Regulation of developing myelin sheath elongation by oligodendrocyte calcium transients in vivo

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How action potentials regulate myelination by oligodendrocytes is uncertain. We show that neuronal activity raises [Ca\textsuperscript{2+}], in developing oligodendrocytes in vivo and that myelin sheath elongation is promoted by a high frequency of [Ca\textsuperscript{2+}], transients and prevented by [Ca\textsuperscript{2+}], buffering. Sheath elongation occurs ~1h after [Ca\textsuperscript{2+}], elevation. Sheath shortening is associated with a low frequency of [Ca\textsuperscript{2+}], transients but with longer duration [Ca\textsuperscript{2+}], bursts. Thus, [Ca\textsuperscript{2+}], controls myelin sheath development.

Neuronal action potentials may regulate myelination, both during development1–3 and during white matter plasticity in adulthood4–6. Possible mediators of neuronal activity include exocytosis7–9 of glutamate10,11 or GABA11, at synapses between unmyelinated axons and oligodendrocyte precursor cells2, or release of growth factors13,14. Within developing oligodendrocytes, these agents may alter the concentration of calcium1.

To investigate whether [Ca\textsuperscript{2+}], regulates myelination in vivo, we studied developing oligodendrocytes in the spinal cords of zebrafish (immobilized in agarose at 3 d after fertilization). Spinal cords were imaged using membrane-associated fluorescent proteins encoded by tg(sox10:mRFP) or tg(mbp:mCherry-CAAX) transgenes to reveal the structures of developing oligodendrocytes and by tg(sox10:GCaMP6s) transgene to monitor [Ca\textsuperscript{2+}]. In some animals, somatic silencing of the tg(sox10:mRFP) transgene in most cells, mediated by CRISPR/Cas9 genome editing, enabled observation of single cells developing. We classified cells into three developmental stages. Premyelinating oligodendrocytes (pre-OLs; Fig. 1a) showed many processes, none of which had made a sheath. Since tg(sox10:mRFP) can be expressed in interneurons, the identity of these cells was confirmed by observing them until they formed myelin sheaths. Early oligodendrocytes (early OLs; Fig. 1b) had started to make sheaths. Oligodendrocytes (OLs; Fig. 1c) had initiated formation of all the sheaths that would be produced (as confirmed by subsequent observation), placing them past the 5 h window15 of sheath initiation, although a small fraction of sheaths could still shorten and disappear in these cells16.

Cells at all stages exhibited [Ca\textsuperscript{2+}], transients (quantified in Methods) in their processes and somata (Fig. 1d–f; interactions between [Ca\textsuperscript{2+}], transients in different locations are presented in Supplementary Fig. 1). The overall transient rate declined with development and was similar (Fig. 1g) in single processes (where transients may regulate local cytoskeletal events and/or protein and lipid synthesis) and in somata (where they may regulate gene expression). Because there are many processes per cell (10.3 ± 0.6 processes analyzed in pre-OLs, 11.9 ± 0.6 in early OLs and 6.9 ± 0.6 in OLs), most transients occurred in processes rather than in somata. However, [Ca\textsuperscript{2+}], transients were more than twofold longer in the somata (Fig. 1h). The amplitude of transients (assessed as ΔF/F, where F is fluorescence of the genetically encoded calcium indicator GCaMP6s) declined in processes over the course of development and was smaller in somata than in processes throughout development (Fig. 1i).

To determine whether neuronal activity generated oligodendrocyte [Ca\textsuperscript{2+}], transients, we stimulated action potentials electrically or blocked them by injecting tetrodotoxin (TTX) (or extracellular solution as a sham injection) into the hindbrain, from where it entered the spinal cord (Fig. 2a–c). Stimulated action potentials were detected as extracellular field potentials, which were stable during sham injections but abolished by TTX (Fig. 2c). Averaging responses to 10-s durations 50-Hz stimulation trains revealed that stimulation significantly increased [Ca\textsuperscript{2+}], both in processes that had not yet made a sheath (‘nonsheath’, part of pre-OLs or early OLs; Fig. 2d) and in sheaths of processes that were already myelinating (‘sheath’, part of early OLs or OLs; Fig. 2e). The [Ca\textsuperscript{2+}], transient amplitude was larger in nonsheath than in sheath processes (Fig. 2d,e). Stimulation increased the frequency of [Ca\textsuperscript{2+}] elevations (Fig. 2f,g) but by less than the 0.6 per min (one per 100 s) increase expected if every stimulation train evoked a [Ca\textsuperscript{2+}] rise. Neuronal stimulation also evoked [Ca\textsuperscript{2+}], rises in somata of cells with processes that had not yet made a sheath, but not in somata of cells that already had a sheath-forming process (Fig. 2h–k), implying that at this later developmental stage, neuronal activity releases factors that raise [Ca\textsuperscript{2+}], in developing sheaths but not in somata.

Injecting extracellular solution had no effect on ongoing [Ca\textsuperscript{2+}], transients in the processes and somata of oligodendrocyte-lineage cells, but TTX injection roughly halved the transient rate in processes (Fig. 2l,m). TTX produced a similar reduction of transient rate in somata of cells with nonsheath processes (Fig. 2n,o). Thus, approximately half of the [Ca\textsuperscript{2+}], transients in developing oligodendrocytes are driven by axonal action potentials, but the remainder occur independently of action potentials.

To determine whether myelin sheath [Ca\textsuperscript{2+}], transients regulate sheath growth, we imaged growing sheaths in OLs for 10.5 min every hour for 5 h (Fig. 3a,b). This allowed measurement of the [Ca\textsuperscript{2+}], transient frequency without excessive fluorophore bleaching or phototoxicity while imaging structural changes over an extended period. Over 5 h, the lengths of myelin sheaths increased (44% of 68 sheaths studied), decreased (26%), showed no change (24%) or increased and then decreased (6%; Fig. 3c–e). Plotting the total change of sheath length against the mean [Ca\textsuperscript{2+}], transient rate in that sheath over 5 h revealed that sheath elongation correlated strongly with [Ca\textsuperscript{2+}], transient rate.
Calcium transient properties change during oligodendrocyte development. a–c. Oligodendrocyte morphology at different developmental stages (3 cells from different CRISPR/Cas9-injected tgsx10:mRFP fish for each stage): (a) pre-OLs, before sheaths have been initiated; (b) early OLs, when some sheaths have been initiated; (c) OLs, when all sheaths have been initiated. d–f. Specimen [Ca\textsuperscript{2+}] \textsuperscript{i} traces for three developmental stages showing (top) sequentially recorded traces for the somata of 6 cells and (bottom) simultaneously recorded traces in different processes of a single cell of each class. A [Ca\textsuperscript{2+}] \textsuperscript{i} rise (red triangle) occurs simultaneously in four processes in d–f. Rate, duration and amplitude of the transients at the three developmental stages (n = 10 pre-OLs, 8 early OLs and 8 OLs from 5, 6 and 6 animals respectively). P values are from two-tailed tests: one-way ANOVA followed by Holm–Bonferroni-corrected t tests in g for ‘all processes’ and in h and i for ‘processes’; Kruskal–Wallis followed by Dunn’s test in g for per-process and for somata and in h, i for somata; two-way ANOVA followed by Holm-Sidak test in h, i for somata vs. processes. Data are mean ± s.e.m.

(Fig. 3f; regression slope is different from zero, P = 10\textsuperscript{-12}). However, for a [Ca\textsuperscript{2+}] \textsuperscript{i}, transient rate lower than ~1 per 30 min, sheath retraction occurred. Thus, a threshold rate of [Ca\textsuperscript{2+}] \textsuperscript{i} transients may be needed to maintain sheath length at this developmental stage.

Similar plots showed that sheath elongation also correlated weakly but significantly (Supplementary Fig. 2a–c) with sheath [Ca\textsuperscript{2+}] \textsuperscript{i}, transient amplitude, duration and area (∫[ΔF/F].dt), but not with the somatic [Ca\textsuperscript{2+}] \textsuperscript{i}, transient rate (Supplementary Fig. 2d). Thus, sheath development is correlated with local [Ca\textsuperscript{2+}] \textsuperscript{i}, changes but not with somatic changes that might alter gene expression. To assess the temporal relationship between [Ca\textsuperscript{2+}] \textsuperscript{i}, elevations and sheath length, we selected sheaths that showed no growth for at least 1 h (Fig. 3c) and aligned their time courses of sheath elongation and of [Ca\textsuperscript{2+}] \textsuperscript{i}, transient rate at the onset of growth (Supplementary Fig. 2e). Plotting sheath growth per hour against mean [Ca\textsuperscript{2+}] \textsuperscript{i}, transient rate (assessed as the average of the rates in imaging periods bracketing that hour) suggested that an increase in transient rate triggers sheath elongation after ~1 h (Fig. 3g).

To demonstrate that the [Ca\textsuperscript{2+}] \textsuperscript{i} transients, including those evoked by neuronal activity (Fig. 2), mechanistically drive sheath elongation (rather than merely being correlated with elongation), we first modulated neuronal activity and hence the [Ca\textsuperscript{2+}] \textsuperscript{i}.
Fig. 2 | Neuronal activity evokes half the calcium transients in developing oligodendrocytes. a, Stimulating the spinal cord rostral to the imaged area and recording evoked activity caudally. In some experiments TTX or sham injections were made into the hindbrain; 15 min was allowed for diffusion to the imaged area. b, Stimulation: 50-Hz pulses for 10 s, repeated seven times every 100 s. c, Field potential recording (at 1 Hz) before and after TTX (9 animals) or sham solution (8 animals) injection showed abolition by TTX but not sham. Calcium imaging was performed before and after the injection (red vertical lines). d–e, [Ca\(^{2+}\)] traces (mean ± s.e.m. in gray) averaged over seven periods without or with stimulation (red rectangles) in d 18 nonsheath-forming or e 45 sheath-forming processes. f–g, Mean ± s.e.m. (points are individual processes) rates of [Ca\(^{2+}\)] transients without and with stimulation in d nonsheath-forming or g sheath-forming processes. h–k, As in d–g but for somata (n = 9 each for j and k). Experiments in d–k are from 5 animals. l–m, Rate of [Ca\(^{2+}\)] transients before and after sham or TTX injections in l nonsheath-forming (n = 27 and 32 processes for sham and TTX respectively) or m sheath-forming processes (n = 59 and 61 processes for sham and TTX). n–o, As in l–m but for somata (n = 18 and 19 somata for sham and TTX in n; n = 25 and 27 somata for sham and TTX in o). Numbers of animals in l–o were as in c. Statistical tests used (all two-tailed): Wilcoxon signed-rank test in f–g; paired t test in j–k; repeated-measures ANOVA followed by Holm–Sidak for l–o. Data are mean ± s.e.m.
by increasing neuronal activity with stimulation or by decreasing it with TTX (Fig. 3h). Stimulation increased sheath elongation by 60% (Fig. 3i), while TTX reduced elongation by 43% (Fig. 3j). Second, we manipulated the [Ca\(^{2+}\)]\(i\), changes occurring within growing myelin sheaths by briefly whole-cell patch-clamping developing oligodendrocytes to introduce into them solutions of low (1 mM EGTA) or high (30 mM BAPTA) [Ca\(^{2+}\)]\(i\) buffering power (with the same free [Ca\(^{2+}\)]\(i\): -63 nM; see Methods). Sheaths dialyzed with the weak calcium buffer continued to elongate over 3 h, while those dialyzed with the strong calcium buffer on average shortened (significantly different; P = 2 × 10\(^{-4}\); Fig. 3k). Thus, myelin sheath elongation is driven by [Ca\(^{2+}\)]\(i\), transients, including those evoked by neuronal activity.

In addition to the temporally isolated [Ca\(^{2+}\)]\(i\), transients associated with sheath lengthening, we observed a longer type of [Ca\(^{2+}\)]\(i\) bursts (Fig. 1l) exhibiting repeated rises and falls of [Ca\(^{2+}\)]\(i\) (duration: 87.5 ± 15.8 s (mean ± s.e.m.), n = 7; mean ∆F/F: 0.52 ± 0.09). These were rare but, strikingly, occurred only in sheaths that were shortening (or transitioning from growing to shortening). Consequently, isolated transients and bursts were inversely correlated in their association with sheath behavior (Fig. 3i). On average, [Ca\(^{2+}\)]\(i\) bursts contributed only 7.8 ± 2.8% of the Ca\(^{2+}\) entry (calculated as \(\int|\Delta F/F|\) dt) in all sheaths; however, this was 0% in lengthening sheaths and 75.6 ± 2.2% in shortening sheaths with bursts. Dialysis with the BAPTA-containing internal solution would be expected to reduce [Ca\(^{2+}\)]\(i\), during these events, so that sheaths shortened in this condition (Fig. 3k) suggests that a BAPTA-induced reduction of magnitude of the brief [Ca\(^{2+}\)]\(i\) transients dominated the effect on sheath length or that the large Ca\(^{2+}\) entry occurring during bursts overwhelmed the BAPTA buffer.

Our data show that, like astrocytes\(^{16}\), developing oligodendrocytes in vivo exhibit spatially restricted [Ca\(^{2+}\)]\(i\), elevations. At early developmental stages, these can occur simultaneously in many cell processes (possibly due to simultaneous neuronal input), but as myelination proceeds they become independent in different growing sheaths (Fig. 1 and Supplementary Fig. 1). Approximately half of these transients are evoked by action potentials, as they are blocked by TTX and their rate is increased by electrical stimulation (Fig. 2). They may be generated by synaptic input from unmyelinated axons\(^{12}\), although this is downregulated as oligodendrocytes mature\(^{17}\). The remainder of the transients appear to be generated spontaneously, perhaps by TRPA1 channels\(^{18}\) in oligodendrocyte sheaths\(^{14}\). Conceivably, since [Ca\(^{2+}\)]\(i\), transients promote sheath growth, these spontaneous and activity-driven transients may underlie the activity-independent and activity-dependent forms of myelination reported previously\(^{19}\).

Lengthening of the myelin sheath is driven by the rate of [Ca\(^{2+}\)]\(i\), transients occurring (Fig. 3) and, below a certain rate, sheaths shorten, so a minimum [Ca\(^{2+}\)]\(i\), transient rate may be needed to maintain sheath length. However, long-duration [Ca\(^{2+}\)]\(i\) bursts imparting a high calcium load were also associated with shortening. These results suggest that modest [Ca\(^{2+}\)]\(i\) elevations trigger sheath elongation, while very low or excessively large rises lead to sheath shortening. The [Ca\(^{2+}\)]\(i\), transients presumably control proteins regulating cytoskeletal growth and myelin assembly. A likely effector producing sheath elongation is the PI3K–Akt–mTOR pathway, which is Ca\(^{2+}\)-activated\(^{20}\) and promotes myelin growth when activated in oligodendrocytes\(^{21}\). Although our observations are in developing oligodendrocytes,
it will be intriguing to determine whether the same is true in established sheaths in adult animals, given that myelin plasticity is increasingly invoked as a mechanism for learning4, as well as during remyelination following stroke, spinal cord injury or multiple sclerosis.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41593-017-0031-y.

Received: 28 August 2017; Accepted: 7 November 2017; Published online: 11 December 2017

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Acknowledgements

We thank I.L. Arancibia-Carcamo, I. Bianco, E. Dreosti, V. Kyrrgryi and W.T. Sherlock for comments, and we thank Fish Facility personnel for care of fish. This work was supported by a Wellcome Trust 4-year PhD studentship (099691/Z/12/Z) to A.M.K., an EU Marie Curie Fellowship (623714) to M.C.F., Wellcome Trust Investigator Awards to D.A. (099222/Z/12/Z) and S.W.W. (104682/Z/14/Z) and an MRC Programme grant (MR/L003775/1) to S.W.W. and G. Gestri.

Author contributions

The work was conceived by A.M.K. and D.A. A.M.K. generated the transgenic lines with help from L.E.V. A.M.K. devised and performed imaging and some electrophysiological experiments with advice from L.E.V. and S.W.W. M.C.F. devised methods for electrical stimulation and whole-cell patch-clamping and performed electrophysiology, whole-cell patch-clamping experiments and some other imaging experiments. A.M.K., M.C.F. and D.A. analyzed the data. S.W.W. and D.A. provided zebrafish, imaging and electrophysiology resources. D.A. and A.M.K. wrote the manuscript, with input from all authors.

Competing interests

The authors declare no competing financial interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41593-017-0031-y.

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Methods

Animals. The following transgenic zebrafish lines were used: tg(sox10:RFP)224 (ref. 21, kindly provided by D. Lyons, Edinburgh) and two lines generated in-house, tg(mbp:mCherry-CAAX)u4011 and tg(sox10:GCaMP6s)w98. The red-fluorescing tg(sox10:RFP5) and (embp:mCherry) lines were used to visualize oligodendrocyte structure early and late in development, respectively, and the green-fluorescing tg(sox10:GCaMP6) line was used to detect [Ca\(^{2+}\)] changes. To visualize the morphology of single cells, CRISPR/Cas9 was used to achieve mosaic expression of the mKFP fluorophore (see below). All experiments were performed in the nonpigmented nacre background (mitfaw2/w2). Embryos were collected by natural spawning and raised at 28.5 °C in fish water. Animal procedures were carried out in accordance with the guidelines of the UK Animals (Scientific Procedures) Act 1986 and European Directive 2010/63/EU. Animals were randomly assigned to different treatments.

Plasmid construction. Constructs were generated with Gateway cloning technology using the clones pSE-mbp5–9; pSE-sox10 (generated by excising the sox10 promoter from p-4.9sox10:eGfp and liigating into pSE-MCS), pME-GCaMP6s (generated in a BP reaction from pGP-CMV-GCaMP6s; Addgene) and components of the Tol2kit: pSE-polA, pME-mCherry-CAAX, pDesiTol2pA2 and pDesiTol2G2. Vectors were recombined using LR Clonase II (ThermoFisher, Invitrogen), Gateway BP Clonase II was used to generate pME-GCaMP6s.

Generation of transgenic zebrafish lines. Fertilized eggs were injected with 1 nl of solution containing 25 ng/μl of plasmid DNA and 100 ng/μl of transposase mRNA. Injected embryos were grown to adulthood and F1 offspring were screened by phenotype (expression of fluorescent protein in the appropriate cells) to identify F0 founders.

CRISPR mosaics. We used CRISPR/Cas9 genome editing to perform somatic inactivation (in most cells) of the mKFP transgene in a stable transgenic fish line (tg(sox10:RFP)224) to allow visualization of single developing oligodendrocytes. Guide RNA design was performed20 with CHOPCHOP (https://chopchop.ucsf.edu/) using the mKFP sequence as input. One-cell to 16-cell stage embryos were injected with 150 pg Cas9 and 30 pg mMrna GRNA (with target sequence GGGAGGCGCCGCGCTACAG), and embryos were selected for imaging based on their mosaic expression of the fluorescent marker.

Live imaging. At 3 d post fertilization (dpf), larvae were mounted in 1% LMP agarose (Sigma, made up in fish water) in 0.5 mg/mL mivacurium chloride (a neuromuscular blocker used to prevent movement; LKT Labs) and imaged at 28–29 °C with a Zeiss LSM780 two-photon/confocal microscope. A confocal stack was taken in an area between segments 8 and 14 of the spinal cord to record the morphology of developing oligodendrocytes (tg(sox10:RFP) or mbp:mCherry-CAAX, excited at 561 nm). In calcium imaging experiments, GCaMP6s was two-photon excited at 940 nm, with an intensity at the preparation of 11 mW and a pixel dwell time of 2.55 μs, using a W Plan-Apochromat 20× objective with NA=1.0. Stacks of five planes, each spaced 2 μm apart, were acquired every 3.5 s for 10.5 min (180 stacks). In Fig. 1 calcium imaging was performed once for each field of view, in Fig. 2 it was performed twice for each field of view (control and stimulation, or control and sham injection) and in Fig. 3a–g it was done six times (every hour for 5 h; see Fig. 3a). The spatial resolution provided by two-photon imaging did not allow us to define the image to the extent to which the sheaths have started compacting at the stage imaged (compaction is a gradual process, starting in the outer wraps, that occurs only as the sheath develops25). This does not affect our conclusions in any way, since the great majority of the sheaths are we are studying are destined to become compacted25 (and only a small fraction of sheaths are removed). Image analysis. Regions of interest (ROIs) were drawn for each nonsheath process (the terminal ~2–5 μm portion of a nonmyelinating process) or sheath.

Electrical stimulation and pharmacology. A stimulation electrode (glass capillary filled with 1 M NaCl) was inserted in the rostral spinal cord. For recording field potentials, a similar electrode (tip resistance 15–35 MΩ inside the spinal cord) was placed in the caudal spinal cord. Stimuli (single pulses applied at 1 Hz, or 50–Hz stimulus trains of 10 s duration) were generated with a GRASS S88 stimulator placed in the caudal spinal cord. Stimuli (single pulses applied at 1 Hz, or 50–Hz stimulus trains of 10 s duration) were generated with a GRASS S88 stimulator triggered with Clamp software (Axon Instruments, Molecular Devices). For these experiments, the fish was surrounded by fish extracellular solution (134 mM NaCl, 10 mM HEPES, 10 mM glucose, 2.9 mM KCl, 2.1 CaCl2, and 1.2 mM MgCl2) rather than fish water, to allow better current flow from the stimulation electrode to the ground electrode. Field potentials (for TTX experiments measured in voltage-clamp mode as currents) were recorded using an Axon Instruments MultiClamp 700B amplifier. Data were digitized at 10 kHz with an Axon Instruments Digidata 1440 (Molecular Devices). Stimulation strength was set to 15–100 V (depending on the electrode resistance) to reliably evoke field potentials. When imaging calcium with and without stimulation (Fig. 2b,d–k), the periods of stimulation and no stimulation (control) were alternated, and [Ca\(^{2+}\)] transient rate was measured in a 40–s time window that included the 10-s stimulation and the 30-s post-stimulation periods (equivalent time windows were analyzed in the control experiments, in which no stimulation was applied). To block action potential propagation in the fish extracellular solution (2.1 mM CaCl2, 1.2 mM MgCl2), 0.24 frames for somata). Results of these analyses, which were carried out in Matlab, were manually checked against the original GCaMP6s recordings to exclude false positives (resulting from increases in background activity or activity in other cell processes). In Fig. 1g the transient rate is shown for somata (averaged over cells), per process (calculated by obtaining the mean rate per process for each cell and then calculating the mean ± s.e.m. by averaging over all cells) and for all processes (calculated by averaging over all cells the product of [mean rate per process for each cell] × (number of processes on the cell)). Duration of transients or bursts (Fig. 1b and Supplementary Fig. 2b) was taken as the time during which they were above the detection threshold. Amplitude (Fig. 1i and Supplementary Fig. 2a) was taken as the magnitude of ΔF/F at the peak of the response relative to the time-smoothed baseline. The transient area (Supplementary Fig. 2c) was taken as the sum of (i) the area of the transient above the threshold and (ii) the base, which was taken as the duration multiplied by the threshold value (i.e., the area of the transient beyond the threshold). Rarely observed [Ca\(^{2+}\)] bursts, defined as periods of raised [Ca\(^{2+}\)], with frequently alternating subthreshold and suprathreshold values of ΔF/F, with suprathreshold events constituting at least 55% of the frames, and with an overall duration greater than 40 s, were excluded from the analysis of transients for Fig. 3f and were analyzed separately for Fig. 3i. The burst area was calculated as the sum of the areas of each suprathreshold component of the burst plus its base. Mean burst amplitude was calculated as the burst area divided by its duration. The average rate (Fig. 3l), amplitude, duration and area (Supplementary Fig. 2a–c) of calcium transients were calculated using the flanking calcium recordings to obtain average values for each hour and for the total size of those and dividing it by the total duration of the experiment.

Whole-cell patch-clamp loading of calcium buffer into oligodendrocytes in vivo. After immobilization using 0.50–0.55 mg/mL mivacurium chloride dissolved in fish external solution, fish were pinned through the notochord to a Sylgard-coated recording chamber using fine tungsten needles. The skin was removed using fine tungsten tools and fine tungsten aspirating microelectrode. The aorta was retracted carefully to allow the membrane to reseal (monitored in voltage-clamp mode). The control (low calcium buffering power) internal solution contained (in mM) 125 potassium gluconate, 2 NaCl, 0.2 CaCl2, 10 HEPEs, 1.2 mM MgCl2, 10 mM glucose, 2.9 mM KCl, 2.1 CaCl2, and 1.2 mM MgCl2). Directly after zapping, the patch pipette was retracted carefully to allow the membrane to reseal (monitored in voltage-clamp mode). The tissue was allowed to relax for approximately 15 min before imaging. Image stacks (image size, 70.7 μm × 41.4 μm; pixel size, 0.14 μm; z-step size, 1 μm) of the patch-clamp loaded oligodendrocytes were taken every hour for a period of 3 h. The control (low calcium buffering power) internal solution contained (in mM) 125 potassium gluconate, 2 NaCl, 0.2 CaCl2, 10 HEPEs, 1.2 MgCl2, 2 Mg-ATP, 0.5 Na2-EGTA, 10 Na2-phosphocreatine and 0.1 Alexa Fluor 647, with pH set to 7.1 using KOH. The high calcium buffering power internal solution

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contained BAPTA (tetrapotassium salt) and was similar but with the CaCl₂ and EGTA replaced with (in mM) 7 CaCl₂ and 30 BAPTA and with osmolarity differences compensated for by lowering the potassium gluconate concentration. The solution contained (in mM) 63 potassium gluconate, 2 NaCl, 7 CaCl₂, 10 HEPES, 30 K₂ BAPTA, 2 MgCl₂, 2 Mg-ATP, 0.5 Na₂-GTP, 10 Na₂-phosphocreatine and 0.1 Alexa Fluor 647, with pH set to 7.1 using KOH. The free [Ca²⁺] was ~63 nM in both internal solutions, calculated using MaxChelator (http://maxchelator.stanford.edu/webmaxc/webmaxc5.htm) with the temperature set to 28 °C and ion strength set to 140 mM.

The experimenter was blinded as to whether the pipette contained the low or high calcium buffering power solution.

**Statistics.** Data are shown as mean ± s.e.m., with individual data points superimposed. N values used for statistics were of cell processes, sheaths or somata, as appropriate. Normality of data was verified using the D’Agostino & Pearson omnibus test and equality of variance tested using the F statistic. Normally distributed data were analyzed using ANOVA and post hoc t tests or using paired t tests, as appropriate. For t tests, data were compared to interleaved controls using a homoscedastic, two-sided Student’s t test (equal variance), except where the variances of the data were unequal, when a heteroscedastic, two-sided Student’s t test was used. Data were corrected for multiple comparisons within figure panels using a procedure equivalent to the Holm–Bonferroni method (for N comparisons, the most significant P value is multiplied by N, the second-most significant by N − 1, the third-most significant by N − 2, etc.; corrected P values are significant if they are less than 0.05). Non-normally distributed data were analyzed with (two-tailed) Kruskal–Wallis tests followed by Dunn’s multiple comparison tests or with Wilcoxon tests, as appropriate (in GraphPad Prism). Differences between processes and somata in Fig. 1h,i were analyzed with graphPad Prism). Differences between processes and somata in Fig. 1h,i were analyzed with two-way ANOVA followed by Holm–Sidak’s multiple comparison test. Data in Fig. 2i–o were analyzed with two-way repeated-measures ANOVA followed by Holm–Sidak’s multiple comparison test. Comparisons of the slopes of linear regressions with a slope of zero were performed using the t statistic for the regression. Comparison of the numbers of [Ca²⁺] transients or bursts in different process categories was done with χ² tests. Further details of statistical tests are available in Supplementary Table 1.

An estimate of the sample size needed for a typical experiment is as follows. For a control response of 100%, a typical response s.d. of 40%, a response at a different developmental stage of 30% (70% less), a power of 80% and P < 0.05, 7 cell processes are needed (http://www.biomath.info/power/ttest.htm) in each of the two groups. The exact numbers depend on the effect size and standard error of the data.

**Materials.** TTX citrate was obtained from Abcam and mivacurium chloride from LKT Labs. Other chemicals were from Sigma.

**Life Sciences Reporting Summary.** Further information on experimental design is available in the Life Sciences Reporting Summary.

**Data and code availability.** The data that support the findings of this study are available from the corresponding author upon reasonable request. The Matlab code used can be obtained from https://github.com/AttwellLab/MyelinCalcium.

**References**

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Life Sciences Reporting Summary

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### Experimental design

1. **Sample size**
   - Describe how sample size was determined.
   - An estimate of the sample size needed for a typical experiment is as follows: For a control response of 100%, a typical response standard deviation of 40%, a response in a drug of 30% (70% inhibition), a power of 80% and p<0.05, 7 processes are needed (http://www.biomath.info/power/ttest.htm) in each of the control and drug groups. The exact numbers depend on the drug effect size and standard error of the data.

2. **Data exclusions**
   - Describe any data exclusions.
   - No data points were excluded.

3. **Replication**
   - Describe whether the experimental findings were reliably reproduced.
   - We performed a minimum of 5 biological replicas for each experiment (i.e. n numbers were obtained from at least 5 different animals).

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - Animals were randomly allocated to groups. In addition there was:
     - (i) random interleaving of controls and stimulation experiments;
     - (ii) random interleaving of controls and BAPTA experiments.

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - No blinding was done for experiments reported in Fig. 2. Methods, section 'Electrical stimulation and pharmacology'.

   For patch-clamping experiments the experimenter was blinded as to whether the pipette contained the low or high calcium buffer solution (Fig. 3k). Methods, section 'Whole-cell patch-clamp loading of calcium buffer into oligodendrocytes in vivo'.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- [ ] The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- [ ] A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- [ ] A statement indicating how many times each experiment was replicated
- [ ] The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- [ ] A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- [ ] The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
- [ ] A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- [ ] Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

Images were processed using: ImageJ 1.49s, including plug-ins: GECIquant1.0, StackReg, Simple Neurite Tracer
Calcium transients were detected using a custom script written for MATLAB 2015b
Graphs and statistical analysis were carried out using GraphPad Prism 6

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No restrictions

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

No antibodies were used in this study

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

No eukaryotic cell lines were used in this study

b. Describe the method of cell line authentication used.

N/A

c. Report whether the cell lines were tested for mycoplasma contamination.

N/A

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

N/A
11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

| The study was performed on zebrafish larvae aged 2.5-3.5 days post fertilization. |

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

| No human research participants were involved in this study |