyte-like functions. However, it is clear that zebrafish radial glia do have at least some additional features in common with mammalian astrocytes. Astrocytes have a well-known role in regulating ionic and water homeostasis in the CNS. The expression of the water-transporting protein aquaporin-4 is characteristic of astrocytes, and it is localized to the characteristic astrocytic endfeet that contact endothelial cells at blood vessels, that is, at the blood–brain barrier, and the pia mater and ependyma at CNS–cerebrospinal fluid (CSF) interfaces (Papadopoulos and Verkman 2013). Zebrafish radial glia express aquaporin-4, although its localization is not polarized in the same way as in mammalian astrocytes (Grupp et al. 2010). Zebrafish radial glial endfeet also express markers of tight junctions much like mammalian astrocytic endfeet (Corbo et al. 2012). Zebrafish radial glial endfeet also associate with neuronal processes (Marcus and Easter 1995). Although the functional significance of this association remains to be determined, zebrafish radial glia also express the glutamate transporter Eaat2b (McKeown et al. 2012), suggesting that they may share with mammalian astrocytes functions related to the regulation of neurotransmitters. Further studies of specific markers, transgenic reporters, and mutants (Barresi et al. 2010; Johnson et al. 2013) will continue to illuminate the roles of radial glia in zebrafish, which may in turn provide novel insight into the biology of mammalian astrocytes.

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## MICROGLIA

Microglia are highly specialized phagocytic cells dedicated to the defense of the CNS (Ransohoff and Cardona 2010). In zebrafish and mammals, mature microglia are present throughout the parenchyma of the brain and spinal cord (Sieger and Peri 2013; Svahn et al. 2013). In the healthy CNS, the cell bodies of microglia are largely stationary, but they actively extend and retract processes as they inspect their environment for any sign of perturbation (Nimmerjahn 2005). After injury or infection, microglia adopt an amoeboid morphology and migrate to the site of the insult, where they engulf pathogens and debris (Davalos et al. 2005; Neumann et al. 2009; Sieger et al. 2012). Microglia are activated in many disease states and they may initiate or exacerbate neurodegeneration (Aguzzi et al. 2013). Several recent analyses have exploited imaging and genetic approaches in zebrafish to increase the understanding of the development and functions of microglia.

### Microglia: Origins and Migration

Unlike neurons, OLs, and radial glia/astrocytes, which originate from neuroepithelial progenitors, microglia derive from primitive macrophages that move into the CNS from the periphery and later differentiate as CNS-resident microglia (Herbomel et al. 2001; Ginhoux et al. 2010; Schulz et al. 2012; Sieger and Peri 2013; Svahn et al. 2013). The first macrophages in zebrafish arise from anterior mesodermal cells and begin to migrate throughout the embryo by 24 hours postfertilization (hpf) (Herbomel et al. 1999, 2001; Le Guyader et al. 2008). As in mouse, the development of these primitive macrophages requires the transcription factors Pu.1 (Spi1) and Irf8 (Rhodes et al. 2005; Li et al. 2011b). Primitive macrophages in the CNS begin to differentiate as microglia as early as 60 hpf (Herbomel et al. 2001; Peri and Nusslein-Volhard 2008). Primitive macrophages and microglia are both actively phagocytic, and microglia maintain expression of some early macrophage genes, such as *mfap4* and *mpeg1* (Zakrzewska et al. 2010; van Ham et al. 2012, 2014; Svahn et al. 2013). Differentiating microglia, however, initiate expression of distinct genes, such as *apoe* and *p2y12*, and they transition from the amoeboid appearance of primitive macrophages to a more complex morphology with highly active processes (Herbomel et al. 2001; Peri and Nusslein-Volhard 2008; Sieger et al. 2012).

In zebrafish and mouse, the initial migration of primitive macrophages from the yolk sac to the CNS and other parts of the embryo requires Csf1r, the receptor for macrophage-colony-stimulating factor (Herbomel et al. 2001; Schulz et al. 2012). In *csf1r* mutants, primitive macrophages appear normal at early stages, but are delayed in leaving the yolk sac to colonize the rest of the embryo (Herbomel et al. 2001). In mutants, the few macrophages that have entered the brain do begin to express *apoe* and clear apoptotic cell debris, suggesting that the *csf1r* is required for migration rather than differentiation of microglial progenitors. Despite the paucity of microglia in *csf1r* mutant embryos, recovery of microglial cells in the mutants is evident by early larval stages, indicating that other yet-to-be identified signaling pathways are also capable of allowing primitive macrophages to enter the CNS in the absence of *csf1r* (Herbomel et al. 2001). A recent study reported that *xpr1b*, which encodes a zebrafish ortholog of the phosphate exporter Xpr1, is required for primitive macrophages to colonize the brain (Meireles et al. 2014). Unlike *csf1r* mutants, the macrophages that do enter the CNS in *xpr1b* mutants do not differentiate as microglia, and microglia do not recover in *xpr1b* mutants at early larval stages. Additional work is needed to determine how Xpr1b functions in the differentiation of microglia.

Another recent study shows that control of the inflammatory phenotypes of primitive macrophages is essential for their normal migration during development. In a screen for microglia mutants, Shiau et al. (2013) identified *nlrc3-like* as a gene essential for development of microglia. Most well-characterized members of the NOD-like receptor (NLR) family encode pattern recognition receptors that trigger inflammation in response to pathogen- and damage-associated molecules (Davis et al. 2011). In contrast, *nlrc3-*

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*like* encodes a noncanonical NLR that is essential to suppress inflammation in the absence of infection or injury. Expression of proinflammatory cytokines is greatly elevated in *nlrc3-like* mutants, and primitive macrophages adopt inflammatory phenotypes that are reminiscent of the response to wounds and infection in wild-type embryos. In the mutants, primitive macrophages actively migrate toward each other and form large aggregates with neutrophils instead of entering the brain to differentiate as microglia as they do in wild type (Shiau et al. 2013). Additional work is required to identify all of the pathways that control the migration of microglial progenitors and to determine how unchecked inflammation overrides these signals in *nlrc3-like* mutants.

## Microglial Functions in Development

Many studies have analyzed the roles of microglia in inflammatory and neurodegenerative disease, and recent studies have begun to focus more on the functions of microglia in the healthy CNS (Ransohoff and Cardona 2010; Aguzzi et al. 2013). Microglia have a widely recognized function in clearing apoptotic cell corpses during development, which prevents the contents of dying cells from leaking and harming neighboring cells or triggering inflammation. In addition, recent studies in mammals have reported that microglia engulf specific synapses in an activity-dependent fashion, raising the exciting possibility that microglia shape connections between neurons (Schafer et al. 2012, 2013).

In zebrafish *csf1r* mutants, numerous apoptotic neuronal corpses accumulate at ~3 days postfertilization (dpf), when the mutant CNS contains few or no primitive macrophages or microglia (Herbomel et al. 2001). Similarly, knockdown of *pu.1* eliminates macrophages and causes a pronounced accumulation of apoptotic cell corpses in the CNS (van Ham et al. 2012). Primitive macrophages and microglia are actively phagocytic cells, and the apoptotic cell corpses are cleared soon after these cells reach the CNS of *csfr1* mutants (Herbomel et al. 2001). Using elegant in vivo imaging approaches, van Ham et al. (2012) followed apoptotic neurons in vivo. Apoptotic neurons are remarkably motile and often move to the basolateral aspect of the developing brain, where they may be extruded from the CNS at early stages before macrophages are present. Starting at ~2 dpf, apoptotic cells are actively engulfed by primitive macrophages and microglia. A recent study reported that *xpr1b*, which encodes a zebrafish ortholog of the phosphate exporter Xpr1, is required for primitive macrophages to colonize the brain (Meireles et al. 2014). Unlike *csf1r* mutants, the macrophages that do enter the CNS in *xpr1b* mutants do not differentiate as microglia, and microglia do not recover in *xpr1b* mutants at early larval stages. Additional work is needed to determine how Xpr1b functions in the differentiation of microglia. In *elmo1* knockdown animals, macrophages approach apoptotic cells but only rarely engulf them (van Ham et al. 2012). Elmo1 is thought to activate Rac1, suggesting that *elmo1* is required for the cytoskeletal reorganization that is involved in engulfment (van Ham et al. 2012).

Another study used in vivo imaging to show that Atp6v0a1, a component of the vaculolar ATPase, is required for the digestion of neurons after engulfment (Peri and Nusslein-Volhard 2008). In Atp6v0a1 knockdown zebrafish, microglia engulf apoptotic neurons, but the phagocytosed material persists for a much longer period of time than in wild type. Studies with indicator dyes showed that phagosomes in the Atp6v0a1 knockdown animals acidify normally but do not fuse with lysosomes to form phagolysosomes, the organelle that digests engulfed material (Peri and Nusslein-Volhard 2008). Thus, the combination of genetic studies and in vivo imaging in zebrafish has provided important new insights into the phagocytic activity of primitive macrophages and microglia.

Li et al. (2012) provided exciting evidence that microglia regulate the activity of neurons in the developing zebrafish CNS. Simultaneous in vivo time-lapse imaging of microglial morphology and neuronal activity (something that would be hugely challenging in most other animal models) revealed that microglia extend the process toward the cell bodies of active neurons. These dynamic microglial processes typically

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contacted a particular neuron for 5–6 min, after which they retracted. Pannexin-1 hemichannels, which are gated by membrane depolarization, are required for directed extension of microglial processes, based on both pharmacological approaches and morpholino knockdown. ATP, which can direct microglial migration after injury in mammals (Davalos et al. 2005), released from pannexin-1 hemichannels apparently guides microglial processes, because elimination of extracellular ATP and pharmacological blockade of puringeric receptors prevents process extension. The spontaneous and evoked activity of neurons was reduced after microglial contact (Li et al. 2012). These experiments provide evidence that microglia detect active neurons in their vicinity and modulate their excitability.

### Microglial Functions in Injury

Many studies have addressed the response of microglia to injury in the CNS in mammals (Ransohoff and Cardona 2010; Aguzzi et al. 2013). In particular, ATP, acting through its receptor P2Y12 on microglia, serves as an attractant that guides microglia to sites of injury (Davalos et al. 2005; Haynes et al. 2006). Other signals may also serve to attract microglia to injuries, and the complete sequence of events by which microglia detect and migrate to distant sites of neuronal damage remains to be elucidated.

A recent study used an elegant combination of in vivo imaging, pharmacology, and knockdown approaches to define a cascade of signals that direct microglia to injured neurons (Sieger et al. 2012). Their injury model used a laser to damage ~200 neurons at a defined position within the zebrafish CNS. Analysis of a genetically encoded calcium indicator revealed that the injury initiates a calcium wave that propagates at ~14 μm/sec over a distance that is proportional to the number of damaged neurons. Microglia orient their processes toward the injury site within a minute of the increase in calcium, and begin translocating their cell bodies in that direction shortly thereafter. ATP and the P2Y12 purinergic receptor are also required for microglia to respond to injury, but the calcium wave propagates independently of ATP. Instead, it seems that glutamate released from damaged neurons may be the cue that initiates the calcium wave via activation of *N*-methyl-D-aspartate (NMDA) receptors. Thus, this work highlights a conserved role of ATP and purinergic receptors in microglia migration, and also defines a mechanism by which microglia are able to detect neuronal damage at distant sites (Sieger et al. 2012).

## CONCLUDING REMARKS

The chief advantages of zebrafish as a model organism, their suitability for high-resolution in vivo imaging and genetic analyses, have underpinned studies that have taught us a great deal about glial cell development and function over the past decade. Work performed to date has discovered widespread conservation in mechanisms of glial development and function between fish and mammalian species, and future studies are likely to take advantages of complementary models. As genetic screens expand to encompass additional glial cell types and functions, important new genes will continue to be discovered and analyzed. The advent of better reverse genetic technologies in zebrafish (including the possibility to generate conditional alleles) will allow zebrafish to be used to quickly examine the functions of genes identified in other studies, such as genome-wide association studies and other efforts that generate candidate genes for functional studies. Generation of new transgenic reporters and imaging approaches will ensure that the zebrafish remains a premier system for study of glia activity in vivo. Screens for chemicals that rescue zebrafish models of glial disease and injury will make important contributions to understanding and treating human disease.

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