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Notch-Regulated Oligodendrocyte Specification From Radial Glia in the Spinal Cord of Zebrafish Embryos

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Abstract

During vertebrate neural development, many dividing neuroepithelial precursors adopt features of radial glia, which are now known to also serve as neural precursors. In mammals, most radial glia do not persist past early postnatal stages, whereas zebrafish maintain large numbers of radial glia into adulthood. The mechanisms that maintain and specify radial glia for different fates are still poorly understood. We investigated formation of radial glia in the spinal cord of zebrafish and the role of Notch signaling in their maintenance and specification. We found that spinal cord precursors begin to express *gfap*⁺, a marker of radial glia, during neurogenesis and that *gfap* cells give rise to both neurons and oligodendrocytes. We also determined that Notch signaling is continuously required during embryogenesis to maintain radial glia, limit motor neuron formation and permit oligodendrocyte development, but that radial glia seem to be refractory to changes in Notch activity in postembryonic animals.

Keywords

spinal cord; oligodendrocyte; motor neuron; radial glia; zebrafish transgenic; Notch

INTRODUCTION

Before initiation of neurogenesis, the vertebrate central nervous system (CNS) is composed of a single layer of dividing, pseudostratified neuroepithelial cells (Hollyday, 2001). Neuroepithelial cells are radially elongated and contact both the ventricular and pial surfaces of the neural tube. During early stages of neural development, they divide symmetrically to increase the pool of neural precursors; later, many neuroepithelial cells divide asymmetrically to give rise to neurons (Mc-Connell, 1995). As neurons are added and the CNS grows in size, neuroepithelial cells transform into cells characterized as radial glia, which maintain some features of neuroepithelial cells, like apical–basal polarity, but also have astroglial characteristics, such as expression of glial fibrillary acidic protein (GFAP) and brain-lipid-binding protein (BLBP; Gotz and Huttner, 2005). Like neuroepithelial cells, the soma of radial glial cells lie close to the ventricular wall and extend long radial processes that contact the pial surface (Gotz and Huttner, 2005; Merkle and Alvarez-Buylla, 2006).

Although viewed historically as serving as a structural element of the CNS, recent studies have shown that radial glial cells generate new neurons in mammals during embryogenesis and into early postnatal stages (Miyata et al., 2001; Noctor et al., 2001; Gotz et al., 2002; Malatesta et

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al., 2003; Anthony et al., 2004). Eventually, radial glial cells disappear, apparently through transformation into astrocytes (Rakic, 2003). This transformation coincides with reduction of neural cell proliferation and neurogenesis. However, a genetic marking strategy revealed that radial glia of the subventricular zone (SVZ) transform into astrocytic neural stem cells that give rise to olfactory bulb neurons in adults (Merkle et al., 2004). Therefore, neuroepithelial cells, radial glia, and SVZ astrocytes represent a continuum of neural precursor and stem cells (Alvarez-Buylla et al., 2001).

In contrast to mammals, adult birds, reptiles, and fish maintain large numbers of radial glia (Garcia-Verdugo et al., 2002; Zupanc and Clint, 2003). Additionally, whereas adult neurogenesis in mammals is apparently restricted to the SVZ and subgranular zone (SGZ) of the hippocampus (Gage, 2000), adult neurogenesis is widespread in the brains of birds, reptiles, and fish (Chapouton et al., 2007). Although radial glia have been implicated as a source of neurons in adult teleost fish (Zupanc and Clint, 2003), direct evidence that neurons arise from radial glia in these animals is lacking. Recently, we identified a discrete population of spinal cord radial glia in adult zebrafish, marked by expression of a transgenic reporter driven by *olig2* regulatory DNA, that divide slowly and, apparently, asymmetrically to continuously produce new oligodendrocytes (Park et al., 2007). Of interest, in adult rodents *Olig2* expression marks dividing cells associated with the SVZ that give rise to oligodendrocytes (Hack et al., 2005; Marshall et al., 2005; Menn et al., 2006).

During development, *Olig2* is expressed by a distinct population of ventral spinal cord precursors, called pMN cells, that produce motor neurons and oligodendrocytes (Lu et al., 2000; Takebayashi et al., 2000; Zhou et al., 2000; Park et al., 2004; Masahira et al., 2006). Therefore, at least in zebrafish, a subset of spinal *olig2*⁺ neuroepithelial precursors is maintained into adulthood as radial glia that have stem cell characteristics. How neural precursors are maintained from embryonic to postnatal stages is poorly understood. Here, we investigate the emergence of *olig2*⁺ radial glia during zebrafish embryogenesis and their maintenance by Notch signaling.

RESULTS

GFAP⁺ Spinal Cord Radial Glia Arise During Zebrafish Development and Persist During Adulthood

GFAP is a class III intermediate filament expressed in astrocytes and radial glial cells of the CNS. To investigate the status of GFAP⁺ radial glia as neuronal and glial precursors we used a transgenic line of zebrafish, *Tg-(gfap:GFP)*, which expresses enhanced green fluorescent protein (EGFP) under the control of *gfap* regulatory DNA (Bernardos and Raymond, 2006). At 36 hours postfertilization (hpf), a late embryonic stage, *egfp* RNA and EGFP are expressed at high level in cells located along the medial septum but mostly absent from cells near the pial surface and in ventral spinal cord (Fig. 1A,B). This pattern is similar to expression of RNA and protein produced by the endogenous *gfap* gene (Fig. 1C,D) with the exception that GFAP antibody only labels radial fibers whereas transgenically expressed EGFP also reveals radial glial cell bodies. Both 3 days postfertilization (dpf) and 7 dpf *Tg-(gfap:GFP)* larvae similarly express EGFP throughout the spinal cord except for a ventral domain (Fig. 1E,F). Notably, Zrf-1 antibody, which marks zebrafish radial glia (Trevarrow et al., 1990) labels EGFP⁺ radial processes in dorsal spinal cord and EGFP⁻ processes in ventral spinal cord suggesting that zebrafish radial glia are heterogeneous with respect to GFAP expression. To learn if GFAP⁺ radial glia persist in adults, we examined 3-month-old *Tg-(gfap:GFP)* animals. Significant numbers of EGFP⁺ Zrf-1⁺ radial fibers are evident throughout the spinal cord (Fig. 1G). EGFP⁺ cells additionally express BLBP, which also has been used as a marker of neural precursors (Hartfuss et al., 2001; Gotz and Barde, 2005; Fig. 1H). Similar to early larval stages, some ventral Zrf-1⁺ and BLBP⁺ fibers do not express EGFP (Fig. 1G,H).

GFAP⁺ Radial Glia Produce Motor Neurons and Oligodendrocyte Lineage Cells During Development

Several experimental approaches have shown that GFAP⁺ radial glia are precursors that give rise to specific subpopulations of neurons in the brain during development (Miyata et al., 2001; Noctor et al., 2002; Malatesta et al., 2003; Anthony et al., 2004). To examine whether GFAP⁺ radial glia in zebrafish spinal cord similarly function as precursors we labeled *Tg(gfap:GFP)* embryos with BrdU to identify dividing cells. EGFP⁺ cells near the central canal and medial septum of the spinal cord incorporated bromodeoxyuridine (BrdU) at 24 hpf (Fig. 2A) and 36 hpf (Fig. 2B), consistent with the possibility that these cells function as neural precursors. We next labeled transgenic embryos with anti-Hu antibody, which marks newly born neurons (Marusich et al., 1994). Because EGFP fluorescence persists even after the transgene is no longer transcribed, it can be used as a short-term lineage marker to determine the fates of radial glia. Many of the most medial neurons, which had relatively low levels of Hu immunofluorescence, were EGFP⁺ (Fig. 2C,D), indicating that radial glia give rise to spinal cord neurons.

Previously, we showed that EGFP expressed under control of *olig2* regulatory DNA marks BLBP⁺ ventral spinal cord radial glia that appear to produce oligodendrocytes in larval and adult animals (Park et al., 2007). However, at these late stages anti-GFAP antibody apparently did not recognize *olig2*⁺ radial glia, consistent with our observations described above that ventral spinal cord radial glia of larvae are heterogeneous with respect to GFAP expression. To investigate whether this heterogeneity is evident during development, we created *Tg(olig2:DsRed2);Tg(gfap:GFP)* double transgenic embryos. At both 1 and 2 dpf, DsRed2⁺ cells near the central canal were EGFP⁺ (Fig. 3A–D). DsRed2⁺ cells located close to the pial surface of the spinal cord had low levels of EGFP or were EGFP[−], consistent with the possibility that they down-regulated *gfap* expression as they differentiated.

olig2⁺ pMN precursors produce motor neurons, some interneurons and oligodendrocytes during development (Park et al., 2004). Based on the above results, we predicted that motor neurons and oligodendrocytes would arise from *gfap*⁺ cells. To test this, we labeled *Tg(gfap:GFP)* embryos with antibody markers of motor neurons and oligodendrocyte lineage cells. Both cell types were EGFP⁺ (Fig. 3E–H), indicating that they arose from cells that expressed *gfap*. At 2 dpf, some of the most ventral anti-Neuroilin⁺ secondary motor neurons were EGFP[−]. One possible explanation is that these were the earliest-born secondary motor neurons, from which EGFP fluorescence had decayed.

Notch Signaling Is Continuously Required During Development to Maintain Radial Glia and Regulate Formation of Motor Neurons and Oligodendrocytes

The above data show that GFAP⁺ radial glia are formed during development and function as precursors giving rise to, among other neural cell types, motor neurons and oligodendrocytes. We showed previously that Notch signaling is necessary for maintenance of precursors during early stages of neural development (Appel et al., 2001) but we have not tested whether Notch signaling is also required for precursor maintenance during late neural development and transition to larval stage. As a first step toward addressing this question, we extended our previous analysis of Notch pathway gene expression. At 2 dpf, during late embryogenesis, medial spinal cord cells express high levels of the receptor-encoding genes *notch1a* and *notch1b* and ligand-encoding genes *dla* and *dld* (Fig. 4A–D). By contrast, in newly hatched 3 dpf larvae, many fewer spinal cord cells expressed *notch1a*, *notch1b*, and *dla*, and *dld* expression was not evident (Fig. 4E–H). Down-regulation of *notch* and *delta* gene expression therefore coincides with the transition from frequent to infrequent cell division at the end of embryogenesis (Park et al., 2007) and may reflect a sharp decline in neuronal and glial cell production.

We showed previously that disruption of Notch signaling during early development results in formation of excess early-born primary motor neurons and deficits of later-born secondary motor neurons and oligodendrocytes (Appel and Eisen, 1998; Park and Appel, 2003). However, we more recently discovered that most primary motor neurons do not arise from the same precursors that produce secondary motor neurons and OPCs (Park et al., 2004), thus calling into question our original interpretation that Notch regulates a choice between primary motor neuron and oligodendrocyte fates. To further investigate the role of Notch signaling in motor neuron and oligodendrocyte specification, we blocked Notch signaling by inducing expression of a dominant negative form of the *Xenopus laevis* Suppressor of Hairless transcription factor by heat-shocking *Tg(hsp70l:dnSu(H)-myc)* embryos (Latimer et al., 2005). Similar to embryos that had a constitutive deficit of Notch signaling (Park and Appel, 2003) transgenic embryos heat-shocked at 30 hpf, just before OPC specification, had few OPCs relative to control embryos at 50 hpf (Fig. 5A,E,I). In contrast to those with a constitutive deficit, which had excess primary motor neurons and a deficit of secondary motor neurons (Appel and Eisen, 1998; Appel et al., 2001), heat-shocked embryos had excess secondary motor neurons relative to control embryos (Fig. 5B,F,J). We noticed that secondary motor neurons occupied medial positions within the ventral spinal cord of heat-shocked transgenic embryos (Fig. 5F), raising the possibility that disruption of Notch signaling during late stages of neural development causes remaining precursors to adopt neuronal fates. Consistent with this, whereas control embryos were devoid of Hu⁺ neurons in medial spinal cord, neurons were distributed throughout the spinal cords of heat-shocked embryos (Fig. 5C,G).

Notch signaling might regulate secondary motor neuron and oligodendrocyte formation by limiting the number of dividing precursor cells specified for motor neuron fate, preserving some of them for an alternative oligodendrocyte fate. To test this, we marked dividing cells in control and heat-shocked transgenic embryos with the thymidine analog BrdU after heat shock at 30 hpf, allowed the cells time to exit the cell cycle and determined their fates. Whereas few motor neurons arose from dividing precursors in control embryos (Fig. 5D,K), a substantial number of motor neurons, which were predominantly in medial positions, incorporated BrdU in heat-shocked embryos (Fig. 5H,K).

The above data show that Notch signaling is continuously required for oligodendrocyte specification and that precursors that give rise to oligodendrocytes have radial glial characteristics. Thus, we next hypothesized that Notch signaling is also necessary to maintain radial glial cells as neural precursors for OPC specification. Therefore, we tested the requirement of Notch signaling in maintaining radial glial cells during late embryonic and early postembryonic stages. In contrast to heat-shock induction during embryonic stages, heat-shocking *Tg(hsp70l:XdnSu(H)-myc)* larvae at early postembryonic stage had little apparent effect on spinal cord cells (data not shown). We reasoned that transient expression of dominant-negative Suppressor of Hairless protein might not be sufficient to alter cell fate if the cell-cycle time of larval neural precursors is substantially longer than that of embryonic precursors. Consequently, we turned to DAPT, a pharmacological agent that effectively blocks Notch signaling in zebrafish embryos (Geling et al., 2002). Similar to heat induction of *Tg(hsp70l:XdnSu(H)-myc)* embryos at 30 hpf, treating embryos with DAPT from 30 – 40 hpf caused formation of excess secondary motor neurons and deficits of spinal cord precursors and OPCs (data not shown). Consistent with the requirement of Notch signaling for OPC specification during late embryonic stage, incubation of *Tg(olig2:egfp)* animals from 32 hpf to 6 dpf resulted in reduction and disorganization in cells with Zrf-1⁺ and EGFP⁺ radial processes and deficits of EGFP⁺ OPCs (Fig. 6B). By contrast, embryos incubated with DAPT from 50 hours post fertilization (hpf) to 6 dpf had apparently normal numbers of radial glial cells and OPCs (Fig. 6C).

As a complementary approach to conditional inhibition of Notch activity, we compared the effects of expressing a constitutively active form of Notch1a during late embryonic and early postembryonic stages. The 3 dpf *Tg(hsp70l:GAL4);Tg(UAS:myc-Notch1a-intra)* (Scheer et al., 2001) larvae that were heat-shocked at 24 hpf had a large increase in the number of radial glia relative to controls (Fig. 6D,E), consistent with our previous demonstration that driving expression of constitutively active Notch during embryogenesis promotes OPC specification (Park and Appel, 2003). However, transgenic larvae that were heat-shocked at 50 hpf had apparently normal numbers of radial glia (Fig. 6F). Thus, neither our loss or gain of function tests had an apparent effect on spinal cord radial glia at late embryonic and early larval stages. To test whether spinal cord cells can respond to changes in Notch activity at late embryonic stage, we initiated DAPT treatment at 2 dpf and examined expression of *her4*, a Notch target gene (Takke et al., 1999) at 3 dpf. DAPT-treated larvae consistently expressed *her4* RNA at lower levels than control larvae (Fig. 6G,H), indicating that Notch signaling remains active during this period and that it can be effectively inhibited by DAPT. Altogether, these data suggest that Notch signaling is continuously required during development for maintaining radial glia as neural precursors but radial glial become refractory to alterations of Notch signaling at late embryonic stage.

DISCUSSION

Zebrafish Spinal Cord Radial Glia Emerge Early in Development and Persist Into Adulthood

Neurogenesis and gliogenesis in the zebrafish embryonic spinal cord is rapid, occurring within approximately 48 hr. The first neurons produced, primary motor neurons and primary sensory Rohon-Beard neurons, arise from neuroepithelial cells of the neural plate beginning at approximately 10–11 hpf (Korzh et al., 1993). Over the next several hours, the neural plate is shaped into a solid rod, which then cavitates to form the spinal cord (Papan and Campos-Ortega, 1994). Production of most neurons, including secondary motor neurons, occurs from approximately 18–30 hpf. OPCs then arise beginning at approximately 36–40 hpf until the end of embryogenesis at 2–2.5 dpf (Park et al., 2002; Kirby et al., 2006).

The transition from neuroepithelial cells to radial glia, as in mammals, begins during neurogenesis. We first detect spinal cord expression of *gfap*, a canonical marker of radial glia, by in situ RNA hybridization at 3- to 5-somite stage in zebrafish embryos (data not shown). This coincides with the time at which the first primary neurons are produced in zebrafish and transition from the neuroepithelial neural plate to a thickened neural keel (Papan and Campos-Ortega, 1994). Expression of intermediate filament proteins such as GFAP might reflect the gradual lengthening of radially oriented precursors spanning the neural tube as it forms. Labeling by anti-GFAP and anti-Zrf-1 antibodies also reveal significant numbers of radial fibers in the embryonic spinal cord, which persist into larval and adult stages. Of interest, larval and adult radial glia might be heterogeneous with respect to gene expression. Some radial glia in the ventral spinal cord that are labeled by anti-Zrf-1 and anti-BLBP antibodies do not appear to express GFAP. Previously, we showed that BLBP⁺ GFAP[−] radial glia in larvae and adults express *olig2*, revealed by transgenic reporter expression, and that these cells seem to give rise to oligodendrocyte lineage cells but not neurons (Park et al., 2007). We speculate that some radial glia might function as fate-restricted precursors for neurons or oligodendrocytes in adult zebrafish.

Consistent with the early onset of *gfap* expression during zebrafish neural development, *gfap*⁺ spinal cord cells divide and produce neurons, including motor neurons. Previous studies showed that neurons in rodent brains and spinal cord arise from radial glial progenitors (Miyata et al., 2001; Noctor et al., 2001, 2002; Tamamaki et al., 2001; Malatesta et al., 2003; Anthony et al., 2004; Fogarty et al., 2005). Therefore, our data now extend evidence of neurogenic radial glia to the spinal cord of zebrafish embryos. Our data also show that *gfap*⁺ spinal cord cells

produce oligodendrocyte lineage cells during development, again consistent with previous data from rodents (Choi and Kim, 1985; Hirano and Goldman, 1988; Fogarty et al., 2005). Together these observations suggest that, at least during early stages of neural development, common populations of *gfap*⁺ neural cells can be multipotent, giving rise to both neurons and oligodendrocytes.

Notch Signaling Is Required Continuously During Embryogenesis for Maintenance of Radial Glia and Production of Oligodendrocytes

What are the mechanisms that maintain radial glia during embryogenesis into postnatal stages? Previous work showed that expression of constitutively active Notch in mouse embryos could promote formation of radial glia (Gaiano et al., 2000). At the time, this was taken as evidence that Notch signaling promotes gliogenesis. Since then, the accumulating evidence that radial glia can give rise to neurons provides a more nuanced view whereby Notch signaling, consistent with its well known role in maintaining neuroepithelial precursors, also maintains radial glia as precursor cells. Our own data support this latter interpretation. We have found that blocking Notch signaling at any time during embryogenesis depletes spinal cord radial glia whereas expression of constitutively active Notch causes formation of a large excess of radial glia. Accordingly, Notch signaling is necessary for specification of cells that arise from precursors that have radial glia characteristics. Previously, we showed that loss of Notch signaling caused formation of excess early-born primary motor neurons at the apparent expense of later-born secondary motor neurons and oligodendrocytes (Appel and Eisen, 1998; Appel et al., 2001; Park and Appel, 2003). By fate mapping, we subsequently learned that most secondary motor neurons and oligodendrocytes are produced by a subset of pMN precursors distinct from those that produce primary motor neurons (Park et al., 2004). Consequently, we extended our investigation of the role of Notch signaling in motor neuron and oligodendrocyte development here. Embryos in which Notch signaling was blocked after the period of primary motor neuron specification, but before oligodendrocyte specification, had elevated numbers of secondary motor neurons, which arose from dividing precursors, and a deficit of oligodendrocytes. All together, our data support a model in which Notch signaling is continuously required during development within neuroepithelial precursors and, subsequently, radial glia precursors to regulate formation of different motor neurons and oligodendrocytes.

We recently showed that a slowly dividing population of radial glia precursors, marked by expression of an *olig2* reporter gene, persists into adulthood (Park et al., 2007). To test if Notch signaling is also required for maintenance of postembryonic radial glia precursors, we both blocked and induced Notch signaling at the transition of embryonic to larval periods. In contrast to manipulations at earlier periods, neither experiment had an appreciable effect on the number and distribution of larval spinal cord radial glia. This finding raises the possibility that spinal cord radial glia become independent of Notch signaling after embryogenesis, coincident with the transition from rapid embryonic division to slow larval division and a dramatic diminution of *notch* and *delta* gene expression. Therefore, distinct mechanisms of precursor maintenance might operate in zebrafish embryos and larvae.

EXPERIMENTAL PROCEDURES

Fish Lines

Wild-type AB, *Tg(olig2:egfp)^{vu12}* (Shin et al., 2003), *Tg(gfap:GFP)* (Bernardos and Raymond, 2006), *Tg(hsp70l: dnSu(H)myc)^{vu21}* (Latimer et al., 2005), *Tg(hsp70:GAL4)* and *Tg(UAS: Notch1a^{ac}-myc)* (Scheer et al., 2001) zebrafish were used for this study.

Generation of *Tg(olig2:DsRed2)* Transgenic Zebrafish

To produce a targeting construct for *Tg(olig2:DsRed2)* transgenic fish, *egfp* cDNA was first removed from a targeting vector that was previously used to generate a *Tg(olig2:egfp)* transgenic line (Shin et al., 2003) by digestion with *NcoI* and *BamHI* restriction enzymes. The targeting vector was then ligated to a *DsRed2* cDNA fragment that was amplified by polymerase chain reaction using *DsRed2*-specific primers containing *NcoI* and *BamHI* restriction sites. A targeting construct containing *DsRed2* and *kanamycin* resistance sequence flanked by FRT sites was removed from the plasmid by *EcoRI/NotI* restriction enzyme digestion and recovered from an agarose gel. Modification of an *olig2* Bacterial Artificial Chromosome was performed according to a *Escherichia coli*-based homologous recombination method (Lee et al., 2001), and the resulting construct was injected into newly fertilized eggs to produce *Tg(olig2:DsRed2)* transgenic fish as described previously (Shin et al., 2003).

In Situ RNA Hybridization

In situ RNA hybridization was performed as described previously (Hauptmann and Gerster, 2000). Previously described RNA probes included *gfap* (Nielsen and Jorgensen, 2003), *her4* (Takke et al., 1999), *notch1a*, *notch1b*, *dla*, and *dld* (Appel et al., 2001).

BrdU Labeling and Immunocytochemistry

Manually dechorionated embryos were labeled with bromodeoxyuridine (BrdU) by incubating them for 20 min on ice in a solution of 10 mM BrdU and 15% dimethyl sulfoxide (DMSO) in embryo medium (EM; 15 mM NaCl, 0.5 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 0.15 mM KH₂PO₄, 0.05 mM NH₂PO₄, 0.7 mM NaHCO₃). The embryos were then placed in EM and incubated 20 min at 28.5°C and fixed using 4% paraformaldehyde in PBS. Embryos were treated 1 h with 2 M HCl, and then processed for anti-BrdU immunocytochemistry.

For immunocytochemistry, we used the following primary antibodies: mouse anti-BrdU (G3G4, 1:1,000, Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA), mouse anti-Hu (16A11, 1:20, Molecular Probes), mouse anti-zrf1 (1:400, University of Oregon Monoclonal Antibody Facility), rabbit anti-BLBP (1:1,500; Feng et al., 1994), rabbit anti-Sox10 (1:1,000) (Park et al., 2005), mouse anti-GFAP (G-A-5, 1:100, Sigma), mouse anti-Neurolin (zn-8, 1:1,000, DSHB), and mouse anti-Isl (39.4D5, 1:100, DSHB).

For fluorescent detection of antibody labeling, we used Alexa Fluor 488 and Alexa Fluor 568 goat anti-mouse or goat anti-rabbit conjugates (1:500, Molecular Probes). Fluorescence images were collected using a Zeiss LSM 510 laser scanning confocal microscope.

Heat-Induced Transgene Expression

To induce expression heat-shocked regulated transgenes, embryos were collected from matings of *Tg(hsp70: GAL4)* and *Tg(UAS:Notch1a^{ac}-myc)* adults, and from matings of *Tg(hsp70l:dnSu(H)myc)* adults and raised at 28.5°C. At appropriate stages, embryos were transferred to EM at 39.0°C for 30 min and then returned to 28.5°C until desired stages. Transgene expression was confirmed by anti-Myc immunocytochemistry (data not shown).

DAPT Treatment

DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl]-S-phenylglycine-t-butyl ester; Calbiochem, La Jolla, CA) treatments were performed as previously described (Geling et al., 2002). DAPT was reconstituted with DMSO to make a stock concentration of 10 mM. For experiments, aliquots were diluted to 100 μM in EM. Embryos were dechorionated with watchmakers

forceps and placed in the DAPT solution at specified times and incubated overnight at 28.5°
C. Control embryos were incubated in an equivalent concentration (1%) of DMSO in EM.

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Fig. 1.

gfap⁺ cells are radial glia in embryonic and postembryonic zebrafish spinal cord. All images are transverse sections of spinal cords, dorsal to top. **A,B:** Spinal cords of 36 hours postfertilization (hpf) *Tg(gfap:GFP)* embryos labeled by *gfap* (A) and *egfp* (B) RNA in situ hybridization. **C:** *gfap* RNA expression at 36 hpf. **D:** Glial fibrillary acidic protein (GFAP) expression detected by immunocytochemistry at 48 hpf. Brackets in A–D mark ventral spinal cord cells that do not express the endogenous *gfap* gene or the reporter transgene. Arrowheads mark floor plate. **E–G:** Anti-Zrf-1 antibody labeling (red) and enhanced green fluorescent protein (EGFP) fluorescence (green) of *Tg(gfap:GFP)* animals at 3 days postfertilization (dpf), 7 dpf, and 3 months. Numerous Zrf-1⁺ EGFP⁺ radial glia appear as yellow fibers. Arrows indicate Zrf-1⁺ GFAP⁺ radial glial cells in ventral most spinal cord. **H:** Anti-brain-lipid-

binding protein (BLBP) antibody (red) labeling of 3-month-old adult *Tg(gfap:GFP)* fish. BLBP⁺ EGFP⁺ radial glia appear yellow. Arrows indicate BLBP⁺ EGFP⁻ radial fibers in ventral spinal cord. Scale bar = 20 μ M in A–F, 80 μ M in G,H.

Fig. 2. *gfap*⁺ radial glial cells divide and produce neurons. All images are transverse sections of spinal cords obtained from *Tg(gfap: GFP)* embryos, dorsal up. Enhanced green fluorescent protein (EGFP) fluorescence is shown as green. **A,B:** Bromodeoxyuridine (BrdU) labeling (red) at 24 hours postfertilization (hpf; A) and 36 hpf (B). **C,D:** Anti-Hu antibody labeling (red) at 24 hpf (C) and 48 hpf (D). Arrows indicate EGFP⁺ cells with relatively weak Hu labeling, which probably are newly born neurons. Scale bar = 20 μM.

Fig. 3. *gfap*⁺ cells produce motor neurons and OPCs. All panels show transverse sections of spinal cord with dorsal upward. **A–D:** *Tg(olig2:DsRed2);Tg(gfap:EGFP)* embryos at 1 dpf (A,B) and 2 dpf (C,D). Arrows and arrowheads indicate DsRed2⁺ EGFP⁺ cells near the central canal and pial surface, respectively. **E:** Arrows mark Isl⁺ EGFP⁺ motor neurons at 1 dpf. **F:** Neurolin⁺ EGFP⁺ secondary motor neurons (yellow) at 2 dpf. **G,H:** Arrowheads indicate Sox10⁺ EGFP⁺ OPCs at 2 dpf. Scale bar = 20 μM.

Fig. 4.

Expression of *notch* and *delta* genes in spinal cord of late embryonic and early postembryonic zebrafish. All panels show in situ RNA hybridized, transverse sections of spinal cord, dorsal up. **A–D:** A 2 days postfertilization (dpf) late embryonic stage. **E–H:** A 3 dpf early postembryonic stage. Scale bar = 20 μM.

Fig. 5. Conditional inhibition of Notch signaling before OPC specification produces excess secondary motor neurons at the expense of OPCs. All images show transverse sections of spinal cord, dorsal to top. **A–D:** Control embryos. **E–H:** *Tg(hsp70l:dnSu(H)myc)* embryos heat-shocked at 30 hours postfertilization (hpf). Inhibition of Notch signaling before OPC specification blocks formation of Sox10⁺ OPCs (A,E,I) and produces excess secondary motor neurons (B,F,J). Arrows mark OPCs and secondary motor neurons and the small, dashed circle marks the area around the central canal, which is not occupied by motor neurons, in control embryos. The medial portion of the spinal cord, which does not have Hu⁺ neurons in control embryos (dashed circle, C) is filled with neurons in Notch-inhibited embryos (G). Pulse labeling with bromodeoxyuridine (BrdU, red) reveals that more dividing spinal cord cells adopt Isl⁺ motor neuron fates in Notch-inhibited embryos relative to controls (D,H,K). The quantitative data were obtained from sections of six control and six heat-shocked embryos for each experiment. Error bars represent SEM. Scale bar = 20 μM.

Fig. 6.

Notch signaling is required to maintain radial glial cells during embryonic development. All panels show transverse sections of spinal cord with dorsal upward. **A–C:** *Tg(olig2:egfp)* larvae labeled with anti-Zrf-1 antibody (red) at 6 days postfertilization (dpf). Control (A), DAPT treatment begun at 32 hours postfertilization (hpf). (B), and DAPT treatment begun at 50 hpf (C). Arrows indicate Zrf-1⁺ enhanced green fluorescent protein-positive (EGFP⁺) radial glial cells, and arrowheads indicate EGFP⁺ oligodendrocyte lineage cells. **D–F:** A 3 dpf *Tg(hsp70l:GAL4);Tg(UAS:myc-Notch1a-intra)* larvae labeled with Zrf-1 antibody; control (D), heat induction at 24 hpf (E), and heat induction at 50 hpf (F). **G,H:** A 3 dpf larvae probed to detect *her4* expression by in situ RNA hybridization. Control (G) and DAPT treatment begun at approximately 50 hpf (H). Scale bar = 20 μM.