Synaptic vesicle release regulates myelinated axon number of individual oligodendrocytes in vivo

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The myelination of axons by oligodendrocytes markedly affects CNS function, but how this is regulated by neuronal activity in vivo is not known. We found that blocking synaptic vesicle release impaired CNS myelination by reducing the number of myelinated sheaths produced by individual oligodendrocytes during their short period of formation. We also found that stimulating neuronal activity increased myelinated sheath formation by individual oligodendrocytes. These data indicate that neuronal activity regulates the myelinating capacity of single oligodendrocytes.

CNS myelination continues into adulthood and previous studies have suggested that neuronal activity may regulate myelination (for example, see refs. 4–6) and, in turn, nervous system plasticity⁷,⁸. Studies in humans have shown that the execution of specific tasks can promote the growth of white matter in relevant brain areas⁹. Furthermore, rodent models have revealed that the social environment influences the amount of myelin made by individual oligodendrocytes¹⁰ and that learning specific tasks stimulates and requires the generation of new myelinating oligodendrocytes¹¹. However, how neuronal activity might regulate the myelinating capacity of individual oligodendrocytes in vivo remains unclear. We have previously shown that the presence of supernumerary axons in the zebrafish spinal cord can stimulate individual oligodendrocytes to generate more myelin sheaths than they would otherwise, indicating that axons can regulate the myelinating potential of individual oligodendrocytes in vivo¹². Here we tested the possibility that neuronal activity might regulate myelinated sheath generation by individual oligodendrocytes during their previously characterized short period of sheath formation in vivo¹³,¹⁴.

Using zebrafish as a model organism, we used tetanus toxin (TeNT) to abrogate synaptic vesicle release in vivo¹⁵. Electrophysiology confirmed that synaptic activity was reduced and brightfield analyses of whole embryos showed that general morphological development was not disrupted by TeNT expression (Online Methods, Fig. 1a and Supplementary Figs. 1 and 2). To visualize myelination in live animals, we used the Tg(mbp:mCherry-CAAX) and Tg(mbp:EGFP-CAAX) fish lines, and found that, although the onset of myelination was not delayed, there was a reduction in the amount of myelin in TeNT-expressing animals (Fig. 1b and Supplementary Fig. 2). Using transmission electron microscopy (TEM), we observed a 39% decrease

Figure 1 Synaptic vesicle release regulates myelinated axon number. (a) Lateral view of control (left) and TeNT-expressing (right) zebrafish at 3 dpf. Boxes indicate areas in b. Scale bar represents 250 µm. (b) Lateral view of Tg(mbp:mCherry-CAAX) spinal cords in controls (left) and TeNT-expressing animals (right). Scale bar represents 25 µm. White lines indicate the areas of ventral spinal cord illustrated in c. (c) Transmission electron micrographs of control (left) and TeNT-expressing (right) animals at 4 dpf. Scale bar represents 2 µm. Unmyelinated axons ≥0.3 µm in diameter are indicated in turquoise. (d) Number of myelinated and unmyelinated axons ≥0.3 µm in diameter in control and TeNT-expressing semi-spinal cords (Student’s two-tailed t-tests; myelinated axon number, P = 0.0017; unmyelinated axon number, P = 0.0005; total axon ≥0.3 µm number, P = 0.4; control, n = 5; TeNT, n = 5). (e) Axon diameter distribution of axons between 0.3 and 1.9 µm (control, n = 5; TeNT, n = 5). **P < 0.01, ***P < 0.001. Error bars represent s.d.

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have implicated neuronal activity in the regulation of oligodendrocyte number.\textsuperscript{6,16} Using Tg(mbp:EGFP) fish, we found a small (10%) reduction in the number of myelinating oligodendrocytes by 4 dpf in TeNT-expressing animals (49 ± 9 control versus 44 ± 9 TeNT; Supplementary Fig. 4). To determine whether this reduction reflected changes in oligodendrocyte precursor cell (OPC) proliferation, survival or differentiation, we carried out time-course and time-lapse analyses from OPC specification onwards (Online Methods). Notably, we found a 10% decrease in cell number from the earliest appearance of OPCs, suggesting that the reduction in cell number results from disruption to specification in the precursor motor neuron (pMN) domain (Supplementary Fig. 5). Indeed, a role for neurotransmitters in modulation of pMN domain behavior in zebrafish has recently been demonstrated with respect to the generation of motor neurons.\textsuperscript{17} Time-lapse analyses revealed no differences in later OPC proliferation or survival in TeNT-expressing animals (Supplementary Fig. 5 and Supplementary Movies 1 and 2, and data not shown). It should be noted that the rates of OPC proliferation and cell death were low in zebrafish at the stages that we examined (Supplementary Fig. 5 and Supplementary Movies 1 and 2). Thus, the modest phenotype in cell number that we observed may reflect a difference in scale, rather than principle, between species.

Given that the 10% reduction in oligodendrocyte number does not account for the 40% reduction in myelinated axons that we observed at 4 dpf, we reasoned that individual oligodendrocytes in TeNT-expressing animals must either have fewer myelin sheaths or substantially shorter sheaths. Thus, we next assessed individual oligodendrocyte morphology using mbp:mCherry-CAAX. We found that the average number of myelin sheaths per oligodendrocyte at 4 dpf was reduced by 30% in TeNT-expressing animals (11.9 ± 3.1 sheaths per cell control versus 8.2 ± 2.9 TeNT; Fig. 2a,b). Of the myelin sheaths that did form in the global absence of synaptic vesicle release, we did not observe a decrease in their length at 4 dpf (Supplementary Fig. 6). To determine whether the reduction in myelin sheath number per cell was a result of a defect in their initial formation or an increase in retraction, we performed time-lapse imaging of individual oligodendrocytes using Tg(nkx2.2a:mEGFP) fish, as previously described;\textsuperscript{13} here we define myelin sheaths as elongate structures with characteristic ensheathing profiles >5 μm in length that are stable for >10 min. By the end of the characteristic short period of myelin sheath generation, which was unchanged in TeNT-expressing animals (Fig. 2c–e), we found that individual oligodendrocytes had generated 30% fewer myelin sheaths (9.5 ± 2.0 control versus 6.7 ± 2.3 TeNT) in TeNT-expressing animals; no difference was observed in the number of myelin sheath rejections (3.1 ± 1.2 control versus 3.5 ± 1.2 TeNT; Fig. 2f–i and Supplementary Movies 3 and 4). Together, the 10% reduction in oligodendrocyte number and the 30% decrease in myelin sheath number per cell accounted for the 40% reduction in myelinated axon number seen by TEM.

To exclude the possibility that the effect on myelin sheath generation is a result of the function of TeNT in oligodendrocytes,\textsuperscript{18,19} we created genetic chimeras (Online Methods). When TeNT-expressing oligodendrocytes were placed into a control axonal environment, we found that they generated a normal number of myelin sheaths (average of 12 ± 2.7 per cell; Supplementary Fig. 7). In contrast, when we analyzed control oligodendrocytes in TeNT-expressing animals with reduced synaptic vesicle release, we observed reduced myelin sheath number (average of 8.6 ± 3.3 per cell; Supplementary Fig. 7). These analyses indicate that TeNT does not function in oligodendrocytes to regulate myelin sheath formation. To demonstrate that disruption of synaptic vesicle release specifically in neurons regulates myelin sheath formation, we drove expression of TeNT in individual oligodendrocytes to regulate myelin sheath formation. To demonstrate that disruption of synaptic vesicle release specifically in neurons regulates myelin sheath formation, we drove expression of TeNT in individual oligodendrocytes to regulate myelin sheath formation. To demonstrate that disruption of synaptic vesicle release specifically in neurons regulates myelin sheath formation, we drove expression of TeNT in individual oligodendrocytes to regulate myelin sheath formation. To demonstrate that disruption of synaptic vesicle release specifically in neurons regulates myelin sheath formation, we drove expression of TeNT in individual oligodendrocytes to regulate myelin sheath formation.
Brief Communications

Figure 3 PTZ increases myelin sheath number per oligodendrocyte in a synaptic vesicle–dependent manner. (a) Individual oligodendrocytes in the ventral spinal cord labeled by mbp:mCherry-CAAX in control (top), PTZ-treated (middle) and PTZ + TeNT–treated (bottom) animals at 4 dpf. Scale bar represents 10 µm. (b) Myelin sheath number per cell (oligodendrocytes in ventral spinal cord) (one-way ANOVA, PTZ versus PTZ+TeNT, cells in 14 animals; PTZ + TeNT, where reticulospinal axons are located (45–630 increase in the number of oligodendrocytes in the ventral spinal cord, bar represents 10 µm. (b) Myelin sheath number per cell (oligodendrocytes in ventral spinal cord) (one-way ANOVA, PTZ versus PTZ+TeNT, cells in 14 animals; PTZ + TeNT, n = 46 cells in 29 animals). ****P < 0.0001. Error bars represent s.d.

Note added in proof: Please see related paper by Hines et al.21 in this issue.

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
S.M. and D.A.L. designed the experiments and prepared the manuscript. S.M., M.B. and R.A. carried out the experiments. T.C. generated transgenic lines. J.A. and A.E.M. conducted and analyzed the electrophysiological recordings. D.A.L. supervised the project.

Competing Financial Interests
The authors declare no competing financial interests.

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Embryos were embedded in 1.3–1.5% low npg
lower dose being paralyzed (data not shown). Therefore 1 nl of 100 ng consistent blockade of synaptic transmission, despite those injected with the potassium gluconate, 5 mM KCl, 10 mM HEPES, 4 mM ATP-Mg2+, 0.3 mM cytes fertilized eggs were injected with 10 ng synaptic vesicle release, fertilized eggs were injected with 1 nl of either 10, 50 or Single oligodendrocyte labeling.
for simplicity we refer to TeNT treatment throughout.
used throughout all experiments. Although encoding a EGFP fusion protein, lines. As controls, we injected UAS:T dT omato into Tg(HuC:Gal4), Tg(mbp:EGFP-CAAX) double transgenic expression and analysis of tent in individual reticulospinal neurons.
To generate genetic chimeras, we carried out cell transplantation at blastula stages. We transplanted cells from control to control, and from control to TeNT-expressing animals. Donor embryos were labeled with either 0.2% Oregon Green Dextran (wt/vol) or 0.2% Cascade Blue Dextran (wt/vol). For control to control and TeNT to control chimeras, donor embryos were Tg(mbp:EGFP-CAAX) to be able to easily quantify myelin sheath number per cell in the host. For control to TeNT chimeras, the donor embryos were Tg(sox10:mRFP) to be able to easily quantify myelin sheath number in the host TeNT animal, which expresses a low level of EGFP because of the TeNT-LC:EGFP fusion protein. Quantification of myelin sheath number was performed while blinded to experimental group.
Expression and analysis of TeNT in individual reticulospinal neurons. To express TeNT in individual neurons, we injected UAS:TeNT-TdTomato (kind gift of M. Meyer) into Tg(Huc:Gal4). We generated a mbp:mCherry-CAAX transgenic construct, we recombined pSE_mbp12 with pME_mCherry-CAAX, p3E_pA, and pDesTol2pa22 (all from Tol2Kit26).
Calibration of tetanus toxin concentration using electrophysiology. To inhibit synaptic vesicle release, fertilized eggs were injected with 1 nl of either 10, 50 or 100 ng µl−1 of mRNA encoding EGFP and the tetanus toxin light chain (tent-lc:EGFP) at the 1–4 cell stage25. Controls were injected at the same developmental stage with 1 nl of nuclease free water. All embryos were manually dechorionated at 1 dpf.
4 dpf fish were anesthetized in 0.03% Tricaine (wt/vol, MS-222, Sigma) in extracellular solution and then pinned down on the side using tungsten pins placed through the notochord in a Sylgard-lined recording chamber. The fish were paralyzed with 6.25 µM tr-bungarotoxin (Sigma-Aldrich) for 10 minutes and muscles were removed over one segment to record from spinal cord neurons. Patch-clamp electrodes were pulled from borosilicate glass (1.5-mm outer diameter, 0.87-mm inner diameter, Hilgenberg). The intracellular solution (120 mM potassium gluconate, 5 mM KCl, 10 mM HEPES, 4 mM ATP-Mg2+, 0.3 mM GTP-Na+, 10 mM Na+−phosphocreatine, pH 7.4 with KOH, 275 mOs), yielding resistances of 8–12 MΩ. Synaptic activity was induced in spinal neurons by electrical stimulation using a glass electrode placed at the level of the otic vesicle. The synaptic activity of spinal cord neurons was examined in current clamp and in voltage clamp. Inward excitatory currents were recorded in neurons held at −65 mV, which corresponds to the reversal potential of chloride-mediated inhibition. Outward inhibitory currents were recorded in neurons held at 0 mV, which corresponds to the reversal potential of excitation.
Only animals injected with 100 ng µl−1 tent-lc:EGFP mRNA exhibited consistent blockade of synaptic transmission, despite those injected with the lower dose being paralyzed (data not shown). Therefore 1 nl of 100 ng µl−1 was used throughout all experiments. Although encoding a EGFP fusion protein, for simplicity we refer to TeNT treatment throughout.
Live imaging and data analysis. Embryos were embedded in 1.3–1.5% low melting point agarose (Invitrogen) in embryo medium with MS-222 (tricaine methane-sulfonate, Sigma-Aldrich). All fluorescent live images and time-lapse movies represent a lateral view of the spinal cord, anterior to the left and dorsal on top. Confocal images and time-lapses were acquired using Zeiss LSM 710 and 780 confocal microscopes. During time lapses the agarose embedded fish were staged on a Tempcontrol 37 temperature controlled microscope stage at 28 °C to keep the fish under optimal conditions. The time-lapse movies were acquired at time intervals of 10 min between each frame.
Images of individual cells in the figure panels are labeled by mbp:mCherry-CAAX, whereas images of stable transgenic lines are labeled by the “Tg” designation indicating a transgenic line, for example, Tg(mbp:mCherry-CAAX). All time-lapse analysis and color coding in the figure panels was carried out using ImageJ and Adobe Photoshop CS3.
Statistical analyses. All data are expressed as a mean ± s.d. or as relative proportions of 100% as indicated in the appropriate legends. For statistical analyses, either a Student’s two-tailed t test, a one-way or two-way ANOVA was used, as indicated in the figure legends. All graphs and statistical tests were carried out using either GraphPad Prism 5 or Microsoft Excel 2010. Samples in our data sets were not randomized and the analysis was not blinded unless otherwise stated above. Data distribution was assumed to be normal, but this was not formally tested.
Sample size was determined by power analysis. 100% power was achieved in the quantification of myelin sheaths per oligodendrocytes in Supplementary Figure 8b. 100% power was achieved in the quantification of myelin sheaths per oligodendrocyte in Supplementary Figure 9e. 99% power was achieved in the quantification of oligodendrocytes in the ventral spinal cord shown in Supplementary Figure 9g. 100% power was achieved in the quantification of myelin sheaths along single axons was performed whilst blinded to experimental group.

Pentylenetetrazole (PTZ) treatment. PTZ is a well-characterized GABA-A receptor antagonist28. Previous studies using zebrafish have shown that PTZ can induce increases in neuronal activity and exacerbated response to touch stimuli at concentrations up to 2.5 mM and can induce seizures at higher concentrations29. Previous studies have also indicated that reticulospinal neurons receive significant inhibitory GABAergic input20,31 making PTZ a strong candidate to induce a gain of function in reticulospinal axons that could in turn regulate oligodendrocyte behavior. We next reasoned that it would be important to induce a gain of function throughout a period that would encompass all stages of oligodendrocyte development from specification from the pMN domain, migration to axonal tracts, proliferation, survival, differentiation and ultimately myelination (from 2 dpf through 4 dpf). We found that zebrafish tolerated long-term treatment with PTZ up to 2.5 mM without substantial deleterious effects to morphological development or maturation, or any evidence of seizing. We selected morphologically normal and healthy animals for cellular analyses. To confirm that animals treated with 2 mM PTZ exhibited an exaggerated response to touch as previously indicated, we quantified the swim distance swim in animals responding to touch using Noldus Ethovision XT and ImageJ. Quantification of oligodendrocyte number in control and PTZ treated Tg(mbp:EGFP) animals as well as the quantification of myelin sheath number in single mbp:mCherry-caax labeled oligodendrocytes in control, PTZ, PTZ and TeNT-treated animals was performed while blinded to the treatment group.

TEM. Tissue was prepared for TEM as previously described32 and sectioned using a Reichert Jung Ultracut Microtome. TEM images were taken with a Phillips CM120 Biotwin TEM. Image analysis was performed using ImageJ and Adobe Photoshop CS3.

Online Methods

Fish husbandry. All animals used in this study were maintained under standard conditions and all experiments performed according to the British Home Office regulations. The following already published transgenic zebrafish lines were used: Tg(nkx2.2a:mEGFP)22,23, Tg(sox10:mRFP)22, Tg(olig2:EGFP)24, Tg(mbp:EGFP-CAAX) and Tg(mbp:EGFP)25, and Tg(mnt1b:skb:mCherry)23, Tg(mbp:mCherry-CAAX) and Tg(Huc:Gal4) was generated for the purpose of this study by screening founders derived following co-injection of 1 nl of 10 ng µl−1 plasmid DNA encoding mbp:mCherry-CAAX and Huc:Gal4 respectively with 25 ng µl−1 tolv2 transposable mRNA.

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time-lapse analysis shown in Figure 2f. 80% power was achieved in the number of oligodendrocytes shown in Supplementary Figure 4b.

A Supplementary Methods Checklist is available.

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