Neuronal activity biases axon selection for myelination
in vivo

Jacob H Hines1,3, Andrew M Ravanelli1, Rani Schwindt1, Ethan K Scott2 & Bruce Appel1

An essential feature of vertebrate neural development is ensheathment of axons with myelin, an insulating membrane formed by oligodendrocytes. Not all axons are myelinated, but mechanisms directing myelination of specific axons are unknown. Using zebrafish, we found that activity-dependent secretion stabilized myelin sheath formation on select axons. When VAMP2-dependent exocytosis was silenced in single axons, oligodendrocytes preferentially ensheathed neighboring axons. Nascent sheaths formed on silenced axons were shorter in length, but when activity of neighboring axons was also suppressed, inhibition of sheath growth was relieved. Using in vivo time-lapse microscopy, we found that only 25% of oligodendrocyte processes that initiated axon wrapping were stabilized during normal development and that initiation did not require activity. Instead, oligodendrocyte processes wrapping silenced axons retracted more frequently. We propose that axon selection for myelination results from excessive and indiscriminate initiation of wrapping followed by refinement that is biased by activity-dependent secretion from axons.

In the developing CNS, oligodendrocytes extend membrane processes that ensheath axons with a lipid-rich myelin membrane. Myelination enables thinner axons to transmit information more rapidly, facilitating evolution of a complex yet compact CNS in vertebrates. Despite this advantage, not all axons are myelinated. For instance, in the corpus callosum, a main white-matter tract connecting cerebral hemispheres, fewer than half of all axons become myelinated1. Although selective mechanisms clearly exist in vivo, cultured oligodendrocytes will myelinate fixed axons or synthetic fibers with a diameter larger than 0.4 µm (refs. 2,3). If axon diameter is sufficient for indiscriminate myelination in vitro, what mechanisms enable oligodendrocytes to make stereotyped decisions and myelinate specific axons in vivo?

Many studies have shown that electrical activity promotes myelination4-9, raising the possibility that action potentials and release of axonal factors instruct nearby oligodendrocyte processes to initiate ensheathment. Unmyelinated axons secrete neurotransmitters and neurotrophic factors extrasynaptically along axons10-12, and pre-myelinating oligodendrocytes express a plethora of receptors that are poised to interpret axonal factors released in response to activity13. Consistent with this possibility, electrical stimulation of cultured neurons triggers local Ca2+ signaling in oligodendrocyte processes, which requires synaptic vesicle exocytosis and glutamate receptors7. The mechanisms mediating axon selection in vivo and the contribution of neuronal activity remain completely unknown.

The inability to visualize and genetically manipulate subsets of axons in vivo while simultaneously visualizing which axons are ensheathed by oligodendrocyte wrapping processes has precluded the discovery of axon selection mechanisms. Here we investigated oligodendrocyte membrane sheath formation on single, identifiable axons under normal and electrically silenced conditions in zebrafish larvae. We found that most oligodendrocyte membrane processes in ventral spinal cord tracts selected and ensheathed axons marked by expression of a phox2b reporter gene. The fidelity of this axon choice was reduced when VAMP2-dependent exocytosis or electrical excitability was suppressed in single axons, indicating that axon selection is biased by activity-dependent secretion. Moreover, myelin sheaths that did form on single silenced axons were shorter in length. This activity-dependent control of sheath length is influenced by other axons, as suppressing all electrical activity restored normal sheath length. Finally, using in vivo time-lapse microscopy, we found that oligodendrocytes wrapping silenced axons retracted sheaths more frequently. Collectively, this study suggests a new model for axon selection whereby oligodendrocytes initially form excess sheaths, detect differences in activity-dependent secretion among ensheathed axons and preferentially maintain sheaths on select axons. These findings extend recent discoveries that social experiences and experimentally altered neuronal activity can influence oligodendrocyte proliferation, differentiation and myelin sheath thickness9,14,15 by demonstrating that neuronal activity can bias which axons become myelinated, an additional means of myelin plasticity. Additionally, our work indicates that molecular and cellular mechanisms that stabilize myelin sheaths following indiscriminate sheath initiation contribute substantially to axon selection in response to activity.

RESULTS

Activity-dependent secretion regulates axon selection

We have developed a genetically tractable system to study mechanisms of axon selection in the zebrafish spinal cord. Testing the activity-dependent myelination hypothesis in vivo necessitated transgenic reporters permitting direct observation of subsets of myelin-fated axons under normal and electrically silenced conditions in vivo. We found that only 25% of oligodendrocyte processes that initiated axon wrapping were stabilized during normal development and that initiation did not require activity. Instead, oligodendrocyte processes wrapping silenced axons retracted more frequently. We propose that axon selection for myelination results from excessive and indiscriminate initiation of wrapping followed by refinement that is biased by activity-dependent secretion from axons.

1Department of Pediatrics, University of Colorado School of Medicine, Aurora, Colorado, USA. 2School of Biomedical Sciences, The University of Queensland, St. Lucia, Queensland, Australia. 3Present address: Biology Department, Winona State University, Winona, Minnesota, USA. Correspondence should be addressed to J.H.H. (jhhines@winona.edu) or B.A. (bruce.appel@ucdenver.edu).

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Figure 1 Axon selection is biased by electrical activity. (a) Confocal images show axon wrapping in Tg(phox2b:EGFP) larvae. In uninjected control larvae (left), phox2b+ axons are frequently wrapped by nascent myelin sheaths marked by sox10:mRFP. TTX treatment (right) reduces the proportion of nascent sheaths wrapping phox2b+ axons. For each condition, the upper panels are lateral spinal cord views and the lower panels show orthogonal projections, which were generated at the dashed lines. Arrowheads point to axons (white) and nascent sheaths (magenta). Scale bars, 1 µm. (b) Quantification of the proportion of sox10:mRFP sheaths wrapping phox2b+ axons. P = 0.0005, t-test. (c) Quantification of the overall number of nascent sheaths per spinal cord hemisegment. P = 0.3426, t-test. For b and c, n = 22 control larvae (283 sheaths) and 23 TTX-treated larvae (283 sheaths). (d) Quantification of nascent sheath length; n = 16 control larvae (406 sheaths) and 12 TTX-treated larvae (296 sheaths). P = 0.7468 (phox2b:EGFP+) and P = 0.1540 (phox2b–), t-test. For all panels, error bars show s.e.m.; ***P < 0.001; n.s., not significant.

axons and drivers enabling genetic manipulation of neuronal activity. In Tg(phox2b:EGFP) zebrafish larvae (hereafter phox2b:EGFP), a subset of hindbrain neurons with axons projecting to ventral spinal cord tracts express EGFP (Supplementary Fig. 1). In transverse spinal cord sections, phox2b+ axons were surrounded by myelin basic protein (MBP), a marker for myelin sheaths (Supplementary Fig. 1). To observe the initial myelination of phox2b+ axons in live zebrafish, we visualized nascent sheaths by confocal microscopy using the Tg(sox10: mRFP) reporter (hereafter sox10:mRFP), which expresses membrane-tethered RFP in oligodendrocyte lineage cells. phox2b+ axons were ensheathed shortly after the onset of myelination, and most nascent sheaths in the ventral spinal cord wrapped phox2b+ axons (Fig. 1a).

If axon selection requires electrical activity, then suppressing activity should abrogate the selection of myelin-fated phox2b+ axons. To test this, we treated phox2b:EGFP; sox10:mRFP larvae with the voltage-gated sodium channel blocker tetrodotoxin (TTX) 48 h post-fertilization, before the onset of myelination. TTX treatment paralyzed zebrafish larvae for at least 3 d without major developmental defects or toxicity, but it did not prevent myelin sheath formation in the spinal cord. We assessed axon selection in phox2b:EGFP; sox10:mRFP reporter larvae and found that TTX treatment reduced the proportion of nascent sheaths wrapping phox2b+ axons (Fig. 1a,b). In contrast, TTX treatment had no effect on the overall number or length of nascent sheaths (Fig. 1c,d) or the number of spinal cord oligodendrocyte progenitor cells or oligodendrocytes (Supplementary Fig. 2).

Together, these data show that TTX-sensitive activity is not required for oligodendrocyte differentiation or formation of nascent sheaths in vivo, but that it biases axon choice.

If activity is necessary for biased axon choice, can heightened activity enhance wrapping? To test this, we treated embryos with the Na+ channel modulator veratridine 72 h post-fertilization, immediately before the onset of myelination. Veratridine prolongs Na+ channel opening, and, as expected, injected embryos showed striking and sustained behavioral phenotypes. Upon touch stimulation, control embryos initiated short bursts of swim movements (Supplementary Video 1). By contrast, veratridine-treated embryos initiated a prolonged swim response that eventually terminated in seizure-like behavior and brief paralysis (Supplementary Video 2). Although veratridine treatment had a pronounced effect on neural behavior (Fig. 2a), we observed no change in the spinal cord in the selection of phox2b+ axons or the overall level of wrapping (Fig. 2b–d). However, nascent sheaths on both phox2b+ and phox2b– axons were slightly shorter in veratridine-treated embryos relative to controls (Fig. 2e).

Figure 2 Veratridine reduces nascent sheath length but not sheath number or axon selection. (a) Quantification of touch response assays on control and veratridine-treated larvae. Data shown represent the proportion of larvae exhibiting either wild-type or prolonged touch-response phenotypes (see Online Methods). Scoring was performed 3 h after treatment (post-tx) and again immediately before confocal imaging (24 h after treatment). For both control and treated groups, n = 80 (3 h after treatment) and 76 (24 h after treatment). (b) Representative confocal images show reporter expression in control and veratridine-treated Tg(phox2b:EGFP); Tg(sox10:mRFP) larvae. M indicates the position of the Mauthner axon and arrowheads point to sites of phox2b+ axon wrapping. Scale bar, 5 µm. (c–e) Summary of axon selection, sheath number and sheath length measurements from b. P = 0.2147 (c), P = 0.2173 (d), P = 0.0011 (e, left) and P = 0.0342 (e, right); t-test. Error bars show s.e.m.; *P < 0.05, **P < 0.01; n.s., not significant. For c–e, n = 30 control and 28 veratridine-treated larvae.
We conclude that TTX-sensitive activity is necessary for selection of axons at high fidelity but that widespread elevated neuronal activity is not sufficient to increase wrapping of spinal cord axons.

To test whether activity-dependent signals that bias axon choice originate in neurons, we next inhibited synaptic vesicle exocytosis selectively in phox2b+ axons by targeted overexpression of tetanus neurotoxin light chain (TeNT), a protease that cleaves vesicle-associated membrane protein (VAMP) family members, including Synaptobrevin/VAMP2. This is a potent inhibitor of vesicle exocytosis in many animal models, including zebrafish16,17. We cloned a 2.1-kb genomic fragment of the zebrafish phox2b gene and generated the Tg(phox2b:GAL4-VP16, myl7::Cerulean) line (hereafter phox2b::GAL4; here, myl7::Cerulean is expressed in heart as a marker for transgenesis). When this line was crossed to Tg(UAS:mCherry-CaaX), which expresses mCherry fluorescent protein tethered to membranes by the CaaX motif, mCherry was faithfully coexpressed in axons marked by phox2b:EGFP, validating the specificity of this new transgenic line (Supplementary Fig. 1). We injected phox2b::GAL4 embryos at early cleavage stage with DNA plasmids encoding UAS:EGFP or UAS:TeNT-EGFP, resulting in mosaic expression in single phox2b+ axons of 4-d post-fertilization larvae. When phox2b+ axons expressed EGFP as a control, 55.3% were myelinated at the time of imaging, whereas only 31.6% expressing TeNT-EGFP were myelinated (Fig. 3a, b). Quantitatively, we found that single TeNT-EGFP+ axons were less well wrapped: the percentage of the length of axons ensheathed by sox10::mRFP+ sheaths was reduced (Fig. 3c). Notably, when oligodendrocytes did wrap TeNT-EGFP+ axons, nascent sheaths were shorter in length (Fig. 3d).

Our finding that nascent sheath length is reduced on single TeNT-EGFP+ axons but unaffected when all axons are silenced by TTX is reminiscent of competition mechanisms that refine topographic maps in the visual system16,18–20. To test for activity-based interactions among axons that could modulate myelination, we next asked whether the effects of blocking vesicular release are influenced, or rescued, when neighboring axons are also silenced. We treated larvae with single UAS:TeNT-EGFP+ axons with TTX to suppress activity in surrounding axons. Nascent sheaths wrapping TeNT-EGFP+ axons in TTX-treated larvae grew to the usual lengths characteristic of single EGFP+ axons in a normal environment (Fig. 3d). However, only 21.4% of TeNT-EGFP+ axons were selected for myelination, and the overall enshraement of phox2b+ axons remained reduced (Fig. 3b, c). These findings demonstrate that control of nascent sheath length involves vesicular release and is influenced by neighboring axons. In addition, the observation that phox2b+ axons remain selected with reduced fidelity when activity in other axons is suppressed suggests that additional activity-dependent, noncompetitive forces participate in axon selection.

To test the requirement for neuronal excitability in axon selection, we used targeted overexpression of the human inward rectifier K+ channel Kir2.1 in single phox2b+ axons. This approach suppresses neuronal excitability in various model systems, including zebrafish neurons18,21,22. We used the viral 2A system to coexpress Kir2.1 and the EGFP-CaaX reporter in the same cells22 (hereafter Kir2.1-2A-EGFP). In axon selection assays, UAS:Kir2.1-2A-EGFP+ axons were selected for myelination less frequently than controls (Fig. 3b, c). However, unlike those wrapping TeNT-EGFP+ axons, nascent sheaths wrapping Kir2.1-2A-EGFP+ axons were normal in length (Fig. 3d).

Collectively, these data reveal distinct activity-dependent forces during axon selection. Synaptic vesicle release biases axon selection and positively regulates myelin sheath length in a manner that can be influenced by activity in other axons. Additional forces mediated by excitability, which are suppressed by TTX and Kir2.1 but not influenced by activity in other axons, are also required for axon selection. Notably, myelin sheath length is reduced on single TeNT-EGFP+ axons that are excitable but cannot perform VAMP2-dependent exocytosis, whereas sheath length is normal on Kir2.1-2A-EGFP+ axons with reduced excitability but normal excytotic function.

We next asked whether ectopic neuronal activity in phox2b+ is sufficient to modulate axon selection and initial enshraement by crossing phox2b::GAL4 to the Tg(UAS:ChR2R-EGFP) line, which can induce activity of zebrafish spinal neurons in response to blue light stimulation23. Before optogenetic stimulation, we performed confocal microscopy to identify the specific somite at which oligodendrocytes were

**Figure 3** Activity-dependent interactions during axon selection. (a) Confocal images show expression of UAS:EGFP or UAS:TeNT-EGFP in single phox2b+ axons and nascent sheaths marked by sox10::mRFP. Images on the left are lateral views and the right panel shows orthogonal projections, generated at the dashed lines. Arrowheads point to ensheathed axons. Scale bars, 1 µm. (b) Summary of the percentage of axons selected for myelination. For each condition, the number selected and overall number of axons analyzed is indicated. (c) Quantitative measurements show the wrapping efficiency of phox2b+ axons expressing from the indicated plasmids. Data are expressed as the percentage of total axon length ensheathed at the time of imaging. *P = 0.0096 (upper asterisk), P = 0.0294 (lower asterisk), P = 0.7257 (lower comparison, n.s.); Mann-Whitney test. For c and d, n corresponds to the numbers of axons indicated in b, derived from 17 (UAS:EGFP), 17 (UAS:TeNT-EGFP), 22 (UAS:TeNT-EGFP + TTX), and 29 (UAS:Kir2.1-2A-EGFP) larvae. Error bars show s.e.m.; *P < 0.05, ***P < 0.001; n.s., not significant.
initiating ensheathment. After blue light stimulation (473 nm), we returned to the same position and found no change in the proportion of wrapped phox2b+ axons or the overall ensheathment of phox2b+ axons after 24 h (Supplementary Fig. 3). These data are consistent with our pharmacologic stimulation results (Fig. 2), further indicating that ectopic neuronal activity in phox2b+ axons is not sufficient to alter selection or ensheathment. However, we cannot rule out the possibility that an undetermined stimulation pattern in phox2b+ axons could bias axon selection or regulate ensheathment.

Our observation that vesicle release from axons regulates selection for myelination raises the possibility that vesicle cargo is locally secreted from axons onto oligodendrocyte processes, acting instructively to initiate or maintain nascent sheaths. We next performed confocal microscopy to directly observe the distribution and behavior of axonal vesicles at and away from sites of ensheathment. When phox2b:GAL4 was crossed to Tg(UAS:Syp-EGFP) (hereafter UAS:Syp-EGFP), which expresses EGFP fused to the zebrafish synaptic vesicle protein Synaptophysin, we observed single labeled axons containing vesicles marked by EGFP (Fig. 4 and Supplementary Video 3). Vesicle puncta were both stationary and motile, with rates of movement consistent with microtubule-based axonal transport. When UAS:Syp-EGFP was crossed to the Tg(sox10:TeNT-EGFP) transgenic reporter, we frequently found accumulations of Syp-EGFP+ vesicle puncta at sites of ensheathment (Fig. 4a).

Syp-EGFP+ puncta at ensheathment sites may represent a stable vesicle pool or could be undergoing transport toward or away from the synaptic terminal. To distinguish between these possibilities, we collected time-lapse images at 10-s intervals and measured the motility of vesicles at unmyelinated segments and ensheathment sites. Whereas Syp-EGFP+

![Image](https://example.com/image1.png)

**Figure 4** Accumulation of Syp-EGFP vesicles at myelin ensheathment sites. (a) Representative confocal images show Syp-EGFP vesicle puncta (white) and nascent myelin sheaths marked by the sox10:mRFP reporter (magenta). Scale bars, 2 µm. (b) Representative time-lapse confocal images show stationary and motile Syp-EGFP+ vesicle puncta at unmyelinated axon segments (left panels) and ensheathment sites (right panels). The upper panels show images acquired 30 s before the onset of time-lapse imaging, and the lower panels show time-lapse images acquired at 10-s intervals. In a and b, Syp-EGFP puncta at unmyelinated segments and puncta at ensheathment sites are indicated by blue and yellow arrowheads, respectively. Images are lateral views with dorsal up and anterior right. The Mauthner axon (M) is marked for reference. Scale bars, 2 µm. (c) Summary of vesicle motility measurements show the proportion of motile and stationary vesicles at unmyelinated segments and ensheathment sites. Error bars represent s.e.m.; n = 272 puncta at unmyelinated segments (12 larvae), 31 puncta at ensheathment sites (7 larvae); ***P = 0.0009, t-test.

![Image](https://example.com/image2.png)

**Figure 5** Activity-dependent secretion is not required for initial axon wrapping. (a) Representative time-lapse confocal images show initiation of axon wrapping in the ventral spinal cord of sibling control (left panels) and Tg(neurod1:TeNT-EGFP) larvae (right panels). Images are lateral views and time relative to wrapping initiation is indicated at the left. Arrowheads point to prospective sheaths that fail to stabilize. Scale bar, 2 µm. (b) Summary of time-lapse measurements show the proportion of prospective sheaths that are stable for at least 90 min. n = 12 control (66 sheaths) and 8 TeNT-EGFP larvae (57 sheaths); P = 0.5624, Mann-Whitney test. (c) Measurements of nascent sheath lifetime among transient ensheathments during time-lapse imaging in a. n = 12 control (50 prospective sheaths) and 8 TeNT-EGFP larvae (41 prospective sheaths); P = 0.0341, Mann-Whitney test. For b and c, error bars show s.e.m., *P < 0.05; n.s., not significant.
Axon secretion is required for myelin sheath maintenance

What are the neuron-oligodendrocyte interactions leading to the preferential myelination of specific axons? We next aimed to determine whether axon secretion regulates myelination before or after initial ensheathment. In a preferential ensheathment model, axons without the proper excitability and secreted factors will not support initial ensheathment. Alternatively, in a preferential maintenance model, silenced axons can be initially wrapped but will not maintain sheaths. To test the contribution of activity-dependent secretion in these models, we generated the transgenic line Tg(neurod1:TeNT-EGFP). In this line, the pan-neuronal driver neurod1 expresses TeNT-EGFP broadly in neurons but not oligodendrocytes (Supplementary Fig. 4). Consequently, Tg(neurod1:TeNT-EGFP) larvae (hereafter neurod1:TeNT) show no spontaneous locomotion or touch response (Supplementary Video 4). neurod1:TeNT expression, which is initiated before oligodendrocyte progenitor cell specification, caused a slight reduction in oligodendrocyte progenitor cell and oligodendrocyte numbers (Supplementary Fig. 5), as also described by Mensch et al.24 (this issue). Because TTX treatments, initiated after formation of oligodendrocyte lineage cells, had no effect on cell numbers, we conclude that activity promotes specification. Oligodendrocytes in neurod1:TeNT+ larvae do form nascent sheaths (Fig. 5a and Supplementary Fig. 5), further supporting our conclusion that neuronal activity and synaptic vesicle exocytosis are not required for initial ensheathment of axons in vivo. Moreover, electron micrographs showed no difference in axon diameter between wild-type and neurod1:TeNT+ larvae (Supplementary Fig. 6), suggesting that phenotypes resulting from TeNT-EGFP overexpression are due to inhibition of exocytosis rather than a change in axon diameter.

To test the preferential ensheathment model, we performed time-lapse microscopy to assess oligodendrocyte membrane sheath initiation and stability in neurod1:TeNT+ larvae. We used the Tg(sox10: mRFP) reporter to observe initial axon wrapping attempts using an imaging procedure with greater temporal resolution than previous studies. Remarkably, this revealed an unanticipated frequency of failed ensheathehs in all samples (Supplementary Video 5). In both sibling control and neurod1:TeNT+ larvae, ~75% of wrapping attempts, or prospective sheaths, had disappeared within 90 min. Thus, 25% of prospective sheaths wrapping normal or silenced axons were initially stabilized in either condition, arguing against the preferential ensheathment model (Fig. 5a,b and Supplementary Videos 6–9). Intriguingly, although the proportion of wrapping attempts initially stabilized was unaffected, the ensheathment failure occurred more rapidly when prospective sheaths attempted to wrap silenced axons (Fig. 5c).

To test the preferential maintenance model, we next performed time-lapse microscopy to visualize the fate of pre-existing sheaths over an extended time period. We again used Tg(neurod1:TeNT-EGFP); Tg(sox10:mRFP) larvae, imaging at 20-min intervals to track individual oligodendrocyte membrane sheaths over a 15-h period. We focused on sheaths that had initiated several hours before and, as in previous reports25, found that existing sheaths were extremely stable. Whereas retractions were rare in control larvae, sheaths were retracted more frequently in neurod1:TeNT+ larvae (Fig. 6a,b and Supplementary Videos 10 and 11). In both conditions, sheaths that retracted started out shorter than stable sheaths (Fig. 6c). Also consistent with the preferential maintenance model, and with the findings of Mensch et al.24, we found that by 5 d post-fertilization, singly labeled oligodendrocytes in neurod1:TeNT+ larvae possessed fewer sheaths (Supplementary Fig. 5). Time-lapse imaging of singly

Figure 6 Naïve myelin sheaths are stabilized by activity-dependent secretion. (a) Representative confocal images show the retraction of existing sheaths during 15-h time-lapse imaging in sibling control and Tg(neurod1:TeNT-EGFP) larvae. Images are lateral views of the dorsal spinal cord and the time relative to the start of image acquisition is indicated for each image. For demonstrative purposes, sheaths stable for the entire time-lapse are shaded in blue. Retracting sheaths are shaded red and are also indicated by red arrowheads. Scale bar, 5 µm. (b) Summary of time-lapse measurements show the frequency of sheath retraction. Bars represent the mean ± s.e.m. n = 7 control (99 sheaths) and 9 Tg(neurod1:TeNT-EGFP) larvae (129 sheaths). P = 0.0071, Mann-Whitney test. (c) Quantitative measurements show the relationship between sheath length and stability. Bars represent the mean ± s.e.m. n = 7 control larvae (72 stable sheaths, 6 retracting sheaths) and 9 Tg(neurod1:TeNT-EGFP) larvae (106 stable sheaths, 19 retracting sheaths); P = 0.0034 (left), P = 0.0002 (right), P = 0.3737 (upper n.s.), P = 0.6105 (lower n.s.), Mann-Whitney test; For b and c, **P < 0.01, ***P < 0.001; n.s., not significant.
labeled oligodendrocytes in neurod1:TeNT+ larvae also supported a relationship between sheath length and retraction. Within 3 h of initiation, prospective sheaths that were later maintained had extended more rapidly and were longer in length. In contrast, prospective sheaths that would be retracted extended more slowly and never exceeded 10 μm in length before being retracted (Supplementary Fig. 7a–c). Collectively, our in vivo time-lapse imaging experiments demonstrate that the maintenance of nascent sheaths is regulated by activity-dependent secretion from axons, whereas initial axon wrapping is activity independent.

DISCUSSION

Taken together, our findings suggest a model whereby, after initial axon wrapping, activity-dependent secretion from axons promotes extension and stabilization of prospective sheaths. In the absence of this input, oligodendrocyte membrane sheaths were able to form but did not extend, and were retracted at a higher frequency. Oligodendrocytes sampled many axons during the first several hours after initiating myelination, but 75% of initial wrapping segments failed to form stable sheaths. In an environment in which all axons are excitatory, VAMP2-dependent secretion from axons promoted myelin sheath growth, and our time-lapse imaging indicated that shorter sheaths were more susceptible to subsequent retractions. In this way, excess axon wrapping is refined and specific axons preferentially maintain myelin sheaths. This highlights a major gap in our current knowledge. If all axons secrete neurotransmitters upon spontaneous and evoked activity, what are the specific factors that distinguish myelin-fated axons from those that will never be myelinated?

Our findings that activity and synaptic vesicle release bias axon selection provide insight into a critical facet of neural development with no previously known mechanism. In culture, oligodendrocytes can myelinate fixed axons and synthetic fibers greater than 0.4 μm in diameter2,3. A minimal diameter may be permissive for myelination, but cannot explain how stereotyped decisions to myelinate occur in vivo. Does axon diameter act instructively before ensheathment and specify axons for myelination? Electron microscopy studies show a strong correlation between axon diameter and myelination. In the corpus callosum, 80% of myelinated axons have a diameter >0.4 μm. Yet there is considerable overlap between the diameters of myelinated and unmymelinated axons, suggesting that axon caliber alone is not sufficient to trigger myelination5. In the absence of causal evidence that axon diameter acts instructively, an alternative possibility is that a minimum diameter of 0.3–0.4 μm is permissive for initial ensheathment and may be followed by radial growth to increase axon diameter1,26,27. Because axon diameter was unaffected by TeNT-EGFP overexpression, we conclude that activity-dependent secretion biases axon choice independently of axon diameter.

Our data are consistent with previous reports that oligodendrocytes have a brief window of myelogenenic potential25. The closure of this developmental window does not involve neuronal activity, because after several hours of initial axon wrapping, oligodendrocytes did not form new sheaths in Tg(neurod1:TeNT-EGFP) larvae (Supplementary Fig. 7d). Is activity-dependent refinement solely responsible for determining which axons are myelinated? Others have suggested that the amount of myelin pruning is insufficient to eliminate all excess or spurious initial wrapping and have proposed that unidentified mechanisms may specify which axons can be wrapped before ensheathment28. Our data do not exclude the possibility that activity-independent forces also contribute to axon selection before initial ensheathment. Such mechanisms seem likely given our findings that phox2b+ axons can still be ensheathed, albeit at a lower frequency, in the presence of TTX or TeNT.

How might new or enhanced brain activity stimulate myelination in vivo? Mice running on a wheel with complex rung spacing increase production of oligodendrocytes29, and optogenetic stimulation within the physiological range promotes proliferation and maturation of oligodendrocyte lineage cells and myelin thickness30. Additionally, Mensch et al.24 found that pharmacologically induced brain activity can promote formation of excess myelin sheaths by individual oligodendrocytes in zebrafish. By contrast, our optogenetic and pharmacological manipulations did not overtly change axon wrapping or axon selection bias in zebrafish spinal cord. One possible explanation is that mechanisms that mediate axon selection require highly specific activity codes that were not replicated in our gain-of-function experiments. Alternatively, activity might not be sufficient to direct myelination of axons that normally remain unmymelinated or to induce myelination prematurely. Because all axons are active, axon selection mechanisms that do not rely solely on activity would prevent ectopic myelination, which could be detrimental. Instead, activity might enhance myelination of predetermined axons, thereby strengthening specific neural circuits in response to experience.

Whether electrical activity and neurotransmitter release regulates myelination is a long-standing question with conflicting results6,7,30,31. Our findings are consistent with models of activity-dependent regulation of myelination and highlight specific roles in axon selection and myelin sheath maintenance. Might neuronal activity influence axon selection via mechanisms resembling synaptogenesis28? Substantial evidence supports extrasynaptic release of axonal factors such as glutamate at sites of oligodendrocyte contact10,11, and our own data show vesicle accumulation at ensheathment sites. This, coupled with evidence for membrane potential shifts and action potential-induced Ca2+ signaling in processes of oligodendrocyte-lineage cells7,32,33, supports a model whereby axon–oligodendrocyte interactions along the axon locally regulate myelination in response to neuronal activity.

Myelination greatly alters action potential conduction velocity, and, in principle, activity-dependent myelination has profound implications for spike timing and Hebbian learning. Therefore, myelination of specific axons, and the parameters of this myelin, may be especially important for normal brain function. Supporting this notion, many neuropsychiatric disorders have been linked to myelin genes and white matter abnormalities34. Do experiences, encoded by neuronal activity, influence myelination? Mice reared in social isolation from postnatal days 21 to 35 show altered prefrontal cortex myelination that corresponds with deficits in social interactions and working memory14. This critical period for experience-dependent myelination corresponds with cortical oligodendrocyte maturation and axon ensheathment, suggesting that experience and neuronal activity may alter the behavior of myelinating oligodendrocytes. Our findings that activity-dependent secretion from axons regulates myelin sheath growth, stabilization and axon selection provide a basis for the stereotyped selection of specific axons and for how altered experience can change the myelogenenic landscape.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.H.H. and B.A. conceived the project. J.H.H. generated all new transgenic zebrafish lines and performed fluorescence microscopy experiments. A.M.R. cloned the phox2b promoter and performed veratridine and Channelrhodopsin experiments. R.S. performed electron microscopy experiments and assisted with cell count experiments. E.K.S. prepared the Tg(UAS:Spy-EYFP) transgenic line. J.H.H. wrote and B.A. edited the manuscript.

COMPETING FINANCIAL INTERESTS

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ONLINE METHODS

Zebrafish lines and husbandry. All animal work performed in this study was approved by the Institutional Animal Care and Use Committee at the University of Colorado School of Medicine. Zebrafish embryos were raised at 28.5 °C in egg water and staged according to hours post-fertilization or morphological criteria. Tg(sox10:GFP)Tg19, Tg(mbp:GAL4-VP16, myl7:Galea)Tg20, Tg(phox2b:GAL4-VP16, myl7:Galea)Tg21 antibody labeling. 7-dpf larvae in paraformaldehyde buffer and generated transverse sections using Tg(sox10:GAL4-VP16, myl7:Cerulean)Tg19 (ref. 35), Tg(sox10:GAL4-VP16, myl7:Cerulean)Tg20, Tg(phox2b:GAL4-VP16, myl7:Cerulean)Tg21, Tg(xnr1US:EGFP-CaaX, myl7:EGFP)Tg21, Tg(xnr1US:mCherry-CaaX, myl7:EGFP)Tg22, Tg(neurod1:TeNT-EGFP)Tg23. This mosaicism is most likely a consequence of UAS transgene silencing in zebrafish. To ensure that all myelin segments emanated from a single soma and not neighboring oligodendrocytes, we only imaged and analyzed isolated oligodendrocytes with no labeled cells in adjacent segments. To determine the number of oligodendrocyte progenitor cells (OPCs) we used the Tg(olig2:EGFP) reporter line. After chemical fixation and embedding, we generated transverse sections of spinal cord. Routine immunofluorescence protocols were used to label Sox10+ spinal cord cells using rabbit anti-Sox10 and goat anti-rabbit Alexa Fluor 568 secondary antibodies. We then counted the number of olig2+ Sox10+ cells per transverse section in the anterior spinal cord. The average OPC number per fish was generated from ten independent sections. Cell counts were performed using a Zeiss Axiosvert 200 microscope equipped with mercury arc lamp and a 20×, 0.8 NA objective.

To determine the number of oligodendrocytes we used the Tg(mbp:GFP) reporter line. Images were collected using the PerkinElmer spinning disk confocal and the number of oligodendrocytes per field was quantified within Volocity software. Only cells with fluorescence intensities twice the mean background pixel intensity were counted in analysis.

To count OPCs and oligodendrocytes in Tg(neurod1:TeNT-EGFP) larvae, we crossed to either Tg(sox10:TagRFP-T) or Tg(mbp:TagRFP-T) reporter lines. Images were collected using the PerkinElmer spinning disk confocal microscope. Confocal stacks at multiple segments were acquired and tiled to generate a large field spanning 1 mm along the anterior-posterior axis. Cell counts were performed within Volocity software.

Time-lapse imaging. All fluorescence time-lapse imaging was performed on the spinning disk confocal system equipped with a heated stage chamber to maintain larvae at 28.5 °C. Larvae were paralyzed by immersion in 0.5 mg/ml α-bungarotoxin (EMD) in 10% Hank’s solution, aided by a small incision in the caudal-most tail epidermis with a fine tungsten needle. After 10-min incubation in α-bungarotoxin, larvae recovered in egg water without anesthetic for at least 1 h. Paralyzed larvae were then embedded in 1% low-melt agarose, without tricaine, and immersed in egg water without tricaine. For time-lapse imaging of initial ensheathment, we collected images at 2.5-min intervals for 4 h. Analysis of sheath initiation was performed by a blind observer. Nascent sheaths were defined morphologically by identifying two parallel sox10mRFP+ membrane processes extending longitudinally along the anterior–posterior axis with lengths exceeding 1 µm. We acquired images at 20-min intervals for retraction assays. When time-lapse durations exceeded 18 h, we recovered larva to egg water without tricaine, then re-embedded in low-melt agarose immediately before the next time point. Time-lapse videos were exported from Volocity as extended z-projection TIFF images. We used ImageJ to rotate, crop and translate in order to correct for x-y drift. Image stacks were then exported in QuickTime (.mov) format using Sorenson 3 compression.

Swim behavior in veratridine-treated larvae. To assess behavioral phenotypes we used a standard touch assay on 3-dpf larvae. A pin tool was used to induce the touch response and any larva with movement persisting greater than 5 s and terminating in seizure-like activity was scored as positive. We collected representative time-lapse videos using a Samsung Galaxy S3 digital camera with 8 megapixel and 30 frames per second acquisition settings. Videos were exported in .mov format in QuickTime Pro 7 using H.264 compression.
Optogenetic stimulation and analysis. We crossed Tg(phox2b:GAL4; myl7: Cerulean); Tg(sox10:mRFP) with Tg(UAS:ChR2R-EGFP)/js3 adults. At 72 hpf, embryos positive for EGFP+ axons and sox10:mRFP were split into control and treated groups. Blue (473 nm, 20 Hz) light was delivered using a Blues 50 laser (473 nm, 3 mW/cm², Cobolt Inc., San Jose, CA, USA) controlled by a Uniblitz shutter (Vincent Associates, Rochester, NY, USA) and LabView software v. 2012 (National Instruments, Austin, TX, USA). We applied 30-s pulses (20 Hz) every 2 min for a total of 3 h. To assess selection of phox2b+ axons we performed confocal microscopy 24 h after stimulation. Images were acquired at the somite position corresponding to where axon wrapping was initiating at the time of treatment (as determined by confocal microscopy).

Electron microscopy. To measure axon size, we fixed 3-dpf Tg(neurod1:TeNT-EGFP) larvae and siblings from two clutches in 2% paraformaldehyde, 2% glutaraldehyde and 0.1 M sodium cacodylate. Microwave stimulation to accelerate fixation of tissue was performed with a Biowave Pro Laboratory Microwave with ColdSpot (Ted Pella, Inc., Redding, CA, USA) at 15 °C. Membranes were enhanced using secondary fixation with OsO₄. We collected the electron micrographs using a FEI Technai G2 BioTwin microscope and images were cropped and contrast-adjusted in Adobe Photoshop. We analyzed 4 or 5 cross-sections of tissue between somites 12 and 14 for each of 3 larvae per condition. On each cross-section, an unbiased observer randomly selected 5 to 10 axons ventral to each Mauthner axon (within a consistent area of 27 µm²) for a total of 204 sib axons and 185 axons from Tg(neurod1:TeNT-EGFP) embryos. Axons were chosen without regard to myelination and were required to have a clearly defined perimeter. The axon area was measured in ImageJ and the axon diameter was calculated from the area value.

Statistical analyses. We plotted all data and performed all statistical analyses in GraphPad Prism software (v6). All data are expressed as mean ± s.e.m. For statistical analysis, we first performed the D’Agostino and Pearson omnibus normality test to address normality. We used Student’s two-tailed t-test for all data with normal distributions. For all other data, we assessed statistical significance using the nonparametric Mann-Whitney test. Statistical significance is indicated as follows: *P < 0.05, **P < 0.01, ***P < 0.001. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications7,9,25.

A Supplementary Methods Checklist is available.

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