Parvalbumin basket cell myelination accumulates axonal mitochondria to internodes

Koen Kole1, Bas J. B. Voesenek1, Maria E. Brinia1,2, Naomi Petersen1 & Maarten H. P. Kole1,3

Parvalbumin-expressing (PV+) basket cells are fast-spiking inhibitory interneurons that exert critical control over local circuit activity and oscillations. PV+ axons are often myelinated, but the electrical and metabolic roles of interneuron myelination remain poorly understood. Here, we developed viral constructs allowing cell type-specific investigation of mitochondria with genetically encoded fluorescent probes. Single-cell reconstructions revealed that mitochondria selectively cluster to myelinated segments of PV+ basket cells, confirmed by analyses of a high-resolution electron microscopy dataset. In contrast to the increased mitochondrial densities in excitatory axons cuprizone-induced demyelination abolished mitochondrial clustering in PV+ axons. Furthermore, with genetic deletion of myelin basic protein the mitochondrial clustering was still observed at internodes wrapped by non-compacted myelin, indicating that compaction is dispensable. Finally, two-photon imaging of action potential-evoked calcium (Ca2+) responses showed that interneuron myelination attenuates both the cytosolic and mitochondrial Ca2+ transients. These findings suggest that oligodendrocyte ensheathment of PV+ axons assembles mitochondria to branch selectively fine-tune metabolic demands.

In the vertebrate central nervous system, oligodendrocytes wrap multilamellar membranes around axons to form myelin sheaths, which reduce local internodal capacitance, speed up action potential propagation and enhance action potential fidelity1,2. Although the myelin sheath strongly facilitates the electrical conductivity of internodes, the insulation also prevents the axolemma from taking up glucose and other metabolites from the extracellular space. Glycolytic oligodendrocytes aid neurons in overcoming this hurdle by providing trophic support to the axon via the myelin sheath in the form of pyruvate and lactate3, and by modulating the function of axonal mitochondria via exosomes4. Mitochondria are versatile organelles that are chiefly committed to the production of adenosine triphosphate (ATP), and in axons require metabolic support from the myelin sheath in order to preserve their integrity5. Previous research, mostly performed in white matter tracts and glutamatergic axons, has shown that demyelination causes dysfunction and shape changes of axonal mitochondria in both multiple sclerosis (MS)7–9 and experimental models10–15. For example, the mitochondrial size and density increase together with electron transport chain protein expression, and mitochondria lose their membrane potential which is critical for ATP synthesis13,14. The accumulation of mitochondria upon demyelination, together with a low mitochondrial content in myelinated axons and vice versa10,19, have given rise to the general notion that myelination reduces the need for mitochondria.

Myelin is also present around inhibitory gamma-aminobutyric acid (GABA)-ergic axons, and in the neocortex is restricted mostly to parvalbumin-expressing (PV+) basket cells20–22. Comprising the most abundant interneuron cell type in the cortex, PV+ basket cells are characterized by a highly complex and extensive axonal organization enabling critical control of the local circuitry and over gamma
These interneurons possess small diameter axons (<0.4 μm, ref. 25) which could make them highly vulnerable to pathology of the myelin sheath.26,27. We recently found that experimental demyelination through toxic or genetic means critically disrupts inhibitory transmission by PV' interneurons, characterized by fewer presynaptic terminals, reduced intrinsic excitability and an abolishment of gamma oscillations.28,29 Interestingly, postmortem studies show that MS is associated with a specific loss in PV' interneurons and their presynaptic terminals.30-32. With a low threshold of action potential generation, sustained high firing frequencies and synchronization with fast gamma oscillations, PV' interneurons use large amounts of energy to fuel the sodium/potassium ATPase to maintain their levels of high-frequency spike generation and activity.33,34 In line with their high energy usage, PV' interneurons have a relatively high mitochondrial content and cytochrome c oxidase levels while deficits in the spatial distribution of mitochondria in PV' axons are associated with impairments of gamma oscillations.35 One leading hypothesis is that myelination provides trophic support to mitochondria in PV' axons to maintain their levels of high-frequency spike generation and activity during gamma oscillations.36

To test how myelination affects mitochondrial distribution and function selectively in PV' interneurons we developed a Cre-dependent viral approach to express mitochondria-targeted genetically encoded fluorescent reporters, mt-GFP and mt-GCamp6f in Cre-driver lines. Two-photon guided patch-clamp recordings from PV' interneurons of the somatosensory cortex followed by confocal microscopy and reconstructions with submicron precision revealed that in normally myelinated axons mitochondria are clustered to myelinated segments, which was disrupted following experimental demyelination. Analyses of a publicly available electron microscopy (EM) dataset confirmed the higher mitochondrial content in myelinated segments of (PV') basket cells but showed homogeneous distribution along partially myelinated layer 2/3 pyramidal neurons. In addition, two-photon imaging of action potential-evoked mitochondrial Ca2+ transients (mt-GCamp6f) showed that mitochondria in myelinated interneurons displayed low or no Ca2+ buffering activity, which increased following demyelination. These results indicate that in neocortical PV' basket cells axons myelination spatially organizes mitochondria, locally increasing the mitochondrial content within internodes, providing evidence for a direct metabolic support by myelination.

**Results**

An AAV-mediated approach for cell type-specific labelling of mitochondria

We first developed a Cre-dependent adeno-associated viral (AAV) vector (AAV-EF1a-mt-GFP-DIO; Fig. 1a) and examined the in vivo labelling of mitochondria. The use of an AAV vector allows control over transduction rates to enable single-cell analyses, an important advantage over brain-wide cell type-specific expression.37 Upon injection into the somatosensory cortex (SI) of either PV-Cre (labelling PV' interneurons) or Rbp4-Cre (labelling cortical layer 5 pyramidal neurons) transgenic mice (Fig. 1b), this viral vector resulted in GFP-labelled mitochondria in molecularly defined cell types (Fig. 1c-f). Expression of mt-GFP-DIO in PV-Cre; AII4 mice (expressing tdTomato in PV' interneurons) labelled interneurons across cortical layers 2 to 6 at high specificity (243/248 cells or 97.94% mt-GFP' cells were tdTomato'; Fig. 1c; ref. 40). Furthermore, two-photon-guided targeted whole-cell recordings from mt-GFP-expressing (mt-GFP') cells revealed that all cells fired action potentials at high frequency (123.41 ± 13.13 Hz upon 500 pA current injection; n = 8 cells from 5 mice). Action potentials were brief in half-width (295.74 ± 2.32 μs; n = 5 cells from 4 mice) and showed a prominent afterhyperpolarization (~15.83 ± 2.25 mV; n = 5 cells from 4 mice, Fig. 1d), consistent with previously described properties of neocortical PV' interneurons.38

To further test the Cre dependency of the mt-GFP-DIO AAV vector, we expressed mt-GFP' in Rbp4-Cre mice, a Cre-driver line commonly used to label cortical layer (L5) pyramidal neurons (PNs).41 In Rbp4-Cre mice, mt-GFP' somata were pyramidal shaped, restricted to L5 and possessed a large apical dendritic characteristic of L5 PNs (Fig. 1e; ref. 42). Mt-GFP' cells in Rbp4-Cre mice always discharged action potentials in low-frequency trains (40.45 ± 4.76 Hz upon 500 pA current injection; n = 13 cells from 4 mice) and of comparatively long half-width (680.85 ± 15.50 μs; n = 11 cells from 4 mice), consistent with the known electrophysiological properties of L5 PN (Fig. 1d; f; ref. 43). Mitochondria are motile organelles but are substantially more stationary in adult mice and in vivo.44 We quantified mitochondrial motility in a subset of our mt-GFP' acutely prepared slices and found that the vast majority (~99%) of mitochondria were stable in both cell types (Supplementary Fig. 1). Together, these findings indicate that mt-GFP-DIO enables the study of mitochondria in a highly cell-type specific manner.

**Mitochondria are clustered at the axonal initial segment (AIS) and are enlarged upon demyelination**

To examine the contribution of myelination to mitochondrial distribution we injected PV-Cre; AII4 mice with AAV-EF1α-mt-GFP-DIO in SI and fed mice either with control food or a diet supplemented with 0.2% cuprizone, which selectively kills oligodendrocytes, leading to widespread cortical demyelination.45 We first focused on the axon initial segment (AIS), a highly excitable domain at the base of the axon where action potentials (APs) are initiated and which is known to adapt to demyelination.46-48 The AIS was identified by immunostaining for the anchoring protein N-cadherin from cortical tissue from mt-GFP-expressing PV-Cre; AII4 mice. By contouring the perimeter of mt-GFP signals we subsequently estimated the shape and size of mitochondria. The results showed that the mitochondrial area was significantly increased in axons from cuprizone-treated mice (nested t-test, P = 0.0241; Fig. 2a-c). In contrast, the mitochondrial aspect ratio (length/width) and density were unaffected (Fig. 2d, e).

Both the AIS length and distance relative to the soma were comparable between groups (Fig. 2f, g). AIS length and location are critical to the current threshold for AP generation.49 To test whether AP initiation was preserved we performed whole-cell recordings from PV' interneurons in acute slices. In all slice experiments, we recorded features of excitability in artificial cerebral spinal fluid containing 5 mM L-lactate and 10 mM D-glucose, resembling the in vivo extracellular environment containing lactate.50 In accordance with the unaffected geometrical properties of the AIS, electrophysiological recordings of PV' interneurons revealed no change in AP wave form, voltage or current threshold, amplitude, half-width, or resting membrane potential (Fig. 2h, i; Supplementary Fig. 2). However, demyelinated PV' interneurons displayed a higher rheobase (Fig. 2j), a lower input resistance (Fig. 2k), and lower firing rate upon sustained somatic current injection (Fig. 2l, m) indicating reduced excitability, in line with previous recordings.51 Together, these results show that following demyelination PV' interneuron excitability is reduced, and AIS mitochondria are larger.

**Mitochondria of the demyelinated PV' axon are increased in size and lost in proximal branches**

To examine the mitochondrial morphology and distribution throughout the entire cytoarchitecture we reconstructed mt-GFP' axons and dendrites of whole-cell recorded biocytin-filled PV' interneurons (Supplementary Figs. 3, 4). Axonal mitochondria in PV' interneurons were often found at branch points and en passant boutons and were smaller in appearance compared to their dendritic counterparts (Supplementary Fig. 3; ref. 49). Quantification showed that compared to dendritic mitochondria those in axons covered significantly less length, were smaller and more spherical (Supplementary
Fig. 3. These subcellular morphological differences are in agreement with previous findings in L2/3 PNs and the observed mitochondrial properties in L5 PNs (Supplementary Fig. 4). Consistent with our observations at the AIS, demyelination significantly increased the size of axonal mitochondria (Fig. 3a, b), with a mean surface area increase of ~60% (nested t-test, \( P = 0.044 \); Fig. 3b, c), without affecting mitochondrial shape (Fig. 3d), suggesting a uniform increase. Importantly, PV+ interneuron myelination is most abundant at lower axonal branch orders (\( \leq 4 \)th order)\(^{20,21,25} \). To test whether demyelination affects axonal mitochondria differently we separated the distal population from the 2nd to 4th branch orders and found that the demyelination-induced mitochondrial size increase was comparable (Fig. 3f).

The PV axons did not show signs of swellings or pathology and consistent with previous work\(^{22} \) the total reconstructed axonal length was similar between control and demyelinated axons (unpaired t-test, \( P = 0.4918 \); Fig. 3g). Interestingly, however, quantification by branch order revealed an increased average segment length in proximal branches of demyelinated PV+ axons (Fig. 3h). The observation of local structural changes, together with previous work showing that demyelination increases mitochondrial density\(^{7,8,12} \), prompted us to investigate the mitochondrial density in PV+ axons. While the total mitochondrial density across all branches was comparable between control and demyelinated PV+ axons (unpaired t-test, \( P = 0.1170 \); Fig. 3i), mitochondria in the proximal branch orders of demyelinated axons were on average ~20% reduced in density (two-way ANOVA, branch order × treatment effect, \( P = 0.0169 \); Fig. 3j). In higher branch orders (\( \geq 5 \)) the mitochondrial densities were similar between groups (two-way ANOVA with Bonferroni’s post hoc test, \( P > 0.9999 \)). In line with these observations, we found an
increased inter-mitochondrial distance specifically in proximal branches of demyelinated axons (≥ 4th order; Kruskal–Wallis test with Dunn’s post hoc test, *P = 0.0241; df = 43; Ctrl, n = 215 mitochondria in 27 AISs from 4 mice; Cpz, n = 144 mitochondria in 18 AISs from 3 mice). Mitochondrial aspect ratio is unaffected (two-sided nested t-test, *P = 0.5656; df = 43; Ctrl, n = 215 mitochondria in 27 AISs from 4 mice; Cpz, n = 144 mitochondria in 18 AISs from 3 mice). e Density of AIS mitochondria is unchanged upon demyelination (two-sided unpaired t-test, *P = 0.0241; df = 43; Ctrl, n = 215 mitochondria in 27 AISs from 4 mice; Cpz, n = 144 mitochondria in 18 AISs from 3 mice). f AIS length is unaffected by cuprizone treatment (two-sided unpaired t-test, *P = 0.2112; df = 42; Ctrl, n = 27 AISs from 4 mice; Cpz, n = 17 AISs from 3 mice). h Example single APs of a control and demyelinated PV+ interneuron upon a 3 ms current injection. i Phase-plane plots of the traces shown in (d); Reduced PV+ interneuron excitability suggested by (j) increased rheobase (two-sided unpaired t-test, *P = 0.0034; df = 33; Ctrl, n = 22 cells from 12 mice; Cpz, n = 13 cells from 7 mice) and k reduced input resistance (two-sided unpaired t-test, *P = 0.0154; df = 33; Ctrl, n = 22 cells from 12 mice; Cpz, n = 13 cells from 7 mice). l Example traces of PV+ interneuron responses after control (black) or cuprizone (red) treatment in response to 400 (left) or 600 pA (right) current injection. m Population data showing a reduced firing frequency upon somatic current injection in demyelinated PV+ interneurons (two-way ANOVA with Bonferroni’s post hoc test; interaction effect, *P = 0.0102, F(6, 156) = 2.910; treatment effect *P = 0.0261, F(1, 26) = 5.569; Ctrl, n = 17 cells from 12 mice; Cpz, n = 11 cells from 7 mice). In truncated violin plots (e, d), solid lines represent the median, dotted lines represent 25th and 75th percentiles, whiskers indicate the maximal and minimal values, solid line represents the mean. Source data are provided as a Source data file.
clustering specifically in the proximal and putatively demyelinated branches of the PV axon.

**Mitochondria are clustered at the myelinated PV axon**

The loss of mitochondrial clustering in proximal axons following demyelination suggests that their distribution is determined, at least in part, by the presence of the myelin sheath. This should be visible in the proximal arbours of PV interneurons, which are characterized by heterogeneous myelin patterns. To test this, we performed a triple staining for myelin basic protein (MBP), GFP and biocytin in sections from control mice and reconstructed PV+ axons to quantify the mitochondrial density in MBP positive (MBP+) and negative (MBP-) axonal segments (Fig. 4a, b, Fig S6). In agreement with previous findings, MBP segments were 19 µm in length (on average 18.59 ± 1.84 µm, range: 2.56–47.62 µm, n = 32 internodes). Interestingly, axonal mitochondria in myelinated segments were larger (nested t-test, P = 0.0121; Fig. 4c), and more elongated compared to mitochondria in unmyelinated branches (nested t-test, P = 0.0388; Fig. 4d). We observed that MBP segments contained more mitochondria (paired t-test, P = 0.0013, Fig. 4e), and were also significantly longer than MBP segments (paired t-test, P = 0.0267, Fig. 4d). After correcting for length, MBP- axonal segments displayed a significantly higher mitochondrial density compared to MBP+ segments of the same axon and comparable branch orders (Fig. 4a, b, g). Furthermore, mitochondrial densities in control MBP+ segments were not different from cuprizone-induced demyelinated PV+ axons (one-way ANOVA, P = 0.0138; Bonferroni’s post hoc test). Mitochondrial density in proximal (<5) but not distal branch orders (two-way ANOVA, Branch order x treatment effect, P = 0.0440; F(6,90) = 2.266; branch order effect, P = 0.0104, F(3,99.6, 8.27) = 3.660; treatment effect, P = 0.0479, F(1,15) = 4.639; Bonferroni’s post hoc test). No change in overall axonal mitochondrial density (two-sided unpaired t-test, P = 0.1170; df = 15; Ctrl, n = 8 axons, 5 mice; Cpz, n = 9 axons, 5 mice). Reduced mitochondrial density in proximal (<5) but not distal branch orders (two-way ANOVA, Branch order x treatment effect, P = 0.0169, F(6,90) = 2.747; branch order effect, P < 0.0001, F(2,65.6, 41.65) = 9.560; treatment effect, P = 0.0143, F(1,15) = 7.679; Bonferroni’s post hoc test Branch order 3, P = 0.0340; Ctrl, n = 8 axons, 5 mice; Cpz, n = 9 axons, 5 mice). Cumulative distribution plot showing increased distance between mitochondria in second to fourth branch orders of demyelinated axons but no change in later branch orders (BO, Kruskal-Wallis test P < 0.0001; Dunn’s post hoc test, All Ctrl vs Cpz comparisons, P < 0.0001, Ctrl BO 2-4 vs BO 5-8, P = 0.8402; Cpz BO 2-4 vs BO 5-8, P = 0.3932. Ctrl, n = 5 axons, 805 mitochondria; 5 cells from 3 mice. Cpz, n = 5 axons, 1218 mitochondria; 5 cells from 4 mice). 

**Fig. 3 | Cuprizone-induced demyelination reduces mitochondrial density in proximal PV+ interneuron axons.** a Example confocal images of mitochondria (mt-GFP) in control (left) and demyelinated axon (right). Similar results were obtained in 7 mice. b 3D rendered images of mt-GFP and biocytin. c Increased mitochondrial size upon demyelination (two-sided nested t-test, *P = 0.0440; df = 8; Ctrl, n = 5 axons, 805 mitochondria; 5 cells from 3 mice, cuprizone (Cpz), n = 5 axons, 1218 mitochondria; 5 cells from 4 mice). d Unchanged mitochondrial aspect ratio in demyelinated axons (two-sided nested t-test, P = 0.2962; df = 8; Ctrl, n = 5 axons, 805 mitochondria; 5 cells from 3 mice; Cpz, n = 5 axons, 1218 mitochondria; 5 cells from 4 mice). e Cumulative distribution plots of mitochondrial size showing uniform increase across all branch orders (BO, Kruskal-Wallis test ***P < 0.0001; Dunn’s post hoc test, All Ctrl vs Cpz comparisons, P < 0.0001, Ctrl BO 2-4 vs BO 5-8, P = 0.8402; Cpz BO 2-4 vs BO 5-8, P = 0.3932. Ctrl, n = 5 axons, 805 mitochondria; 5 cells from 3 mice. Cpz, n = 5 axons, 1218 mitochondria; 5 cells from 4 mice). f Example axonograms of a control (left) and a cuprizone-treated (right) PV interneuron. Red arrowheads indicate examples of second, third or fourth branch orders where mitochondria appear relatively sparse. g Cuprizone treatment induces no change in axonal length (two-sided unpaired t-test, P = 0.4918; df = 15; Ctrl, n = 8 axons, 5 mice; Cpz, n = 9 axons, 5 mice). h Increased segment length in demyelinated PV+ axons as a function of branch order (two-way ANOVA; branch order x treatment effect, *P = 0.0440; F(6,90) = 2.266; branch order effect, P = 0.0104, F(3,99.6, 8.27) = 3.660; treatment effect, P = 0.0479, F(1,15) = 4.639; Bonferroni’s post hoc test n.s.; Ctrl, n = 8 axons, 5 mice; Cpz, n = 9 axons, 5 mice). i No change in overall axonal mitochondrial density (two-sided unpaired t-test, P = 0.1170; df = 15; Ctrl, n = 8 axons, 5 mice; Cpz, n = 9 axons, 5 mice). j Reduced mitochondrial density in proximal (<5) but not distal branch orders (two-way ANOVA, Branch order x treatment effect, P = 0.0169, F(6,90) = 2.747; branch order effect, P < 0.0001, F(2,65.6, 41.65) = 9.560; treatment effect, P = 0.0143, F(1,15) = 7.679; Bonferroni’s post hoc test Branch order 3, *P = 0.0340; Ctrl, n = 8 axons, 5 mice; Cpz, n = 9 axons, 5 mice). k Cumulative distribution plot showing increased distance between mitochondria in second to fourth branch orders of demyelinated axons but no change in later branch orders (BO, Kruskal-Wallis test P < 0.0001; Dunn’s post hoc test, Ctrl vs Cpz BO2-4, ***P < 0.0001; Ctrl BO2-4 vs Ctrl BO5-8, P < 0.0001; Cpz BO2-4 vs Cpz BO5-8, P = 0.6008; Ctrl, n = 8 axons, 5 mice; Cpz, n = 9 axons, 5 mice). Solid lines in truncated violin plots (c, d) represent the median, dotted lines represent 25th and 75th quartiles. Line graphs (h, j) indicate means, error bars indicate SEM. Horizontal bars (g) represent the mean, individual data points represent axons, error bars represent SEM. Source data are provided as a Source data file.
Mitochondria are clustered to myelinated segments of PV⁺ axons.

a Confocal images of a control PV⁺ axon branch point with one myelinated (MBP⁺) and one unmyelinated (MBP⁻) daughter branch. Similar results were obtained in 6 mice. b 3D surface rendering of the confocal images in (a). c Mitochondrial area is higher at the myelin sheath (two-sided nested t-test, \(P = 0.0121\); df = 14; unmyelinated, \(n = 68\) mitochondria; myelinated, \(n = 114\) mitochondria, 8 axons from 6 mice). d Mitochondrial aspect ratio is significantly higher in myelinated axons (two-sided nested t-test, \(P = 0.0358\); df = 14; unmyelinated, \(n = 68\) mitochondria; myelinated, \(n = 114\) mitochondria, 8 axons from 6 mice). e More mitochondria in MBP⁺ segments (two-sided paired t-test, **\(P = 0.0013\), df = 8; \(n = 9\) axons from 6 mice). f Myelinated PV⁺ axonal segments are significantly longer (two-sided paired t-test, *\(P = 0.0267\), df = 8; \(n = 9\) axons from 6 mice). g Mitochondrial density in the myelinated segment is higher compared to unmyelinated ones within the same axon (two-sided paired t-test, **\(P = 0.0099\); \(n = 9\) axons from 6 mice). Solid lines in truncated violin plots (c, d, f, g) represent the median, dotted lines represent 25th and 75th quartiles. Individual data points (e–g) represent axons (1–4 segments per axon), horizontal bars indicate means, error bars indicate SEM. Source data are provided as a Source data file.

Myelination clusters mitochondria selectively to baskets cell internodes

The unexpected axonal mitochondrial clustering within myelinated PV⁺ BC internodes could be a common feature of axon-glial signalling along intermittently myelinated axons. To test this with alternative methods we examined a fully annotated and minable 3D electron microscopy (EM) dataset of the mouse visual cortex. We identified (putative PV⁺) basket cells based on their morphological properties and presence of myelinated arbour (Fig. 5a; see ‘Methods’) and compared axonal mitochondrial distribution with those of the primary axons of L2/3 PNs which are known to exhibit patchy distribution of myelin along the primary axon (Fig. 5b).

Consistent with our immunofluorescence analysis, basket cell myelinated segments were significantly longer (two-way ANOVA with Bonferroni’s post hoc test, \(P = 0.0004\), Supplementary Fig. 6). Within basket cell axon mitochondria were found in ~60% of branch points with at least one myelinated branch (presumed nodes; Supplementary Fig. 6). Interestingly, mitochondria typically avoided ~2 µm regions near the edges of the myelin sheath corresponding to the paranodal loops, but were with high probability and high densities distributed within the internode (Supplementary Fig. 6). Myelinated segments of basket cells, both quantitatively and qualitatively in line with the immunofluorescence data, had an increased mitochondrial density compared to unmyelinated ones (two-way ANOVA with Bonferroni’s post hoc test, \(P = 0.0130\), Fig. 5c). Interestingly, mitochondrial density in myelinated internodes of L2/3 PNs was not different compared to upstream or downstream segments that were unmyelinated (two-way ANOVA with Bonferroni’s post hoc test, \(P = 0.1665\), Fig. 5c).

Furthermore, in keeping with our immunofluorescence data (c.f. Fig. 4), mitochondria within myelinated basket cell segments were significantly larger \((P = 0.0087\), Fig. 5d, Supplementary Fig. 6). Mitochondrial size was, however, not affected in L2/3 PN axonal segments (two-way ANOVA with Bonferroni’s post hoc test, \(P > 0.9999\), Fig. 5d). Strikingly, when comparing the mitochondrial distribution between the two cell types, those in basket cells were ~7-fold larger (two-way ANOVA with Bonferroni’s post hoc test, \(P = 0.0009\), Fig. 5d). Consistent with previous reports, we noticed that unmyelinated segments were typically thinner. A lower cytoplasmic volume could reduce the need for mitochondria and explain the lower density and smaller size of mitochondria. However, plotting the ratio of mitochondrial to axonal volume showed that mitochondria in myelinated basket cell axons occupied a significantly larger volume compared to unmyelinated branches (two-way ANOVA with Bonferroni’s post hoc test, \(P = 0.0044\), Fig. 5e). In contrast, in L2/3 PNs the...
The relative volume of mitochondria was comparable between myelinated and unmyelinated segments (two-way ANOVA with Bonferroni’s post hoc test, $P > 0.9999$, Fig. 5e).

Taken together, the 3D EM results provide independent support of our AAV immunofluorescence results, revealing at ultrastructural detail that mitochondria selectively cluster to myelinated PV$^+$ basket cell internodes. Furthermore, they suggest that the clustering of large mitochondria at myelinated axonal segments is not merely a consequence of patchy myelination but may be specific to PV$^+$ basket cells.
Compact myelin is not required for mitochondrial clustering to myelinated PV⁺ axons

Our data so far suggest that PV⁺ oligodendroglia myelination directly controls mitochondrial clustering. Axonal mitochondria are supplied with metabolites from glycolytic oligodendrocytes via a system of cytoplasmic channels within myelin and transported across the axonal membrane and axolemma. To test the role of compact and noncompact myelin we used the Shiverer mouse line. These mice harbour a deletion in the Shiverer gene severely reducing MBP protein levels and resulting in myelin wrapping with only a few noncompacted noncompact myelin we used the PV-Cre; Ai14 mice with AAV-EF1a-mt-GFP-DIO to target GFP to mitochondria of normal or dysmyelinated PV⁺ interneurons.

Mitochondrial distribution in PV⁺ interneurons from MbpWT mice showed a comparable pattern as found in control mice (Fig. 6 c.f. Fig. 5). In brief, mitochondria were both larger and longer in MOG⁺ segments (Fig. 6d, e). Mitochondria were present in higher numbers in myelinated segments, and myelinated axons were significantly longer than their unmethylated counterparts (Fig. 6f, g). Plotting mitochondrial per unit length showed that myelinated axons displayed higher mitochondrial densities compared to those lacking myelin (Fig. 6h).

In the MbpShi group, mitochondrial morphology was also myelin-dependent with larger and more tubular mitochondria in MOG⁺ segments (two-way ANOVA with Bonferroni's post hoc test; area, \( P = 0.0001 \), aspect ratio, \( P = 0.0069 \); Fig. 6d, e). In contrast, the number of mitochondria in MOG⁺ and MOG⁻ axonal arbours were comparable (two-way ANOVA with Bonferroni's post hoc test, \( P = 0.2549 \); Fig. 6f). Segments of PV⁺ axons possessing noncompacted myelin were also shorter in MbpShi mice (two-way ANOVA with Bonferroni's post hoc test, \( P = 0.0315 \); Fig. 6g), and there was no difference in length between MOG⁺ and MOG⁻ segments (two-way ANOVA with Bonferroni's post hoc test, \( P > 0.9999 \); Fig. 6g). Interestingly, when correcting for segment length, there was no difference in mitochondrial density between noncompact myelinated and unmethylated axons (two-way ANOVA with Bonferroni's post hoc test, \( P = 0.1070 \); Fig. 6h), due to a

---

**Fig. 6** Noncompacted myelin suffices for internodal mitochondrial clustering. a, b Example 3D renders of a PV⁺ axon of (a) MbpWT or (b) MbpShi mouse (see Supplementary Fig. 7). c Schematic representation of mitochondrial distributions in MbpWT and MbpShi PV⁺ axons. d Mitochondria under myelin sheaths are larger in both MbpWT and MbpShi mice (two-way ANOVA, myelination x genotype effect, \( P = 0.1481 \); Fig. 6i, j) = 2.504; myelination effect, \( P < 0.0001 \); Fig. 6i, j) = 125.5; genotype effect, \( P = 0.6304 \); Fig. 6i, j) = 0.2480; Bonferroni's post hoc test, MbpWT MOG vs. MOG⁻, \( P < 0.0001 \); MbpWT MOG vs. MOG⁺, \( P < 0.0001 \); MbpWT MOG vs. MbpShi, \( P = 0.9999 \); MOG⁺ MbpWT vs. MbpShi, \( P = 0.4792 \); MbpWT; \( n = 5 \) cells from 3 mice, MOG⁻; \( n = 72 \) mitochondria, MOG⁺; \( n = 25 \) mitochondria; MbpShi; \( n = 6 \) cells from 3 mice, MOG⁻; \( n = 78 \) mitochondria, MOG⁺; \( n = 59 \) mitochondria). e Internode mitochondria in both MbpWT and MbpShi mice are longer compared to those in unmyelinated axonal segments (two-way ANOVA, myelination x genotype effect, \( P = 0.5428 \); Fig. 6i, j) = 0.4000; myelination effect, \( P = 0.0002 \); Fig. 6i, j) = 35.16; genotype effect, \( P = 0.0045 \); Fig. 6i, j) = 14.13; Bonferroni's post hoc test, MbpWT MOG vs. MOG⁺, \( P < 0.0032 \); MbpWT MOG vs. MOG⁺, \( P = 0.0069 \); MbpWT MOG vs. MbpShi, \( P = 0.0825 \); MOG⁺ MbpWT vs. MbpShi, \( P = 0.0125 \); MOG⁺; \( n = 5 \) cells from 3 mice, MOG⁻; \( n = 72 \) mitochondria, MOG⁺; \( n = 28 \) mitochondria; MbpShi; \( n = 6 \) cells from 3 mice, MOG⁻; \( n = 78 \) mitochondria, MOG⁺; \( n = 59 \) mitochondria). f Number of mitochondria in PV⁺ axons of MbpWT and MbpShi mice (two-way ANOVA, myelination x genotype effect, \( P = 0.0027 \); Fig. 6i, j) = 14.77; myelination effect, \( P = 0.0001 \); Fig. 6i, j) = 37.00; genotype effect, \( P = 0.1406 \); Fig. 6i, j) = 2.522; Bonferroni's post hoc test, MbpWT MOG vs. MOG⁻, \( P = 0.00486 \); Fig. 6i, j) = 4.914; myelination effect, \( P = 0.0145 \); Fig. 6i, j) = 8.330; genotype effect, \( P = 0.1348 \); Fig. 6i, j) = 2.605; Bonferroni's post hoc test, MbpWT MOG vs. MOG⁺, \( P = 0.0104 \); MbpWT MOG vs. MOG⁻, \( P = 0.0099 \); MbpShi MOG vs. MbpShi, \( P = 0.0099 \); MOG⁺ MbpWT vs. MbpShi, \( P = 0.0315 \); MbpShi; \( n = 6 \) cells from 3 mice; MbpWT; \( n = 7 \) cells from 3 mice). g Segment lengths of PV⁺ interneuron axons in MbpWT or MbpShi mice (two-way ANOVA, myelination x genotype effect, \( P = 0.0486 \); Fig. 6i, j) = 4.914; myelination effect, \( P = 0.0145 \); Fig. 6i, j) = 8.330; genotype effect, \( P = 0.1348 \); Fig. 6i, j) = 2.605; Bonferroni's post hoc test, MbpWT MOG vs. MOG⁺, \( P = 0.0104 \); MbpWT MOG vs. MOG⁻, \( P = 0.0099 \); MbpShi MOG vs. MbpShi, \( P = 0.0099 \); MOG⁺ MbpWT vs. MbpShi, \( P = 0.0315 \); MbpShi; \( n = 6 \) cells from 3 mice; MbpWT; \( n = 7 \) cells from 3 mice). h Mitochondrial density is higher in internodes in both MbpWT and MbpShi mice (two-way ANOVA, myelination x genotype effect, \( P = 0.0034 \); Fig. 6i, j) = 13.87; myelination effect, \( P = 0.0001 \); Fig. 6i, j) = 44.39; genotype effect, \( P = 0.4762 \); Fig. 6i, j) = 0.5441; Bonferroni's post hoc test, MbpWT MOG vs. MOG⁺, \( P < 0.0001 \); MbpWT MOG vs. MOG⁻, \( P = 0.0170 \); MbpWT MOG vs. MbpShi, \( P = 0.0168 \); MbpWT MOG vs. MbpShi, \( P = 0.1919 \); MbpWT; \( n = 6 \) cells from 3 mice; MbpShi; \( n = 7 \) cells from 3 mice). Solid horizontal bars indicate means, error bars indicate SEM, individual data points indicate cells. Source data are provided as a Source data file.
significantly increased mitochondrial density in the unmyelinated segments (two-way ANOVA with Bonferroni’s post hoc test, $P = 0.0168$, Fig. 6h). These results provide further evidence that mitochondria are heterogeneously distributed along the proximal arborization of PV⁺ axons as a function of myelination. In addition, the presence of non-compacted membranes suffices for mitochondrial assembly at internodes.

**Myelination attenuates mitochondrial Ca²⁺ buffering**

The clustering of mitochondria to internodes may be important for the buffering of Ca²⁺ during neuronal activity. Our recent work showed that myelin strongly attenuates but not completely blocks depolarization of the axolemma enabling activation of internode voltage-gated channels near the node of Ranvier. Action potential generation has been shown to cause internodal Ca²⁺ influx spreading far from the node of Ranvier but to which extent this holds true for PV⁺ axons and how mitochondria within and outside internodes respond to activity-dependent Ca²⁺ influx is unknown.

To examine mitochondrial buffering of Ca²⁺ in PV⁺ interneurons, we employed AAV-mediated and Cre-dependent expression of the mitochondrion-targeted, genetically encoded calcium indicator mt-GCaMP6f in PV⁺ interneurons (Fig. 7). In acute slices of PV⁺Cre; Ai14 mice we targeted mitochondria with whole-cell patch-clamp recordings and filled them with Atto594, visualizing the arborization of axons during 2 P recordings and enabling targeted mitochondrial imaging (Supplementary Fig. 7a). In the experiments we imaged mt-Ca²⁺ fluorescence responses following a train of ~100 APs (~143 Hz). In line with previous findings, we observed that during AP trains mitochondria were found at putative passant boutons of the distal PV⁺ axon showed strong Ca²⁺ responses, which were unaffected upon demyelination (Supplementary Fig. S8; refs. 50, 59). Similarly, the large amplitude of mt-Ca²⁺ transients at the AIS of PV⁺ interneurons, was unchanged after demyelination (Supplementary Fig. 8).

To test whether there are differences between internodes, unmyelinated and/or demyelinated branches we imaged mt-GCaMP6f along the proximal arbors (≤3th branch order) and post hoc immunostained the slices for GFP, MBP and biocytin, allowing unequivocal assessment of the location of mitochondria with respect to myelin sheaths (Fig. 7b–d). We first focused on segments (i.e. excluding branch points) and found that mitochondria in control axons showed AP-evoked Ca²⁺ responses with greater amplitudes within MBP⁺ segments compared to those in internodes (Kruskal–Wallis test with Dunn’s post hoc test, $P = 0.0001$; Fig. 7d, Supplementary Movie 1). We next asked whether myelin loss would lead to changes in mt-Ca²⁺ buffering in demyelinated PV⁺ interneurons. When we compared all mt-Ca²⁺ responses in control axons (including both MBP⁺ and MBP⁻ segments) with those in putatively demyelinated axons we found no difference between the two groups (nested t-test, $P = 0.6055$). However, mt-Ca²⁺ transients in cuprizone-treated axons were on average larger compared to MBP⁺ control segments (Kruskal–Wallis test with Dunn’s post hoc test, $P = 0.0039$; Fig. 7d) but smaller in amplitude compared to MBP⁺ segments (Kruskal–Wallis test with Dunn’s post hoc test, $P = 0.0195$; Fig. 7d). Mitochondrial Ca²⁺ uptake is known to depend on the influx of extracellular Ca²⁺ (refs. 59, 60), which in turn is controlled by myelination. To examine a possible source for the changes in mitochondrial buffering in PV⁺ internurons, we expressed GCaMP6f in the cytosol of putative mitochondria in PV⁺ interneurons (Fig. 7). In acute slices of PV⁺Cre; Ai14 mice we targeted mt-GCaMP6f⁺ PV⁺ interneurons for whole-cell patch-clamp recordings and investigated how the nodal mt-Ca²⁺ transients were affected upon demyelination-induced changes in cytoplasmic Ca²⁺ in putative internodes remain unknown. 

**Myelin loss impairs mitochondrial Ca²⁺ responses at branch points**

Upon closer inspection of the localization of the mitochondria with large mt-Ca²⁺ responses we found that they were near the outer edge of the myelin sheath at or branch points (Figs. 7b, 8a). To further identify the precise localization of the myelin borders we immunostained for Caspr, a marker for paranodes, which typically had a length of 2.82 ± 0.18 µm (Fig. 8b; $n = 36$ Caspr⁺ segments of 3 cells from 2 mice). The Caspr⁺ segments flanked branch points if their branches were myelinated (n = 19 branch points of 4 cells from 3 mice, Fig. 8b). In line with our initial observation, mitochondria were located near Caspr signals (range 0.00–16.57 µm, on average 2.46 ± 0.96 µm distance, $n = 20$ paranodes of 4 cells from 3 mice; Fig. 8c). These data suggest that in PV⁺ axons branch points are sites of nodes of Ranvier and mitochondria are often found at the nodal domains (Supplementary Fig. 7).

Finally, demyelination is associated with a disassembly of proteins of the node of Ranvier. In PV⁺ axons cuprizone-induced demyelination caused a complete loss of Caspr⁺ segments flanking branch points (Caspr⁺ segments in 4 cells from 3 mice: Fig. 8d). To investigate how the nodal mt-Ca²⁺ transients were affected upon demyelination-induced changes in cytoplasmic Ca²⁺ influx, limiting downstream mt-Ca²⁺ responses in control PV⁺ axons.

**Discussion**

We used a Cre-recombinase-mediated viral approach combined with single-cell reconstructions and found that myelinated segments of PV⁺ axons have a high mitochondrial content (Figs. 4, 5). The increased number of internodal mitochondria is in striking contrast with the general notion that myelinated axons contain fewer mitochondria. In a previous study based on saturated ultrastructural reconstructions of deep layers of the mouse neocortex it was estimated that the mitochondrial content in myelinated axons was a -30-fold lower compared to unmyelinated axons. However, in that study there was no cell type-specific differentiation. Using a publicly available EM database we found that the mitochondrial distribution in intermittently myelinated axons differed between (PV⁺) basket cell interneurons and L2/3 PNs. In contrast to the mitochondrial heterogeneity in interneron axons, mitochondria are homogeneously distributed across myelinated and unmyelinated segments in excitatory axons. Furthermore, cell-type-specific mitochondrial changes were also present following demyelination. While increased numbers of mitochondria were found in excitatory axons (Supplementary Fig. S5), consistent with a wealth of data in experimental demyelination and multiple sclerosis, demyelinated PV⁺ axonal branches however showed reduced mitochondrial density.

The cellular and molecular mechanisms mediating myelin-dependent clustering of mitochondria to PV⁺ internodes remain unknown. Somatostatin-expressing interneurons are also occasionally myelinated and demyelinated PV⁺ axonal branches however showed reduced mitochondrial density.
mitochondrial distribution. Interestingly, expression of the fast Ca²⁺
chelating protein parvalbumin alone most likely does not explain
clustering as in PV⁺ Purkinje axons mitochondria are homogenously
distributed and demyelination increases mitochondrial density like in
excitatory axons. Locally projecting PV⁺ basket cell axons are small
in diameter; on average ~0.3 μm for unmyelinated and ~0.6 μm for
myelinated segments. Small diameter axons possess a relatively
large membrane surface/cytoplasm ratio limiting axial conductivity for
AP propagation but may also require higher energetic costs for axonal
transport and impede metabolic supply. The energetic demand of
the small diameter PV⁺ interneuron axons is further augmented by the
high-frequency firing rates and their generation of local gamma fre-
quency oscillations. Taken together, it is tempting to speculate that
PV⁺ basket cell axis mitochondria efficiently consume the metabolic
substrates supplied externally from oligodendrocytes. A direct exter-
nal metabolic supply is consistent with the multiple distinctive features
in their cellular and molecular organization compared to myelinated
excitatory axons, including relatively high expression of mitochondrial

Fig. 7 | Myelin attenuates internode mt-Ca²⁺ responses. a Example 2P image of an
Atto594-filled mt-GaMP6f+ PV⁺ interneuron. Dashed box indicates the first branch
point of the axon, with a characteristic large angle (>90°). Similar results were
obtained in 17 mice. b Example control PV⁺ axon with mt-GaMP6f-labelled
mitochondria. Mt-Ca²⁺ responses are stronger in MBP⁺ mitochondria (mito 1 and 2)
compared to those in MBP⁻ segment (mito 3 and 4). Dashed lines indicate time-
points corresponding to the heatmap, yellow arrowhead indicates branch point.
Scale bars indicate (top to bottom) 100% ΔF/F, 50 mV, 0.5 nA and 0.5 s. c Schematic
overview of sampled axonal regions. In d, for cuprizone, axonal segments in
between branch points were selected. Similar results were obtained in 17 mice.
d Left, example Ca²⁺ responses of mitochondria in MBP⁺, MBP⁻ or putatively
demyelinated segments. Right, stronger mitochondrial Ca²⁺ responses in MBP⁻
segments compared to MBP⁺ axon and increased Ca²⁺ responses after demyelina-
tion (Kruskal-Wallis test, *P < 0.0001; Dunn’s post hoc test MBP⁻ vs. MBP⁺,
**P < 0.0001; MBP⁻ vs. cuprizone, *P = 0.0195; MBP⁺ vs. cuprizone, ***P = 0.0039;
MBP⁺, n = 101 mitochondria of 12 cells from 7 mice; MBP⁻, n = 27 mitochondria of 10
cells from 6 mice; cuprizone, n = 36 mitochondria of 10 cells from 5 mice). Scale
bars indicate (top to bottom) 25% ΔF/F, 50 mV, 1 nA and 0.5 s. e Top: Example
confocal image of the AIS, myelinated internode and first branch point of a PV⁺
axon Bottom: time lapse of a two-photon cytosolic (cyt-)GaMP6f recording of the
same axon in response to ~100 APs. Notice only a slight Ca²⁺ response at the MBP
edge but practically none in the rest of the internode. Scale bars indicate (top to
bottom) 100% ΔF/F, 50 mV, 0.5 nA and 0.5 s. f Cyt-Ca²⁺ responses are stronger in
unmyelinated and demyelinated axonal segments (Kruskal-Wallis test, *P < 0.0001;
Dunn’s post hoc test MBP⁻ vs MBP⁺, ***P < 0.0001, MBP⁺ vs cuprizone, P = 0.0004,
MBP⁻ vs cuprizone, P = 0.4351; MBP⁺, n = 18 segments of 6 cells from 3 mice; MBP⁻,
n = 24 segments of 8 cells from 3 mice; cuprizone, n = 28 segments of 6 cells from 3
mice). Solid bars in d and f represent the mean, error bars indicate SEM. Individual
data points in d represent mitochondria, individual data points in f represent
segments. Source data are provided as a Source data file.
proteins in the axon (reviewed in ref. 67). The myelin sheath of interneuron axons is characterized by higher levels of 2′,3′-cyclic nucleotide 2′-phosphohydrolase (CNPase)37. This enzyme is found in the cytoplasmic channels in myelin and the noncompacted inner cytoplasmic loops, which may be involved in lactate and pyruvate supply to the periaxonal space, where these nutrients are shuttled into the axon via monocarboxylate transporters3,4,6,37 (Fig. 9). Indeed, our findings in Shiverer mice indicate that noncompacted myelin wrapping sufficed to cluster mitochondria to internodes. Whether oligodendrogial lactate and pyruvate supply acts as a trophic factor to immobilize mitochondria remains to be tested. Interestingly, high glucose availability has been shown to negatively regulate mitochondrial motility68. Furthermore, live imaging in zebrafish axons showed that near paranodes there is myelin-dependent vesicle release69,70, a process which requires high levels of ATP71. ATP consumption by vesicle release, the Na+/K+ ATPase72 or axonal transport73 might cause high local cytoplasmic adenosine diphosphate (ADP) levels, which are known to reduce mitochondrial motility74 and thereby may potentially cluster from control or cuprizone-treated axon branch point mitochondria. Amplitude of branch point mt-Ca2+ responses was reduced upon demyelination (two-sided Mann–Whitney test, *P = 0.0123; control, n = 38 mitochondria of 11 cells from 7 mice; cuprizone, n = 20 mitochondria of 9 cells from 5 mice). In demyelinated PV+ axons, mt-Ca2+ responses at branch points are indistinguishable from those in segments (two-sided Mann–Whitney test, P = 0.8106; segment, n = 36 mitochondria of 10 cells from 5 mice, same data as Fig. 7d; branch points, n = 20 mitochondria of 9 cells from 5 mice, same data as in (f)). BP, branch point. Horizontal bars indicate means, error bars indicate SEM. Solid bars in d and e represent the mean, individual data points represent mitochondria. Source data are provided as a Source data file.

proteins in the axon (reviewed in ref. 67). The myelin sheath of interneuron axons is characterized by higher levels of 2′,3′-cyclic nucleotide 2′-phosphohydrolase (CNPase)37. This enzyme is found in the cytoplasmic channels in myelin and the noncompacted inner cytoplasmic loops, which may be involved in lactate and pyruvate supply to the periaxonal space, where these nutrients are shuttled into the axon via monocarboxylate transporters3,4,6,37 (Fig. 9). Indeed, our findings in Shiverer mice indicate that noncompacted myelin wrapping sufficed to cluster mitochondria to internodes. Whether oligodendrogial lactate and pyruvate supply acts as a trophic factor to immobilize mitochondria remains to be tested. Interestingly, high glucose availability has been shown to negatively regulate mitochondrial motility68. Furthermore, live imaging in zebrafish axons showed that near paranodes there is myelin-dependent vesicle release69,70, a process which requires high levels of ATP71. ATP consumption by vesicle release, the Na+/K+ ATPase72 or axonal transport73 might cause high local cytoplasmic adenosine diphosphate (ADP) levels, which are known to reduce mitochondrial motility74 and thereby may potentially cluster from control or cuprizone-treated axon branch point mitochondria. Amplitude of branch point mt-Ca2+ responses was reduced upon demyelination (two-sided Mann–Whitney test, *P = 0.0123; control, n = 38 mitochondria of 11 cells from 7 mice; cuprizone, n = 20 mitochondria of 9 cells from 5 mice). In demyelinated PV+ axons, mt-Ca2+ responses at branch points are indistinguishable from those in segments (two-sided Mann–Whitney test, P = 0.8106; segment, n = 36 mitochondria of 10 cells from 5 mice, same data as Fig. 7d; branch points, n = 20 mitochondria of 9 cells from 5 mice, same data as in (f)). BP, branch point. Horizontal bars indicate means, error bars indicate SEM. Solid bars in d and e represent the mean, individual data points represent mitochondria. Source data are provided as a Source data file.

Fig. 8 | The mt-Ca2+ responses are amplified at nodal domains in PV+ axons. a Relation between distances from myelin sheaths and mt-Ca2+ response amplitude. Red line indicates exponential decay fit (y = (3.588 – 0.1117) × exp(−0.894 × x) + 0.1117); n = 62 mitochondria of 9 cells from 6 mice. b Example confocal images of PV+ axon branch points flanked by paranodes (Caspr). Similar results were obtained in 3 mice. c Mitochondria are frequently found close to paranodes. Red line indicates exponential decay fit (y = (60.73 – 1.415) × exp(−0.5692 × x) + 1.415); n = 20 mitochondria of 4 cells from 3 mice. d Example of PV+ axon branch points of a cuprizone-treated mouse. Notice the overall lack of both MBP and Caspr immunostaining. Similar results were obtained in 3 mice. e Left: schematic representation of the compartments investigated in (e) and (f); Right: example traces from control or cuprizone-treated axon branch point mitochondria. f Amplitude of branch point mt-Ca2+ responses was reduced upon demyelination (two-sided Mann–Whitney test, *P = 0.0123; control, n = 38 mitochondria of 11 cells from 7 mice; cuprizone, n = 20 mitochondria of 9 cells from 5 mice). g In demyelinated PV+ axons, mt-Ca2+ responses at branch points are indistinguishable from those in segments (two-sided Mann–Whitney test, P = 0.8106; segment, n = 36 mitochondria of 10 cells from 5 mice, same data as Fig. 7d; branch points, n = 20 mitochondria of 9 cells from 5 mice, same data as in (f)). BP, branch point. Horizontal bars indicate means, error bars indicate SEM. Solid bars in d and e represent the mean, individual data points represent mitochondria. Source data are provided as a Source data file.

Fig. 9 | Summary of the impact of myelin on PV+ axonal mitochondria. In normally myelinated PV+ axons (top), mitochondria are clustered at high densities to myelinated internodes (blue), possibly due to local delivery of nutrients such as pyruvate or lactate (orange) from cytoplasmic noncompacted myelin (light blue) to the axon. Internodal mitochondria avoid the first ~2 µm of the myelin sheath (paranodal domain, yellow), are relatively large, and display no or very weak activity-dependent mt-Ca2+ transients (grey). In branch points and unmyelinated segments, mitochondria are smaller but AP-evoked mt-Ca2+ transients are large (dark green). Following myelin loss (bottom), mitochondria become larger and distribute uniformly along proximal axons. The mt-Ca2+ responses are uniform and low in amplitude (light green) and nodes of Ranvier are lost.
mitochondria to PV’ interneurons. An alternative mechanism for the mitochondrial clustering may be the absence of local activity-dependent cytoplasmic mitochondrial Ca\(^{2+}\) influx within the internode (Figs. 7, 8). Since mitochondrial ATP synthesis is in part regulated by Ca\(^{2+}\) (see ref. 75), the mitochondrial clustering at internodes could be viewed as a compensatory mechanism to maintain a sufficient concentration of intracellular ATP. To experimentally examine this requires directly imaging ATP within the PV’ internodes and testing the contribution of internodal mitochondria to local ATP synthesis.

We cannot exclude the possibility that cuprizone affects mitochondria directly\textsuperscript{76}, but the increase in mitochondrial size upon demyelination (Fig. 3) is in keeping with previous studies in axons of other cell types and different models of demyelination\textsuperscript{77,78}, suggesting its toxicity does not play a role in the present analysis. To determine the features of motility, Ca\(^{2+}\) buffering, anatomical properties of size and shape as well as the distribution of the mitochondria in PV’ interneurons we used two-photon and confocal microscopy together with 3D EM data, which have been used previously to study mitochondria\textsuperscript{79,82}. Confocal imaging has the advantage of allowing the mapping of mitochondria in large parts of single cells from multiple animals and distinct genetically modified mice but with the tradeoff of a lower spatial resolution. The EM data independently verified and confirmed our results at the ultrastructural level that mitochondria are larger and more densely distributed at the myelinated PV’ axon. Moreover, the ultrastructural data enabled us to directly investigate mitochondria in different cell types from the same brain.

Our cry-Ca\(^{2+}\) data show that the heterogeneous mt-Ca\(^{2+}\) responses along the PV’ basket cell axons reflect local differentiation of the axon membrane. In normally myelinated axons we observed strong mt-Ca\(^{2+}\) influx near branch points which were flanked by Caspr\(^+\) signals, suggesting that branch points are sites of nodes of Ranvier, consistent with previous work in GABAergic axons\textsuperscript{80}. At the nodal domains large amplitude sodium spikes are generated, opening voltage-gated Ca\(^{2+}\) channels mediating local cytoplasmic Ca\(^{2+}\) responses\textsuperscript{75,76}. The lower Ca\(^{2+}\) influx at these sites in both the axonal cytoplasm and mitochondria after demyelination might therefore reflect loss of nodal voltage-gated ion channel proteins and/or frequency-dependent failures during our trains of ~100 spikes. AP-evoked failures of release during PV’-mediated inhibition of PNs has been reported during recording of sympathetically coupled PV’ basket cells and PNs\textsuperscript{81}. However, during AP trains we still observed large mt-Ca\(^{2+}\) transients in the downstream presynaptic terminals of the PV’ axons (Supplementary Fig. 8). Our cry-Ca\(^{2+}\) imaging data suggest that mt-Ca\(^{2+}\) transients at unmethylated and putatively demethylated segments are caused by AP-evoked membrane depolarizations opening voltage-gated Ca\(^{2+}\) channels. Although we cannot exclude a role for presynaptic GABAergic terminals in proximal unmethylated (MBP\(^+\)) opening voltage-gated Ca\(^{2+}\) channels\textsuperscript{75,76}, such mt-Ca\(^{2+}\) transients at release sites typically show much larger Ca\(^{2+}\) responses (Supplementary Fig. 8). To better understand the biophysical properties of interneuronal myelination and the axolemmal membrane it will be critical to employ high temporal resolution voltage sensitive dye imaging and quantify absolute changes in membrane potential during axonal spiking\textsuperscript{86,87}.

The finding that myelin clusters mitochondria and defines mitochondrial Ca\(^{2+}\) buffering (which also drives ATP synthesis\textsuperscript{82}) indicates that myelin patterns may critically regulate energy homeostasis in the PV’ axon arborization. Such a link also has implications for interneuron myelin plasticity\textsuperscript{85}. Recent work has revealed that experience-dependent myelin remodelling occurs prominently around PV’ interneuron axons. If in PV’ interneurons newly formed myelin sheaths attract mitochondria this could represent a glial mechanism to strengthen axonal pathways and promote specific circuits of inhibitory transmission. Both myelination and mitochondrial distribution in PV’ interneurons are known to be important for gamma oscillations, indicating they may represent converging pathways that shape PV’ interneuron inhibitory functions\textsuperscript{82,83}. More research will be necessary to test this hypothesis, by for instance using longitudinal mitochondrial imaging during myelin development or activity-induced myelination. Finally, the present findings may shed light on the vulnerability of PV’ basket cells to myelin loss in MS\textsuperscript{29-30}. If mitochondria in PV’ axons depend on a glial source of metabolic support, myelin loss might acutely and negatively influence neuronal transport, ion homeostasis and other critical processes in PV’ axons. Moreover, the small diameter of basket cell axons could make them more vulnerable to excess Ca\(^{2+}\) influx upon demyelination\textsuperscript{81}, in particular given that mt-Ca\(^{2+}\) buffering is insufficient to maintain normal cyt-Ca\(^{2+}\) levels during AP trains (Fig. 7). Together, the data presented here reveal a critical role for interneuronal myelin to diversify mitochondrial distribution and function in PV’ axons and encourage future research into mitochondrial dysfunction in PV’ interneuron axons in neurodegenerative diseases.

**Methods**

**Animals**

All procedures were performed after evaluation by the Royal Netherlands Academy of Arts and Sciences (KNAW) Animal Ethics Committee (DEC) and Central Authority for Scientific Procedures on Animals (CCD, license AVD010020172426). The specific experimental designs were evaluated and monitored by the Animal Welfare Body (ND, protocols NIN19.21.01, NIN19.21.09 and NIN19.21.12). Male and female PV-Cre; Ai14, Rbp4-Cre, Rbp4-Cre; ChETA mice were used in this study. PV-Cre × Ai14 mice were obtained by crossing B6.129S6-Gt(Rosa)26Sortm1(CAG-COP4*E123T*H134R,-tdTomato)Gfng and B6.129S6-Gt(Rosa)26Sortm1(CAG-COP4*E123T*H134R,-tdTomato)Gfng/Prkca-Cre; Ai14 × Shiverer mice were used in this study. PV-Cre × Ai14 mice are defined before the phenotype prevented them from reaching food ad libitum. Cages were open or IVC cages with corncob bedding. Wherever possible, animals were housed together with at least one cage mate. Ambient temperature was maintained at 20–24°C, humidity at 45–65%. For cuprizone experiments, at 8 weeks of age, mice were fed powdered food either with or without 0.2% cuprizone (biscyclohexane oxaldihydrazone, Sigma-Aldrich) supplement (range: 5–6 weeks). Animals on cuprizone diet that showed persistent >30% weight loss compared to control litter mates were excluded from the study. Shiverer mice were sacrificed before the phenotype prevented them from reaching food or water (persistent epileptic seizures, hind limb paralysis). The weight, locomotion and overall welfare of all mice were closely monitored.

**Plasmids**

Cre-dependent mt-GFP and mt-GCaMP6f AAV plasmids were created by replacing the mCherry open reading frame (ORF) in pAAV-EF1a-mCherry-DIO (Addgene plasmid #20299, RRID: Addgene_20299) with mt-GFP or 4mt-GCaMP6f (‘mt-GCaMP6f in the main text). High-Fidelity Phusion Taq polymerase (Thermo Fisher Scientific, F-530XL) was used to amplify mt-GFP from Addgene plasmid #44385 (RRID: Addgene_44385) or 4mt-GCaMP6f (plasmid was generously donated by Diego de Stefanis). For mt-GFP, PCR fragment and plasmid were digested with AseI and Nhel (New England Biolabs). Because 4mt-GCaMP6f contains an Nhel digestion site, to create the mt-GCaMP6f-DIO plasmid, pAAV-EF1a-mCherry-DIO was digested with Nhel, blunted using T4 DNA Polymerase (New England Biolabs), digested with AseI and then dephosphorylated using Antarctic Phosphatase (New England Biolabs). The 4mt-GCamP6f PCR product was phosphorylated
using T4 Polynucleotide Kinase (New England Biolabs) and digested using Ascl. Plasmid and insert were then ligated overnight at 16 °C (T4 DNA ligase, New England Biolabs). After transformation into chemically competent E. coli and subsequent plasmid purification, insertion and double inverted orientation (DIO) were confirmed using restriction enzyme analysis and DNA sequencing. The plasmids were deposited to Addgene (mt-GFP-DIO #17412; mt-GCaMP6f-DIO: #1179529). To test Cre-dependent expression, HEK293-T cells (HEK 293T/17 cell line, CRL-11268 obtained from ATCC; RRID:CVCL_1926) were transfected with pAAV-EF1a-mt-GFP-DIO or pAAV-EF1a-mt-GCaMP6f-DIO only or pAAV-EF1a-mt-GFP-DIO and pCAG-Cre (Addgene #13775; RRID: Addgene_13775) using polyethyleneimine (PEI). Briefly, plasmid DNA (500 ng) was diluted in saline and then mixed with PEI, allowed to incubate for 20–25 min and then applied dropwise to HEK293-T cells in a 6-well plate. The next day a fluorescein microscope, mt-GFP or mt-GCaMP6f expression was assessed, which was absent in the cells lacking or expressing only pCAG-Cre, confirming Cre-dependence.

mt-GFP primers: FW: 5′-AATAGCTACATCATGCGTAGCTTGACAGC-3′
RV: 5′-TAATTGGCCGCCGGAAGTCTGGATTTT-3′

mt-GCaMP6f primers: FW: 5′-ACTTCGTCTCACTATCAATGG-3′
RV: 5′-GCTGACACATCTGCGT-3′

Sequencing primers: FW: 5′-GAATTGTAGCTGCTATTAGC-3′
RV: 5′-GCGACCACATCTGCTATTAGG-3′

AAV production
HEK293-T cells of low passage (<25) were kept in Dulbecco’s modified Eagle’s medium (DMEM, Thermo Fisher Scientific #31966-047) containing 10% foetal calf serum (FCS, Thermo Fisher Scientific A1766801) and 5% penicillin-streptomycin (Pen-Strep, Thermo Fisher Scientific #15140122) at 37 °C and 5% CO2. For virus production, cells were plated on 15 cm dishes at a density of 1–2.5 x 10^5 cells per dish (12 dishes per virus). The next day, medium was replaced with Iscove’s modified Dulbecco’s medium (IMDM, Sigma Aldrich I-3390) containing 10% FCS, 5% Pen-Strep and 5% glutamine (Thermo Fisher Scientific #25030081) 1–2 h before transfection. For transfection, AAV rep/cap, AAV helper (mt-GFP: AAV5; mt-GCaMP6f: AAV4) and transfer plasmids were mixed and diluted in saline before mixing with saline-diluted polyethyleneimine (PEI, Polysciences #23966-047) containing 10% foetal calf serum (FCS, Thermo Fisher Scientific #31966-047) and brief vortexing. After 20–25 min of incubation, the transfection mix was added in a dropwise fashion to the culture plates. The next day, medium was refreshed after which the cells were left for an additional two nights (i.e. 72 h after transfection). Medium was discarded and cells were then collected and subsequently lysed with 3 freeze-thaw cycles to release AAVs. Cell lysate was then loaded on an iodixanol gradient (60, 40, 25 and 15% iodixanol, ELITechGroup #1114542) in Beckman Quick- Seal Polylamellar tubes (Beckman-Coulter #342414) which were sealed and centrifuged in a Beckman-Coulter Optima XE-90 Ultracentrifuge using a Type 70 Ti rotor at 16 °C and 69,000 rpm (488727.6 x g) for 1 h and 10 min. The virus-containing fraction was extracted from the tubes and AAVs were then concentrated in Dulbecco’s Phosphate Buffered Saline (D-PBS) + 5% sucrose using Amicon Ultra-15 (100 K) filter units (Merck Millipore UFC910024) at 3220 x g. At least 4 rounds of centrifugation were used to ensure complete replacement of iodixanol with D-PBS + 5% sucrose. Typical yields were ~150 μl of virus, the titre of which was determined using quantitative PCR (titres: mt-GFP-DIO, 4.43 x 10^13 gc/ml; mt-GCaMP6f-DIO, 3.44 x 10^13 gc/ml)48. Viral aliquots were stored at ~80 °C until further use.

qPCR primers, recognize AAV2 inverted terminal repeats (ITRs): 5′-GGACCCCTTAATGTAGGATGT-3′ 5′-CGGCCCTCAGTGACCGA-3′

Viral injection
Viral injections were typically performed during the 3rd week of control powder food or cuprizone treatment. Shiverer mice were injected at 3–4 weeks of age. Mice were anaesthetized using isofluran (3% induction, 1.2–1.5% maintenance) after which they subcutaneously received 5 mg/kg Metacomb. Body temperature was monitored and maintained at 37 °C using a heating pad and eyes were prevented from drying out using eye ointment. The head was then shaved and placed in a stereotaxic frame (Kopf) and an incision was made in the skin along the midline. Lidocaine (10%) was administered on the periorbital area before removing it. Small (<1 mm) bilateral craniotomies were made at ~0.5 mm caudally from Bregma and 2.5 mm laterally from the midline without damaging the dura mater. With a sharp glass pipet attached to a Nanject III (Drummond), 40–50 nl of virus was injected at 1 nl/s and at a depth of 450 μm. Approximately 3 min after finishing the injection, the needle was retracted slowly. Bone wax was applied to the craniotomies and the skin was sutured before mice were allowed to recover. Animals were monitored closely in the 3–5 days following surgery, during which their weight, locomotion and overall wellbeing were checked.

Acute slice preparation
After 5–6 weeks of cuprizone or control treatment or, in the case of Shiverer mice, at 5–7 weeks of age (i.e. 2–3 weeks after virus injection), mice were deeply anaesthetized using pentobarbital (50 mg/kg, intraperitoneal injection) and transcranially perfused with ice-cold carbogenated (95% O2, 5% CO2) cutting artificial cerebrospinal fluid (aCSF; 125 mM NaCl, 3 mM KCl, 6 mM MgCl2, 1 mM CaCl2, 25 mM glucose, 1.25 mM NaHPO4, 1 mM kynurenic acid and 25 mM NaHCO3). After quickly dissecting out the brain 400 μm thick parasagittal slices were cut in ice-cold carbogenated aCSF using a Vibratome (1200S, Leica Microsystems). Slices were transferred to a holding chamber containing carbogenated aCSF where they were kept at 35 °C for 35 min to recover and then allowed to return to room temperature for at least 30 min before starting experiments.

Electrophysiology and two-photon imaging
To perform electrophysiological experiments, slices were transferred to a recording chamber with continuous in- and outflow of carbogenated recording ACSF (rACSF; 125 mM NaCl, 3 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM glucose, 5 mM L-Lactate, 1.25 mM NaHPO4, and 25 mM NaHCO3) at a rate of 1–2 ml per minute at 32 °C. Glass pipettes with an open tip resistance of 6–7 MΩ were filled with an intracellular solution containing (in mM, 130 K-Gluconate, 10 KCl, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, 10 Na2-phosphocreatine; pH ~7.25, osmolality ~280 mosmol/kg) supplemented with 5 mg/ml biocytin (Sigma-Aldrich, B4261) and 50 μM Atto-594 (Sigma-Aldrich, A08637). Whole-cell recordings were made using a patch-clamp amplifier (Multiclamp 700B, Axon Instruments, Molecular Devices, RRID: SCR_018455) operated by AxoGraph X software (version 1.5.4; RRID: SCR_014284). Action potential trains were evoked using 700 ms step pulses, from ~250 pA to +700 nA with increments of 50 pA. Single action potentials were evoked using 3 ms incremental (2.5–5) pA steps with a starting amplitude below spike threshold. Using an AD/DA converter (ITC-18, HEKA Elektronik GmbH), voltage was digitally sampled at 100 Hz. The access resistance during current-clamp experiments (range: 15–30 MΩ) was fully compensated using bridge balance and capacitance neutralization of the amplifier. Somatic single-cell recordings were made from mt-GFP+ cells, which were visualized using a two-photon (2P) laser-scanning microscope (Femto3D-RC, Femtonics Inc., Budapest, Hungary). Imaging was controlled using MES software (Femtonics Inc., Budapest, Hungary, version 6.3.7902). A Ti:Sapphire pulsed laser (Chameleon Ultra II, Coherent Inc., Santa Clara, CA, USA) was tuned to 770 nm for two-photon excitation to visualize mt-GFP/mt-GCaMP6f and tdTomato. Fluorescent signals were detected using two photomultipliers (PMTs, Hamamatsu Photonics Co., Hamamatsu, Japan), one for mt-GFP and for Atto-594 (20 μM) to assess its diffusion, as an indication of biocytin diffusion. For motility

Article
https://doi.org/10.1038/s41467-022-35350-x
Brains were then dissected out and allowed to DIO (mixed 1:1) were 
Cre mice injected with AAV1-EF1a-mCherry-DIO and AAV5-EF1a-mtGFP-

Bodies was 2 h at room temperature. All steps are performed with 
processed into 40 

Cerol, 0.05 M phosphate buffer) until further use. Immunostaining 
sections, which were either placed in PBS for immediate use or stored 
was adjusted: blocking was done 1 h at 37 °C and 1 h room temperature 

Mounting using FluorSave mounting medium (Merck-Millipore 

Sigma B4261). Next, 

Stabilizer plugin for FIJI was used to account for drift. For Ca2+ imaging, 

imaging, z-stacks were acquired every 10 s for 8–12 min. The Image 

Stabilizer plugin for FIJI was used to account for drift. For Ca2+ imaging, 

OBJECTIVE CONTROL FOR DRIFT. For Ca2+ imaging, 

3D EM data analysis 
3D EM data was obtained from the Microns dataset20 (www.microns- 
explorer.org), which entails a 1 mm3 EM block of primary visual cortex 
of one P87 male mouse. Basket interneurons were identified based on 
their morphology, i.e. their relatively round soma (as opposed to a pyramidal shape), the lack of spines on dendrites and a thin and highly 
branched axon that was partly myelinated. From each cell, one to two 

Volumes were selected that either contained an axonal branch point 
with one myelinated and one unmymelinated branch, or a single branch 
with intermittent myelination. The EM volume of interest and cytosolic 
segmentation were then downloaded, after which the mitochondria 
were segmented and the segment length traced manually using 

Volume Annotation and Segmentation Tool (VAST) software (version 

14.1.0). Because we found that branch points in PV+ interneurons are 
often nodes of Ranvier (a specialized axonal compartment), branch 
points and mitochondria that resided in them were not included in 
the analysis. Boutons were excluded for the same reason. If a mitochondria 
spanned a border between myelinated and unmyelinated axon or 
beyond a branch point, they were not included. To analyse the mitochondrial content in L2/3 PNs, we used the same approached as 
described above. These cells were readily identified based on their pyramidal shape, spiny dendrites and distance from the pia. We 
selected L2/3 PNs that displayed patchy myelin. Fully or unmyelinated 
L2/3 PN axons were not included. Length and volume measurements 
were performed and exported using VAST Tools Matlab scripts. 
For examples used in figures, 3D models were exported as OJB 
files using VAST software and rendered using 3ds Max (Autodesk, 
version 25.0.0.997, SCR_014251). See Supplementary Table 2 for cells used in 
the analysis.

Statistics and reproducibility
All statistical comparisons were done using Prism (Graphpad, 
version 8.4.3, RRID: SCR_002798). Normality of datasets was determined using 
D’Agostino & Pearson or Shapiro-Wilk tests. We applied non-
parametric tests if data deviated significantly from a normal distribution. For comparisons between groups we used a two-tailed unpaired 
t-test (normal data) or a two-tailed Mann–Whitney test (non-normal data). For t-tests degrees of freedom (df) are reported in the figure 
legends. One-way ANOVA (normal data) or Kruskal–Wallis test
(non-normal data) was applied for comparisons of three groups; two-way ANOVA was used to test interactions between groups and treatments; Bonferroni’s post hoc test (normal data) or Dunn’s post hoc test (non-normal data) was used for multiple comparisons; nested t-tests or nested one-way ANOVAs were used when large numbers of datasets were involved (i.e. mitochondrial contours) to avoid over-powering of non-nested statistical tests (cells were nested inside their respective treatment groups). Figure legends contain $P$-values and $n$-numbers and whether the latter signify mice, cells or mitochondria. Means are presented with SEM.

Data was collected from multiple cells from multiple animals to ensure reproducibility. Experiments were replicated in a minimum of 4 cells from a minimum of 3 mice with the exception of the 3D EM reconstructions which were done using data obtained from one mouse. For exact $n$ numbers see figure legends. Reconstructions and recordings from different subcellular compartments of the same cell were obtained wherever possible. Age-matched mice were randomly allocated to either the control or cuprizone treatment. For comparisons of subcellular compartments (e.g. myelinated vs unmyelinated axon, axon vs dendrite, segment vs branch point), randomization is not possible as the group data points belong to are determined by their subcellular localization. Therefore, randomization was not performed in these cases. However, selection of cells for patching prior to e.g. cellular reconstruction and calcium imaging was done randomly (i.e. virally transduced cells with a healthy appearance were targeted but otherwise no selection was made). Blinding was not performed in this study as the difference between control and cuprizone-treated brain tissue (or brain tissue from Shiverer mice) can be readily observed during the performance of the experiments (i.e. due to the absence of myelin). In control cells, calcium imaging was done blind to the myelination state of axonal segments, which was revealed only later by immunohistochemistry. To determine the density of mitochondria in MBP/MOG- and MBP/MOG- segments, during tracing of the axon and mitochondria the MBP or MOG channel was disabled and vice versa. This was not possible in the 3D EM dataset, where the presence of myelin is readily observed in the image data that is required to perform the segmentation. Electrophysiological data was excluded if cells had an unstable resting membrane potential between sweeps. Calcium data was excluded when the number of action potentials deviated by more than 15% ($<$85 or $>$115 APs) or if the subsequent immunostaining was not reliable.

**Reporting summary**

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

**Data availability**

All data are available upon request to the corresponding authors. The Microns dataset used for 3D EM analysis is available at www.microns-explorer.org. Source data are provided with this paper.

**Code availability**

Custom-written Matlab scripts for Ca$^{2+}$ imaging analyses along with pseudocode and example data can be found at https://github.com/Kolelab.

**References**

1. Cohen, C. C. H. et al. Saltatory conduction along myelinated axons involves a periaxonal nanocircuit. Cell 180, 311–322.e15 (2020).
2. Nave, K.-A. & Werner, H. B. Myelination of the nervous system: mechanisms and functions. Annu. Rev. Cell Dev. Biol 30, 503–533 (2014).
3. Lee, Y. et al. Oligodendroglia metabolically support axons and contribute to neurodegeneration. Nature 487, 443–448 (2012).
4. Fünfschilling, U. et al. Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. Nature 485, 517–521 (2012).
5. Chamberlain, K. A. et al. Oligodendrocytes enhance axonal energy metabolism by deacetylation of mitochondrial proteins through transcellular delivery of SIRT2. Neuron https://doi.org/10.1016/j.neuron.2021.08.011 (2021).
6. Philips, T. et al. MCT1 deletion in oligodendrocyte lineage cells causes late-onset hypomyelination and axonal degeneration. Cell Rep 34, 108610 (2021).
7. Zambonin, J. L. et al. Increased mitochondrial content in remyelinated axons: implications for multiple sclerosis. Brain 134, 1901-1913 (2011).
8. Witte, M. E. et al. Enhanced number and activity of mitochondria in multiple sclerosis lesions. J. Pathol. 219, 193–204 (2009).
9. Mahad, D. J. et al. Mitochondrial changes within axons in multiple sclerosis. Brain 132, 1161–1174 (2009).
10. Kinyu-So, S., Ohno, N., Kidd, G. J., Komuro, H. & Trapp, B. D. Demyelination increases axonal stationary mitochondrial size and the speed of axonal mitochondrial transport. J. Neurosci. 30, 6658–6666 (2010).
11. Nikic, I. et al. A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis. Nat. Med. 17, 495-499 (2011).
12. Licht-Mayer, S. et al. Enhanced axonal response of mitochondria to demyelination offers neuroprotection: implications for multiple sclerosis. Acta Neuropathol. 140, 143–167 (2020).
13. Ohno, N. et al. Mitochondrial immobilization mediated by syntaphilin facilitates survival of demyelinated axons. Proc. Natl Acad. Sci. USA 111, 9953–9958 (2014).
14. Licht-Mayer, S. et al. Axonal response of mitochondria to demyelination and complex IV activity within demyelinated axons in experimental models of multiple sclerosis. Neuropath. Appl. Neuro. 12851 https://doi.org/10.1111/nan.12851 (2022).
15. Andrews, H. et al. Increased axonal mitochondrial activity as an adaptation to myelin deficiency in the Shiverer mouse. J. Neurosci. Res. 83, 1533–1539 (2006).
16. Balaratnasingam, C., Morgan, W. H., Johnstone, V., Cringle, S. J. & Yu, D.-Y. Heterogeneous distribution of axonal cytoskeleton proteins in the human optic nerve. Invest. Ophth Vis. Sci. 50, 2824–2838 (2009).
17. Perge, J. A., Koch, K., Miller, R., Sterling, P. & Balasubramanian, V. How the optic nerve allocates space, energy capacity, and information. J. Neurosci. 29, 7917–7928 (2009).
18. Kasthuri, N. et al. Saturated reconstruction of a volume of neocortex. Cell 162, 648–661 (2015).
19. Kageyama, G. & Wong-Riley, M. The histochemical localization of cytochrome oxidase in the retina and lateral geniculate nucleus of the ferret, cat, and monkey, with particular reference to retinal mosaics and ON/OFF-center visual channels. J. Neurosci. 4, 2445–2459 (1984).
20. Stedefouder, J. et al. Fast-spiking parvalbumin interneurons are frequently myelinated in the cerebral cortex of mice and humans. Cereb. Cortex 27, 5001–5013 (2017).
21. Micheva, K. D. et al. A large fraction of neocortical myelin ensheathes axons of local inhibitory neurons. Elife 5, e15784 (2016).
22. Dubey, M. et al. Myelination synchronizes cortical oscillations by consolidating parvalbumin-mediated phasic inhibition. Elife 11, e73827 (2022).
23. Kepecs, A. & Fishell, G. Interneuron cell type fits are fit to function. Nature 505, 318–326 (2014).
24. Hu, H., Gan, J. & Jonas, P. Fast-spiking, parvalbumin+ GABAergic interneurons: From cellular design to microcircuit function. Science 345, 1255263 (2014).
25. Stedehouder, J. et al. Local axonal morphology guides the topography of interneuron myelination in mouse and human neocortex. *Elife* 8, e48615 (2019).

26. Edgar, J. M. et al. Early ultrastructural defects of axons and axon-glia junctions in mice lacking expression of Cnp1. *Glia* 57, 1815–1824 (2019).

27. Evangelou, N. et al. Size-selective neuronal changes in the anterior optic pathways suggest a differential susceptibility to injury in multiple sclerosis. *Brain* 124, 1813–1820 (2001).

28. Ramaglia, V. et al. Complement-associated loss of CA2 inhibitory synapses in the demyelinated hippocampus impairs memory. *Acta Neuropathol.* 142, 643–667 (2021).

29. Dutta, R. et al. Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients. *Ann. Neurol.* 58, 478–489 (2000).

30. Zoupi, L. et al. Selective vulnerability of inhibitory networks in multiple sclerosis. *Acta Neuropathol.* 141, 415–429 (2021).

31. Hu, H., Roth, F. C., Vandaël, D. & Jonas, P. Complementary tuning of Na+ and K+ channel gating underlies fast and energy-efficient action potentials in GABAergic interneuron axons. *Neuron* 98, 156–165.e6 (2018).

32. Li, T. et al. Action potential initiation in neocortical inhibitory interneurons. *PLoS Biol.* 12, e001944 (2014).

33. Kann, C., Papageorgiou, I. E. & Draguhn, A. Highly energized inhibitory interneurons are a central element for information processing in cortical networks. *J. Cereb. Blood Flow. Metab.* 34, 1270–1282 (2014).

34. Kageyama, G. H. & Wong-Riley, M. T. T. Histochemical localization of cytochrome oxidase in the hippocampus: correlation with specific neuronal types andafferent pathways. *Neuroscience* 77, 2337–2361 (1992).

35. Kontou, G. et al. Mirot1-dependent mitochondrial dynamics in parvalbumin interneurons. *Elife* 10, e65215 (2021).

36. Stedehouder, J. & Kushner, S. A. Myelination of parvalbumin interneurons: a parsimonious locus of pathophysiological convergence in schizophrenia. *Mol. Psychiatry* 22, 4–12 (2017).

37. Micheva, K. D. et al. Distinctive structural and molecular features of myelinated inhibitory axons in human neocortex. *Eneuro* 5, ENEURO.0297–18.2018 (2018).

38. Consortium, Mic. et al. Functional connectomics spanning multiple areas of mouse visual cortex. Preprint at BioRxiv https://doi.org/10.1101/2021.07.28.454025 (2022).

39. Fecher, C. et al. Cell-type-specific profiling of brain mitochondria reveals functional and molecular diversity. *Nat. Neurosci.* 22, 1731–1742 (2019).

40. Meyer, H. S. et al. Inhibitory interneurons in a cortical column form hot zones of inhibition in layers 2 and 5A. *Proc. Natl. Acad. Sci. USA* 108, 16807–16812 (2011).

41. Gerfen, C. R., Paletzki, R. & Heintz, N. GENSAT BAC Cre-recombinase driver lines to study the functional organization of cerebral cortical and basal ganglia circuits. *Neuron* 80, 1368–1383 (2013).

42. Hamada, M. S., Goethals, S., Vries, S. I. D., Brette, R. & Kole, M. H. P. Covariation of axon initial segment location and dendritic tree normalizes the somatic action potential. *Proc. Natl. Acad. Sci. USA* 113, 14841–14846 (2016).

43. Hamada, M. S. & Kole, M. H. P. Myelin loss and axonal ion channel adaptations associated with gray matter neuronal hyperexcitability. *J. Neurosci.* 35, 7272–7286 (2015).

44. Smit-Rijter, L. et al. Mitochondrial dynamics in visual cortex are limited in vivo and not affected by axonal structural plasticity. *Curr. Biol.* 26, 2609–2616 (2016).

45. Kipp, M., Clamer, T., Dang, J., Copray, S. & Beyer, C. The cuprizone animal model: new insights into an old story. *Acta Neuropathol.* 118, 723–736 (2009).

46. Kole, M. H. P., Letzkus, J. J. & Stuart, G. J. Axon initial segment Kv1 channels control axonal action potential waveform and synaptic efficacy. *Neuron* 55, 633–647 (2007).

47. Kole, M. H. & Brett, R. The electrical significance of axon location diversity. *Curr. Opin. Neurobiol.* 51, 52–59 (2018).

48. Zilberter, Y., Zilberter, T. & Brégestovski, P. Neuronal activity in vitro and the in vivo reality: the role of energy homeostasis. *Trends Pharm. Sci.* 31, 394–401 (2010).

49. Cserép, C., Posfai, B., Schwarcz, A. D. & Denes, A. Mitochondrial ultrastructure is coupled to synaptic performance at axonal release sites. *Eneuro* 5, https://doi.org/10.1523/ENEURO.0390-17.2018 (2019).

50. Lewis, T. L., Kwon, S.-K., Lee, A., Shaw, R. & Polleux, F. MFF-dependent mitochondrial fission regulates presynaptic release and axon branching by limiting axonal mitochondria size. *Nat. Commun.* 9, 5008 (2018).

51. Tomassy, G. S. et al. Distinct profiles of myelin distribution along single axons of pyramidal neurons in the neocortex. *Science* 344, 319–324 (2014).

52. Edgar, J. M., McCulloch, M. C., Thomson, C. E. & Griffiths, I. R. Distribution of mitochondria along small-diameter myelinated central nervous system axons. *J. Neurosci. Res.* 86, 2250–2257 (2008).

53. Saab, A. S. & Nave, K.-A. Myelin dynamics: protecting and shaping neuronal functions. *Curr. Opin. Neurobiol.* 47, 104–112 (2017).

54. Roach, A., Takahashi, N., Pravtcheva, D., Rudlle, F. & Hood, L. Chromosomal mapping of mouse myelin basic protein gene and structure and transcription of the partially deleted gene in shiverer mutant mice. *Cell* 42, 149–155 (1985).

55. Snaidero, N. et al. Myelin membrane wrapping of CNS axons by PI(3,4,5)P3-dependent polarized growth at the inner tongue. *Cell* 156, 277–290 (2014).

56. Meschkat, M. et al. White matter integrity in mice requires continuous myelin synthesis at the inner tongue. *Nat. Commun.* 13, 1163 (2022).

57. Hanemaaijer, N. A. et al. Ca2+ entry through NAv channels generates submillisecond axonal Ca2+ signaling. *Elife* 9, e54566 (2020).

58. Zhang, Z. & David, G. Stimulation-induced Ca2+ influx at nodes of Ranvier in mouse peripheral motor axons. *J. Physiol.* 594, 39–57 (2016).

59. Ashraf, G., Juan-Sanz, J., de, Farrell, R. J. & Ryan, T. A. Molecular tuning of the axonal mitochondrial Ca2+ uniporner ensures metabolic flexibility of neurotransmission. *Neuron* 105, 678–687.e5 (2020).

60. Stoler, O. et al. Frequency- and spike-timing-dependent mitochondrial Ca2+ signaling regulates the metabolic rate and synaptic efficacy in cortical neurons. *Elife* 11, e74606 (2022).

61. Crawford, D. K., Mangiardi, M., Xia, X., López-Valdés, H. E. & Tiwari-Woodruff, S. K. Functional recovery of callosal axons following demyelination: a critical window. *Neuroscience* 164, 1407–1421 (2009).

62. Dupree, J. L. et al. Oligodendrocytes assist in the maintenance of sodium channel clusters independent of the myelin sheath. *Neuron Glia Biol.* 1, 179–192 (2004).

63. Wortman, J. et al. Axonal transport: how high microtubule density can compensate for boundary effects in small-caliber axons. *Biophys. J.* 106, 813–823 (2014).

64. Cail, C. L. & Bergles, D. E. Cortical neurons exhibit diverse myelination patterns that scale between mouse brain regions and regenerate after demyelination. *Nat. Commun.* 12, 4767 (2021).

65. Micheva, K. D., Kiraly, M., Perez, M. M. & Madison, D. V. Conduction velocity along the local axons of parvalbumin interneurons correlates with the degree of axonal myelination. *Cereb. Cortex* 31, hhab018- (2021).
Acknowledgements
The authors are thankful to Fred de Winter and Joost Verhaagen (NIN–KNAW) for the support for developing and producing the AAV constructs. We thank Christian Lohmann for critical reading of the manuscript and feedback during this research. We thank Diego de Stefani for generously sharing the 4mt-GCaMP6f plasmid with us. We thank Christiana Levent for generously sharing the AAV1-CAG-Flex-mRuby2-GSG-P2A-GCaMP6f virus with us. We thank Arnoldo Zaldivar Castro for optimizing the immunohistochemistry protocol. This study was, in part, supported by The Dutch Research Council (NWO, Vici 865.17.003) to M.K., a ZonMW Off Road grant 04510012010066 to K.K. and an Erasmus scholarship G ATHINE 01 to M.E.B.

Author contributions
Conceptualization: M.K. and K.K. Methodology: M.K., K.K., M.E.B. and B.V. Investigation: K.K., M.E.B., B.V. and N.P. Formal analysis, M.K., K.K., M.E.B. and B.V. Visualization: M.K. and K.K. Funding acquisition: M.K. and K.K. Project administration: M.K. Supervision: M.K. and K.K. Writing—original draft: K.K. Writing—review & editing: M.K. and K.K.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-35350-x.

Correspondence and requests for materials should be addressed to Koen Kole or Maarten H. P. Kole.

Peer review information Nature Communications thanks Julia Edgar and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permissions information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022