Neuronal activity regulates remyelination via glutamate signalling to oligodendrocyte progenitors

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Myelin regeneration can occur spontaneously in demyelinating diseases such as multiple sclerosis (MS). However, the underlying mechanisms and causes of its frequent failure remain incompletely understood. Here we show, using an in-vivo remyelination model, that demyelinated axons are electrically active and generate de novo synapses with recruited oligodendrocyte progenitor cells (OPCs), which, early after lesion induction, sense neuronal activity by expressing AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid)/kainate receptors. Blocking neuronal activity, axonal vesicular release or AMPA receptors in demyelinated lesions results in reduced remyelination. In the absence of neuronal activity there is a ~6-fold increase in OPC number within the lesions and a reduced proportion of differentiated oligodendrocytes. These findings reveal that neuronal activity and release of glutamate instruct OPCs to differentiate into new myelinating oligodendrocytes that recover lost function. Co-localization of OPCs with the presynaptic protein VGlut2 in MS lesions implies that this mechanism may provide novel targets to therapeutically enhance remyelination.

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Remyelination of the central nervous system (CNS) axons is essential for functional recovery after demyelinating injuries. It can occur as a spontaneous regenerative process in multiple sclerosis (MS) and other neurological conditions, including spinal cord injury. Oligodendrocyte progenitor cells (OPCs), which comprise ~5% of all cells in the adult CNS and are the principal proliferative cell type, respond to demyelinating injury by differentiating into myelinating oligodendrocytes to restore function\(^2,3\). This regenerative process is controlled by a precisely orchestrated but still incompletely understood array of signalling events\(^4\). However, remyelination often fails, primarily owing to failure of OPC lineage progression rather than a depletion of OPCs, many of which persist in chronic demyelinated lesions\(^5\). Thus, many attempts at promoting remyelination therapeutically have focused on the control of OPC differentiation. A critical feature of the injury environment that has been largely overlooked is the demyelinated axon and the role it might play in regulating OPC lineage progression during remyelination.

In development, white matter OPCs express glutamate receptors\(^6\) and receive synaptic input\(^6,8–11\) from unmyelinated axons\(^9,10\), enabling them to monitor and respond to neuronal activity. Both the synaptic input and glutamate signalling regulate OPC proliferation\(^1,11,12\) and myelination\(^13–16\), although neuronal activity is not essential for myelination\(^13,17\). In demyelinated lesions of the corpus callosum OPCs and progenitor cells recruited from the subependymal zone receive synaptic input from axons\(^18,19\). However, it is unclear whether this input is from unmyelinated corpus callosum axons, as up to 70% of callosal axons are unmyelinated\(^20\) or from demyelinated axons that generate de novo synapses to recruited OPCs. Even less is known of the effects of such signalling on remyelination. Moreover, it is uncertain of the extent to which glutamate exacerbates demyelination via excitotoxicity\(^21,22\) or promotes remyelination\(^13,23\).

Here we show that demyelinated axons generate synaptic contacts to OPCs, and that neuronal activity regulates remyelination, by synaptic release of glutamate, instructing OPCs to differentiate into new myelinating oligodendrocytes.

Results

The toxin-induced demyelination model. To investigate the signalling between demyelinated axons and OPCs within a demyelinated area, we used a toxin-induced focal demyelinating lesion (Fig. 1a,b). In this model, there is little axonal pathology and remyelination proceeds to completion with a clear temporal separation between the acute demyelination phase and the subsequent stages of remyelination\(^24,25\). Moreover, the adult rat caudal cerebellar peduncle (CCP) is fully myelinated at the time of toxin injection; thus, all bare axons are demyelinated axons (Supplementary Fig. 1 and see Methods)\(^24\).

First, we addressed whether demyelinated axons in the CCP conduct action potentials (Fig. 1c–g). During developmental myelination of the CCP, there is a clear separation between the conduction speed of myelinated and unmyelinated axons (Fig. 1d). However, at 3 months of age, at the time of lesion induction, the latency peak associated with conduction in unmyelinated axons was undetectable (Fig. 1e, black trace), indicating that all CCP axons are myelinated at this time. To determine whether the demyelinated axons can still conduct, we recorded from the lesions at 7 days post lesion (dpl), at the time when OPC recruitment is at its maximum\(^25\) (Fig. 1f, j). We found that demyelinated axons conducted action potentials with a speed \((0.29 \pm 0.04 \text{ m s}^{-1})\) similar to that of unmyelinated axons during development \((0.45 \text{ m s}^{-1})\) at p19; Fig. 1d,e), thus demonstrating that all the axons within the focal CCP lesion are demyelinated but, most importantly, are capable of impulse conduction. Recovery of fast action potential propagation was established by 21 dpl, when remyelination is largely complete (Fig. 1f,g), with conduction speeds identical to those of the pre-lesion adult CCP \((1.12 \pm 0.13 \text{ versus } 0.99 \pm 0.12 \text{ m s}^{-1})\), respectively; \(P = 0.48\), one-way analysis of variance (ANOVA) followed by Holm–Bonferroni test, \(n = 5\) and significantly faster than at 7 dpl \((P = 0.008\), one-way ANOVA followed by Holm–Bonferroni test, \(n = 3\)). Therefore, we have clearly established that in this lesion model demyelinated axons can conduct, with the speed of unmyelinated axons, and there is a full functional recovery of conduction speed on remyelination. Although remyelination generates thinner myelin than developmental myelination\(^26\), the resulting internodes are shorter\(^27\), which, along with other changes such as nodal geometry and voltage-gated sodium channel density, may compensate for the thinner myelin\(^28\) and restore the conduction velocity to its normal value\(^29\).

Second, we investigated the cellular profile of the demyelinating lesion as it progresses from demyelination to remyelination. Ethidium bromide (EB) is cytotoxic and will damage all nucleated cell types but leaves the axons intact (Supplementary Fig. 1 and see Methods)\(^24\); thus, all cells within the repairing lesion are recruited from surrounding tissue. As previously demonstrated for early timepoints\(^24\), we found that the lesions lack astrocytes (Fig. 1h,i,j); however, at 14 dpl astrocytes emerge from the lesion edge and at 21 dpl they have fully re-colonized the lesion, but appear slightly more immunoreactive to glial fibrillary acidic protein (GFAP) than in the adjacent normal-appearing white matter. OPCs (NG2\(^+\) and Olig2\(^+)\) are present at 3 dpl\(^25,30\) and reach a much higher density than in normal-appearing adult

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Figure 1 | The toxin-induced demyelination model. (a) Demyelinated lesions were created by injection of 0.01% EB into the rat CCP (Crb, cerebellum). (b) Acute cerebellum and brainstem section containing a lesion in the CCP (delineated by dashed lines). The needle track going through the cerebellum is visible on the left of the vertical dashed line. Scale bar, 2 mm. (c) Fixed section after compound action potential recording, the lesion (delineated by dashed lines) is visible with 4,6-diamidino-2-phenylindole (DAPI) staining due to increased macrophages and OPC infiltration; the location of stimulating (stim, dark blue DiD) and recording (rec, red DiI) electrodes are marked. Scale bar, 200 μm. (d–f) TTX-subtracted compound action potential recordings. (d) At p19, peaks for both myelinated (M) and non-myelinated axons (NM) were detected. (e,g) At 7 dpl, demyelinated axons (grey trace) have a peak latency similar to non-myxelinated axons, whereas (f,j) when remyelinated at 21 dpl (grey trace) the peak latency is similar to adult myelinated axons (black trace). Numbers of brain slices are shown on bars. Data represent means ± s.e.m. The P-value is from Holm–Bonferroni post-hoc test after a one-way ANOVA (\(P = 0.003\)). (h) Timeline of GFAP expression in CCP lesions at 3, 7, 14 and 21 dpl; scale bar, 200 μm. (i) Magnified images from h of the regions indicated by the white box. (j) Timeline of oligodendrocyte lineage cells in the lesion, with Olig2\(^+\) and NG2\(^+\) OPCs, and (k) CC1\(^+\) oligodendrocytes. Scale bars, 20 μm. (l) Diagram showing the peak appearance of oligodendrocytes, OPCs and astrocytes in the CCP following EB lesion. The red shaded area marks the timing of the EB injection and the onset of demyelination with the associated death of all nucleated cell types within the lesion. Re-colonization of the lesion occurs as follows: OPCs arrive in the lesion and proliferate before differentiating into oligodendrocytes. Astrocytes start to repopulate the lesion in week 2. The orange shading indicates the remyelination process; darker orange corresponds to more remyelination.
white matter at 5–7 dpl (Fig. 1j,l), but by 21 dpl their numbers have declined to near-normal adult white matter levels. Staining for CC1, a marker of differentiated oligodendrocytes, at 3 dpl is punctate/globular and is likely to be the postdemyelination remnants of antigen present within phagocytic macrophages. We detected few differentiated oligodendrocytes (CC1+) at 7 dpl (Fig. 1k,l), but their numbers overshot at 14 dpl before decreasing to reach a density similar to that in normal-appearing white
matter at 21 dpl (Fig. 1k,l). This indicates that there is an overproduction of newly formed oligodendrocytes (CC1⁺) that presumably never reach targets and die, similar to as occurs during development²¹.

Together, these data reveal that the lesion provides an excellent model to study the interactions between demyelinated axons and OPCs in vivo, because at early timepoints, it lacks astrocytes and is only populated by OPCs and macrophages; all OPCs within the lesion have been recruited from surrounding areas; the axons are healthy and electrically active; and all axons lacking myelin within the lesion were previously myelinated.

Recruited OPCs express glutamate receptors. To test whether adult OPCs recruited to areas of demyelination can sense glutamate released from active axons, as they do during development, we voltage-clamped OPCs within the lesion at 5–8 dpl in acute brain slices containing the lesion (Fig. 1b). OPCs were identified by their dye-filled morphology, electrophysiological properties and by post-recording immunohistochemistry⁸ (Fig. 2a,b and see Methods). OPCs were both morphologically and electrophysiologically distinct from macrophages (Supplementary Fig. 2), which are large circular cells that lack voltage-gated sodium currents typical of OPCs⁸ but have a slowly

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**Figure 2 | Glutamate signalling in remyelinating lesions.** (a) Voltage-clamped OPC, filled with lucifer yellow (LY) and identified after recording by labelling for NG2 and Olig2 (scale bar, 10 μm) with voltage-gated sodium currents (20 mV steps from −74 mV; inset: leak subtracted trace). (c) Response of a recruited OPC to 100 μM glutamate (Glu). (d) Mean peak current for glutamate-evoked currents in recruited OPCs (a specimen trace shown in c) and macrophages (e–g). The glutamate-evoked current is unaffected by 200 μM AP5 (P = 0.4, n = 6) but inhibited by 25 μM NBQX (P = 5 × 10⁻⁶, n = 7, one sample t-test). (h) AMPA (20 μM), kainate (30 μM) and NMDA (60 μM) evoked inward currents in OPCs. (j) Proportion of OPCs with AMPA-evoked currents (in the first week post lesion, 6 out of 6 cells recorded showed AMPA-evoked currents; in the second week, 13 out of 13 cells responded to AMPA) and NMDA-evoked currents; NMDA-evoked currents only became detectable in OPCs at the start of the second week post lesion induction, P = 0.026 (χ²-test with Yates correction, compared with the first week; in the first week, 0 out of 8 cells recorded had detectable NMDA-evoked currents; for the second week, 8 out of 14 had detectable NMDA receptor responses). (k) Current–voltage relationship (voltage ramp from −134 to +26 mV) for glutamate-evoked current in OPCs (n = 7). (l–n) Glutamate-evoked [Ca²⁺⁺]ᵢ rises in OPCs but not in macrophages (P = 6.2 × 10⁻⁵, Welch’s corrected unpaired t-test, n = 11 and 6 cells, respectively, from 3 independent experiments) measured by taking the fluorescent intensity ratio of Fluo-4/ FuraRed (low Ca²⁺⁺: red; high Ca²⁺⁺: green). Scale bar, 10 μm. Numbers of cells are indicated on bars, data represent means ± s.e.m. in d.g.i.n.
activating outward current at potentials above 40 mV, characteristic of microglia\(^22,33\) (Supplementary Fig. 2b,d). Despite a small proportion of macrophages expressing NG2, we never observed co-expression of Olig2 in these cells (Supplementary Fig. 2e). Glutamate evoked an inward current in NG2\(^+\) and Olig2\(^+\) OPCs (100 \(\mu\)M, 25.1 \(\pm\) 5.8 pA at \(-74\) mV; Fig. 2c,d) but not in macrophages (Fig. 2d and Supplementary Fig. 2c). The glutamate-evoked current in NG2\(^+\) and Olig2\(^+\) OPCs was reduced by the selective AMPA (2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid)/kainate receptor antagonist NBQX (2.3-dihydroxy-6-nitro-7-sulfamolybenzo[f]quinoxaline-2,3-dione; 25 \(\mu\)M)\(^34\), but not the selective NMDA (N-methyl-D-aspartate) receptor antagonist D-AP5 (\(\alpha\)-)-2-amino-5-phosphonovaleric acid; 200 \(\mu\)M)\(^34\) (Fig. 2e–g; \(P = 5 \times 10^{-6}\) and \(P = 0.4\), respectively, one sample \(t\)-test, \(n = 7\) and 6, respectively). Similarly, both AMPA (20 \(\mu\)M) and kainate (30 \(\mu\)M), but not NMDA (60 \(\mu\)M), evoked an inward current in OPCs (Fig. 2h,i). However, at the beginning of the second week post lesioning, NMDA-evoked currents in OPCs could be detected (Fig. 2j); NMDA currents were detected in 0 out of 8 OPCs in the first week but in 8 out of 14 OPCs in the second week: \(P = 0.026\), \(\chi^2\)-test with Yates correction), along with AMPA receptor currents (detected in 6 out of 6 OPCs in the first week and in 13 out of 13 OPCs in the second week). This suggests that, at the time of recruitment (the first week post lesioning), OPCs in the lesions mainly express AMPA and kainate receptors, making them capable of sensing glutamate released from active neurons.

The positive rectification of glutamate-evoked currents in the recruited OPCs varied, with the rectification index \(([I_{+20mV} - I_{-20mV}] / I_{rev}) / ([I_{+20mV} - I_{-20mV}] / I_{rev})\) ranging from 0.18 to 2 (Fig. 2k), indicative of OPCs expressing a variable mixture of non-calcium-permeable and calcium-permeable AMPA receptors\(^35\). Glutamate (100 \(\mu\)M) elicited a rise in intracellular calcium concentration \([(Ca^{2+})]_i\) in ~60% of OPCs (11 out of 18 cells) in the lesions (Fig. 2l–n and Supplementary Fig. 3d,e) and only in these cells were spontaneous \([Ca^{2+}]_i\) spikes detected (Fig. 2m). No \([Ca^{2+}]_i\) changes or glutamate-evoked responses were detected in macrophages (\(P = 6 \times 10^{-5}\), Welch’s corrected unpaired \(t\)-test, \(n = 6\) for macrophages, \(n = 13\) for OPCs; Fig. 2l–n) or demyelinated axons (Supplementary Fig. 3d,e).

Given that at the time of OPC recruitment the lesion is devoid of astrocyte cell bodies\(^24\) (Fig. 1h,i), these data indicate that at this point the OPCs are the only cells within the lesion capable of responding to glutamate.

### Demyelinated axons generate de novo synapses with OPCs.

During development, white matter OPCs receive synaptic input\(^8,9,10\) from unmyelinated axons\(^9,10\), via postsynaptic AMPA receptors\(^36\), and after demyelinating injury corpus callosal axons generate synapses with recruited sub-ependymal zone progenitors\(^18\) and OPCs\(^19\). However, as the majority of corpus callosal axons are unmyelinated, we assessed whether bona fide demyelinated axons are capable of generating synaptic inputs to OPCs by voltage clamping OPCs within the EB lesion, where all axons were previously myelinated. We detected spontaneous excitatory synaptic currents \(\tau_{decay} = 0.78 \pm 0.03\) ms, \(n = 15\); Fig. 3a,d; in some OPCs, outward currents were also detected, presumably mediated by GABAergic input\(^8\), reminiscent of the EPSCs detected at a similar frequency \((P = 0.91,\text{ unpaired } t\text{-test, } n = 6)\) in OPCs in the mouse CCP at p7, at the start of myelination\(^57\) (Fig. 3b,d). In contrast, when myelination was complete, in 3-month-old mice, synaptic inputs were barely detected (in 1 out of 8 cells, a single event above noise levels was detected compared with 7 out of 8 cells in young CCP with multiple events per cell; \(P = 0.01\), \(\chi^2\)-test with Yates correction; Fig. 3b–d) and the total frequency of synaptic events detected when myelination was complete was 0.16 \(\pm\) 0.16 mHz compared with 10.8 \(\pm\) 3.7 mHz during development \((P = 0.012,\text{ unpaired } t\text{-test, } n = 8)\), indicating that OPCs receive relatively few, if any, synaptic inputs in a white matter tract that is fully myelinated. The events detected in lesions were abolished by blocking action potentials with 1 \(\mu\)M tetrodotoxin (TTX; Fig. 3e) and increased in frequency with 1 \(\mu\)M \(\alpha\)-latrotoxin, which, as expected for this concentration\(^38\), promoted presynaptic neurotransmitter release by \(\sim 2.5\)-fold \((3.1 \pm 0.6 \text{ versus } 7.8 \pm 0.8 \text{ mHz}; P = 0.02,\text{ paired } t\text{-test, } n = 4); Fig. 3f,g\)\(^10\). The pharmacological properties and the fast kinetics of these inputs are identical to those of the synaptic inputs that OPCs receive from unmyelinated axons\(^9,10\) and differ greatly from what would be expected if generated by reversed cycling of glutamate transporters\(^39,40\). Thus, these results indicate the existence of defined synaptic structures between recruited OPCs and demyelinated axons, which form after demyelination.

To examine the pattern of expression of presynaptic terminals within demyelinated lesions, we labelled for VGluT2, a marker for glutamatergic presynaptic release sites known to be expressed by neurons whose axons pass through the CCP\(^40\). In the demyelinated lesion, there was clear punctate labelling for VGluT2 (observed for seven lesions). In contrast, VGluT2 labelling was undetectable within the nearby normal-appearing white matter (Fig. 3i) and in the adult white matter of unlesioned animals (Fig. 3h). The punctate VGluT2 labelling seen in the lesion was similar to that seen in the grey matter (Fig. 3b,i). The intensity of neurofilament labelling was unchanged between the lesion and normal white matter areas (Fig. 3k), demonstrating equal detection of intra-axonal proteins between myelinated and demyelinated fibres. Similar to what occurs for unmyelinated axons during development, presynaptic formations occurred along the demyelinated axons independent of OPC proximity\(^9\) (Fig. 3q, top panel), although strikingly, OPC processes seemed to be preferentially located near VGluT2 puncta on the demyelinated axons (Fig. 3l–q; observed for 196 NG2\(^+\) cells with an average of 10 \(\pm\) 2 VGluT2 puncta per cell). Together, these data strongly suggest that demyelinated axons establish de novo synapses with recruited OPCs.

### Synaptic transmission regulates remyelination.

To examine the functional importance of the synaptic input from the demyelinated axons to the OPCs, we infused either 0.9% saline or an antagonist to specifically block distinct stages of synaptic transmission into the lesion via an intracerebral cannula, connected to an osmotic minipump. Infusions into the lesion were from 3 dpl, when OPCs are being recruited, to 21 dpl\(^25\) (see Methods and Fig. 1j,l). We have previously shown that the delivery is local to the lesion, as infusing dye into the lesion via osmotic minipump results in the dye being confined within the lesion\(^41\). First, we infused 11 \(\mu\)M \(\alpha\)-conotoxin MVIIIC, a selective N-, P- and Q-type calcium channel blocker\(^42\), to block vesicular release of neurotransmitter, from 3 dpl. As the lesion is devoid of astrocyte cell bodies\(^24\) (Fig. 1h,i), apart from axons only OPCs and (monocyte- and microglia-derived) macrophages are present and neither express functional N-, P- and Q-type voltage-gated calcium channels\(^43,44\); thus, we attribute the effects of \(\alpha\)-conotoxin MVIIIC to its action on demyelinated axons. Blinded ranking analysis of remyelination, an established histological analysis that assesses cross-sections of the entire lesion, revealed that blocking vesicular neurotransmitter release significantly impaired remyelination compared with saline controls \((P = 0.01,\text{ Mann–Whitney rank analysis, } n = 6\) per group; Fig. 4a,b,h), with fewer axons remyelinated (saline: 63 \(\pm\) 4%,
Figure 3 | OPCs in remyelinating lesions receive synaptic inputs. (a) Recruited OPCs receive synaptic input from demyelinated axons (15/45 cells; inset: specimen of a fitted synaptic event), similar to (b) OPCs at the start of developmental myelination in the CCP of NG2-EYFP knock-in mice (seven out of eight cells recorded; inset: specimen of a fitted synaptic event), whereas (c) after myelination of the CCP is complete OPCs barely receive synaptic inputs (one out of eight cells recorded; P = 0.01, χ²-test with Yates correction compared with young CCP). (d) Average frequency of detected synaptic input in OPCs in lesion (5–8 dpl), in the CCP at the start of myelination (p5–8 NG2-EYFP mice) and once myelination is complete (p98–105 NG2-EYFP mice). Data are means ± s.e.m., significance tested by unpaired t-test, n = 6. The circles underneath the bar graph depict the proportion of OPCs with (black) and without (white) synaptic inputs. (e) The synaptic inputs are blocked by 1 μM TTX. (f,g) α-Latrotoxin (α-LTX; 1 nM) increases the frequency of synaptic events detected in OPCs (P = 0.02, paired Student’s t-test, all cell numbers are indicated on each bar graph, data are means ± s.e.m.). (h) The presynaptic marker VGluT2 is present in the adult cerebellar grey matter (GM), but not in normal white matter (WM). Scale bar, 50 μm. (i,j) At 7 dpl, NG2 and VGluT2 staining are increased in demyelinating lesions. Scale bar, 500 μm. (k) Neurofilament (NF) is equally detectable in normal and lesioned white matter. Scale bar, 200 μm. (l-n) OPCs establish synapses with demyelinated axons. Scale bar, 20 μm. (o) Magnifications of the cell in l-n generated using Imaris software, showing synapses located on the OPC processes. Scale bar, 20 μm. (p) Iso-surface 3D deconvolution of the cell in l-n generated using Imaris software, showing synapses located on the OPC processes. Scale bars, 5 μm. (q) Top: synaptic vesicles along a demyelinated axon; bottom: OPC extending processes towards VGluT2 vesicles. Scale bars, 5 μm.

ω-conotoxin MVIIIC: 23 ± 5%; P = 5 × 10⁻⁴, unpaired t-test, n = 6), but had no effect on lesion size (saline: 0.53 ± 0.05 mm², ω-conotoxin MVIIIC: 0.53 ± 0.05 mm²; P = 0.97, unpaired t-test, n = 6 per group). This indicates that blocking vesicular release of neurotransmitter reduces remyelination, similar to that during development for myelination15,16.

Second, we blocked AMPA/kainate receptors, which are expressed on the OPCs (Fig. 2) and are mainly located postsynaptically at the axon-OPC synapse36, by infusing NBQX from 3 dpl (250 μM; Fig. 4c,d,i), a selective AMPA/kainate receptor antagonist that does not affect NMDA and metabotropic glutamate receptors34, into the lesion. As macrophages do not respond to glutamate (Fig. 2d,l–n and Supplementary Fig. 2c) and demyelinated axons have not been reported to express AMPA receptors (nor did we detect glutamate-evoked calcium changes in demyelinated axons), we attribute the action of NBQX to be on OPCs, presumably at the postsynaptic site. Blocking AMPA/kainate receptors reduced remyelination (P = 0.039,
Mann–Whitney rank analysis, \( n = 7 \) per group) without affecting lesion size (0.52 ± 0.06 mm\(^2\) (saline) versus 0.66 ± 0.08 mm\(^2\) (NBQX); \( P = 0.20 \), unpaired t-test, \( n = 7 \) per group).

This reduction in remyelination significantly affected conduction speed through the lesion. We detected latency peaks associated with conduction of myelinated fibres in all lesions infused with saline but detected only in 25% of lesions infused with NBQX (Fig. 5b), which had a slightly slower conduction speed (0.78 ± 0.02 ms\(^{-1}\) (saline) versus 0.67 ± 0.02 ms\(^{-1}\) (NBQX); \( P = 0.037 \), unpaired t-test, \( n = 4 \)). The majority of axons in NBQX-treated lesions were demyelinated, as 100% of lesions had a latency peak associated with demyelinated axons (a conduction speed of 0.43 ± 0.01 ms\(^{-1}\) ); \( P = 0.027 \), unpaired t-test, \( n = 4 \); Fig. 5b). The reduced recovery of saline-treated lesions indicates that saline dilutes some essential factors for remyelination, as a full recovery of the action potential propagation is not fully established by 21 dpl in saline, unlike in uninfused lesions (Fig. 1f,g). Nevertheless, the demyelinated latency peak in NBQX-treated lesion had a far larger area (0.03 ± 0.00 mV*ms (saline) versus 0.19 ± 0.03 mV*ms (NBQX); \( P = 2.8 \times 10^{-5} \), unpaired t-test, \( n = 4 \)). The peak area reflects the activity of all the stimulated axons and is thus directly proportionate to the number of demyelinated axons, indicating that there are many more demyelinated axons in NBQX-treated samples. Together, these results demonstrate that AMPA/kainate receptors are important for efficient remyelination.

Previously, we have shown that blocking NMDA receptors with MK-801, a highly selective open channel NMDA receptor antagonist\(^3\), reduces remyelination\(^1\), similar to blocking AMPA receptors or vesicular release of glutamate. As we only detect NMDA receptor expression in OPCs at the second week post

Figure 4 | Remyelination is dependent on neuronal activity. Toluidine blue-stained semi-thin sections of CCP 21 dpl, infused with saline, or from 3 dpl (a), \( \omega \)-conotoxin (11\( \mu \)M; blocking neurotransmitter vesicular release), (c) NBQX (250\( \mu \)M; blocking AMPA/kainate receptors), or (e) TTX (50nM) (scale bar, 20\( \mu \)m) and (b,d,f) scored by blinded ranking analysis; higher ranks represent more remyelination, each symbol represents one animal, \( P \)-values are from Mann–Whitney U-test. (g–j) High-magnification images of (g) normal-appearing white matter and (h–j) saline (left), and (h) \( \omega \)-conotoxin-, (i) NBQX- and (j) TTX (right)-infused lesion. Scale bar, 5\( \mu \)m.
Figure 5 | Remyelination depends on activation of AMPA receptors within the lesion at early timepoints. (a) TTX-subtracted compound action potential recordings from saline (black trace) and NBQX-treated lesions from 21 dpl (grey trace). (b) Average conduction speed for the latency peak associated with myelinated axons (M peak) and non-myelinated axons (NM), for saline- and NBQX-treated samples (data from four slices for each condition). Circles underneath depict the proportion of recorded lesions with the latency peak detected (white fraction: no detection of peak; black/grey: proportion of lesion underneath depict the proportion of recorded lesions with the latency peak detected (white fraction: no detection of peak; black/grey: proportion of lesion where the latency peak is detected in saline/NBQX-treated lesions). (c) The average latency peak area reflects the number of axons contributing to each peak. It is noteworthy that there are fewer axons contributing to the latency peak associated with myelinated axons and far more contributing to the peak associated with demyelinated axons in NBQX-treated samples (P-values are from unpaired t-test). (d) The timeline diagram of the lesion remyelination process (Fig. 1l), shaded with grey box to indicate when NBQX infused at later timepoints is present. (e) Toluoyline blue-stained semi-thin sections of CCP 21 dpl, infused with saline or (f) NBQX (250 μM; blocking AMPA/kainate receptors) from 10 dpl (scale bar, 10 μm) (g) scored by blinded ranking analysis; higher ranks represent more remyelination, each symbol represents one animal, P-values from Mann–Whitney U-test.

lesioning (Fig. 2), at a time when OPCs are starting to differentiate25 (Fig. 1k,l), but AMPA/kainate receptors are expressed at early timepoints and directly involved in synaptic transmission36, we hypothesized that AMPA/kainate receptors are important for early stages of the remyelination process and NMDA receptors for the later stages13. To test this hypothesis, we blocked AMPA receptors at the second week post lesioning (Fig. 2j), at a time when OPCs are starting to differentiate and NMDA receptors start to be expressed. Blocking AMPA receptors at the second week post lesioning did not affect remyelination (Fig. 5e–g; P = 0.86, Mann–Whitney rank analysis, n = 4) unlike when blocking AMPA receptors from 3 dpl. These data along with our previous results13 indicate that AMPA/kainate receptor activation is essential for the early stages of remyelination, whereas NMDA receptors are important for later stages of remyelination13.

Lastly, blocking neuronal activity in the lesion with 50 nM TTX, from 3 dpl, reduced remyelination (Fig. 4e,f,j; P = 0.03, Mann–Whitney rank analysis, n = 5) without affecting lesion size (0.64 ± 0.03 mm² (saline) versus 0.48 ± 0.13 mm² (TTX); P = 0.29, unpaired t-test, n = 5). At the ultrastructural level, it was clear that significantly fewer axons were remyelinated when neuronal activity was blocked (P = 0.005, unpaired t-test, n = 3; Fig. 6a,b). Blocking neuronal activity with TTX did not affect total axonal number in the lesion (saline: 805161 ± 93822 axons per mm²; TTX: 649462 ± 111669 axons per mm²; P = 0.31, unpaired t-test, n = 3), but it did affect remyelination of smaller diameter axons more than larger diameter axons (P < 0.001, Kolmogorov–Smirnov test, n = 3; Fig. 6f). In the few axons that were remyelinated when neuronal activity was blocked, the g-ratio (the ratio of axon diameter to the outside diameter of the myelin) was higher compared with saline, indicating a thinner myelin sheath (P = 0.003, unpaired t-test, n = 3; Fig. 6c–e); although the myelin thickness was near identical for larger-diameter axons (axonal diameter ≥ 2 μm, g-ratio saline: 0.93 ± 0.005, TTX: 0.93 ± 0.002; P = 0.95, unpaired t-test, n = 3 lesion), it was markedly thinner for small-diameter axons (axonal diameter < 2 μm, g-ratio saline: 0.86 ± 0.002, TTX: 0.88 ± 0.002; P = 4 × 10⁻⁶, unpaired t-test, n = 3 lesions). Together, this
shows that efficient remyelination, especially at smaller-diameter axons, depends on neuronal activity and the synaptic communication between demyelinated axons and OPCs.

**Neuronal activity regulates OPC differentiation.** OPC migration and proliferation can be regulated by neuronal activity\(^{18,45,46}\) and glutamate\(^{12,47,48}\). Therefore, we investigated whether blocking neuronal activity with TTX affected OPC recruitment by staining lesions for NG2 and Olig2. Remarkably, there was a 6-fold increase in the number of OPCs (defined as NG2\(^+\) and Olig2\(^+\) cells) in the TTX-treated lesions compared with saline controls (33 ± 9 versus 222 ± 22 cells per mm\(^2\); \(P = 4 \times 10^{-8}\), Welch’s corrected unpaired \(t\)-test, TTX: \(n = 4\) and saline: \(n = 3\)) and a 2.7-fold increase in Olig2\(^+\) cells (Fig. 7a,b; \(P = 5.8 \times 10^{-7}\), Welch’s corrected unpaired \(t\)-test, TTX: \(n = 4\) and saline: \(n = 3\)).

With a higher proportion of Olig2\(^+\) cells being NG2\(^+\) OPCs (Fig. 7c,d; \(P = 0.016\), Welch’s corrected unpaired \(t\)-test, TTX: \(n = 4\) and saline: \(n = 3\)), there was a 6-fold increase in the number of OPCs (defined as NG2\(^+\) and Olig2\(^+\) cells) in the TTX-treated lesions compared with saline controls (33 ± 9 versus 222 ± 22 cells per mm\(^2\); \(P = 4 \times 10^{-8}\), Welch’s corrected unpaired \(t\)-test, TTX: \(n = 4\) and saline: \(n = 3\)).

Axonal diameter (\(\mu m\))

\[g-Ratio = \frac{Axonal diameter}{Axonal girth}\]

Figure 6 | Neuronal activity regulates remyelination. (a,b) Ultrastructural analysis reveals that fewer axons are remyelinated (green) in lesions treated with TTX (from 3 dpl) compared with saline-treated lesions; unmyelinated axons are coloured in yellow. Scale bar, 1 \(\mu m\). (c,d) The g-ratio is larger in TTX-treated samples than in saline-treated lesions. Number of animals are indicated on each bar graph. Data represent mean ± s.e.m. \(P\)-values are from unpaired \(t\)-test for b,c. For d, each symbol represents an axon and \(P\)-value from analysis of covariance (ANCOVA). (e,f) Blocking synaptic input induces a shift in the cumulative frequency distribution of g-ratio and axonal diameter, \(P\)-values from Kolmogorov-Smirnov test.

**Human demyelinated axons upregulate synaptic proteins.** To determine the significance of our finding for human white matter disease, we examined the expression of VGluT2 in human MS lesions and adjacent normal-appearing white matter (or non-demyelinated white matter). Similar to the rodent demyelinated lesions, there was an approximately sixfold increase in VGluT2 expression in the human MS lesions compared with the normal-appearing white matter (Fig. 8a-e; \(P = 4.1 \times 10^{-7}\), unpaired \(t\)-test, \(n = 5\) lesions from 5 patients) and the VGluT2 puncta were observed in close proximity of NG2\(^+\) cells present in the MS lesions (Fig. 8f; detected for 150 NG2\(^+\) cells), with NG2\(^+\) processes nearly always located near the VGluT2 puncta. Together, these results strongly suggest that, after white matter injury, demyelinated axons establish de novo synapses with recruited OPCs in humans.
Discussion
Remyelination is essential for functional recovery after demyelinating injury. However, spontaneous remyelination often fails, primarily due to failure of OPC differentiation rather than OPC depletion\(^5\). Here we have shown that glutamatergic synaptic transmission between demyelinated axons and OPCs, acting via AMPA receptors, regulates remyelination, and that blocking neuronal activity keeps OPCs in a proliferative stage, preventing them from fully differentiating into myelinating oligodendrocytes. This may explain why remyelination fails in disease.

Figure 7 | Neuronal activity regulates OPCs differentiation. (a,b) At the time of differentiation (14 dpl), there was an increase in the number of Olig2\(^+\) (marking all cells of the oligodendrocyte lineage) cells in TTX-treated lesions (from 3 dpl) compared with saline controls due to (c,d) a higher proportion of Olig2\(^+\) cells that were NG2\(^+\) OPCs (filled arrowheads) and (e,f) a reduced proportion of Olig2\(^+\) cells that were CC1\(^+\) differentiating oligodendrocytes (open arrowheads), indicating that synaptic input is needed for efficient differentiation. (g,h) At 5 dpl, there are more proliferating Olig2\(^+\) cells in TTX-treated lesions, as shown by EdU incorporation (g) and by Ki-67 staining (h). (i) No change in proliferation was detected by Ki-67 staining in Olig2\(^+\) cells between TTX and saline-treated lesions at 14 dpl. However, at 14 dpl there is an (j) increase in apoptotic Olig2\(^+\) cells labelled for caspase 3. All n numbers represent animals (three sections per animal) and are indicated on each bar graph. Data are means ± s.e.m. P-values are from unpaired t-test, Welch’s corrected for (d) and (f). Scale bars, 20 μm.
We found that following demyelination of a CNS white matter tract where all the axons were previously myelinated, recruited OPCs receive synaptic inputs from demyelinated axons. On demyelination, axons increase their expression of the pre-synaptic protein VGlut2 and generate de novo synapses with recruited OPCs. We found that a similar mechanism may also occur in human MS lesions. At early timepoints after entering the lesion, the recruited OPCs express mainly AMPA and kainate receptors, presumably at the postsynaptic site. The role of the synaptic input to OPCs seems to be instructing them to remyelinate, as when we infused antagonists specific to distinct stages of synaptic transmission (o-conotoxin MVIIC to block vesicular release of neurotransmitter, NBQX to block postsynaptic AMPA receptors on the OPCs and TTX to block voltage-gated Na+ channels and thus neuronal activity) into the lesion, all of them impaired remyelination.

Two lines of evidence show that the antagonists are acting locally within the lesion. First, when a dye is infused into a lesion with an osmotic minipump (even with a faster flow rate and larger volume delivered than used in this study) the dye remains within the lesion. Second, no side effects were detected, such as severe axonal loss, thereby identifying a mechanism of white matter regeneration rather than of white matter damage.

Blocking neuronal activity with TTX, which blocks voltage-gated Na+ channels, reduced remyelination in a similar manner to blocking vesicular release of neurotransmitter with o-conotoxin MVIIC or to blocking with NBQX the AMPA/kainate receptors, located postsynaptically at the axon-OPC synapse. This resulted in fewer axons being remyelinated, with smaller diameter axons being more affected, just as blocking vesicular release of neurotransmitter preferentially affects myelination of small-diameter axons. We assume that the effects of TTX are predominantly to block axonal action potentials. However, OPCs also express voltage-gated Na+ channels and it is currently unknown what role they have in OPC biology. Although, we cannot exclude an effect of TTX on voltage-gated Na+ channels in OPCs, activation of OPC Na+ channels requires the membrane to become depolarized to ~ −40 mV, which is unlikely to occur without activity-driven input.

The lack of activity-driven signals in the TTX-treated samples caused an up to 3.5-fold increase in proliferation of OPCs at 5
oligodendrocytes, many of which die. This possibly reflects a too early development where there is an overproduction of oligodendrocytes, many of which die. This possibly reflects a spontaneous differentiation of OPCS, which occurs at high OPC density. Thus, a higher proportion of untargeted differentiation, owing to higher OPC density, could explain why equal numbers of CC1+ cells still lead to impaired remyelination in TTX-treated lesions.

The effect of blocking the activity of the demyelinated axons is likely to be mediated via synaptic input and glutamate release, as blocking vesicular release and AMPA receptors on OPCS also resulted in impaired remyelination. Previously, we showed remyelination depended on activation of NMDA receptors, but NMDA-evoked currents in OPCS only appear at the second week post lesion induction, which suggests that NMDA receptors only play a role at later stages of remyelination. Conversely, AMPA/kainate receptors are expressed throughout the regenerative process and activation of AMPA receptors during the period of proliferation is essential for remyelination, whereas activation of AMPA receptors later, at the onset of differentiation (a time when NMDA-evoked currents start to be detected), is not. Thus, using this remyelination model we have revealed a potential distinct role for different glutamate receptors expressed on OPCS. Presumably, glutamate released at the axon-OPC synapse activates the AMPA receptors directing OPCS towards the axon to myelinate, as synaptic inputs are important for axonal selection.

Once the axon-OPC contact is made, NMDA receptors (as they tend to be expressed on the OPCs) become important to direct myelination. Our data therefore suggest that neuronal activity fine-tunes OPC proliferation and times the onset of OPC differentiation by acting on AMPA receptors and regulates remyelination by acting on NMDA receptors. Thus, when OPCS do not receive synaptic inputs from active demyelinated axons they remain in a proliferative state, because they lack their inductive signal to differentiate.

Remyelination efficiency decreases with age, as a result of a failure of differentiation rather than OPC depletion. Our data indicate that for remyelination to occur the demyelinated axons must recapitulate early development and direct OPC differentiation by maintaining electrical activity to release glutamate onto OPCS. In fact, on demyelination, axons switch their isofrom of voltage-gated sodium channels (Na Channels) from Na Channels (found at the nodes of myelinated axons) to Na Channels (found in unmyelinated axons during development). Furthermore, in active MS lesions (many of which undergo remyelination), axons express Na Channels and upregulate synaptic proteins, whereas axons in chronic lesions that have failed to remyelinate do not. This, together with our results, suggests a commonality of response by injured axons: the demyelinated axons undergo a similar response to that of transected peripheral axons, which by reactivating silent growth programmes revert to a developmental state for efficient regeneration. Our results indicate that any perturbation of signals that induce demyelinated axons to remain active and/or regain synaptic contacts with recruited OPCS will result in a failure of OPC differentiation and repair. Consistent with our data, OPCS are present in chronic lesions, presumably lacking an inductive signal from the demyelinated axons for differentiation.

Hence, it is conceivable that the use of agonists that promote impulse propagation, vesicular release and/or AMPA receptor currents in OPCS may promote OPC differentiation and myelin repair in disease. Thus, our results reveal a missing link for understanding myelin regeneration and open up new and pharmacologically tractable avenues by which the arrest of OPC differentiation in chronic MS lesions could be therapeutically reversed.

Methods

Toxinduced focal demyelination. Female Sprague-Dawley rats aged 10–14 weeks of age (190–250 g) were used. Experiments were performed in compliance with the Office regulation on animal experiments and the German Animal Experiment Act. In this toxic model, lesions were created by an intracortical injection of 30 µl 3% CCI4 (if higher, cells were excluded from the analysis) and animals were culled by decapitation. Briefly, anesthesia was induced (4%) and maintained (1.5–2.5%) with isoflurane (Henry Schein, Animal Health, Dummerstorf, UK) and the animal placed in a stereotaxic frame. After incision of the skin, the holes were drilled at stereotaxically defined locations, then a Hamilton syringe lowered in the brain at an injection volume of 0.01% EB (in H2O) in 0.9% saline (Vetfixis) in a 0.4 M/s and the brain (in 0.4 M/s). The brain was sutured subcutaneously and the cannula was fixed to the skull with cyanoacrylate gel adhesive (applied under the base of the cannula head before insertion), as well as two anchoring screws and dental acrylic cement (a 1:1 volume mix of Palacos MG+ G powder and liquid, Heraeus Kulzer). Drug delivery into the lesion did not occur until 3 dpl (the start of the OPC recruitment stage), Fig. 11) or at 10 dpl (at the start of OPC differentiation). For the recordings of drug delivery into the lesion was controlled by varying the length of the tube that connects the micropipette to the cannula and by inserting a sterile corn oil drop (Sigma) to prevent diffusion of the drug solution into the initial saline solution. For proliferation studies at 5 dpl, EdU was administered by osmotic minipump, dissolved at 5 µg ml−1 of saline solution, from 3 dpl. We have previously shown that the delivery is local with no significant dye diffusion detectable outside the lesion. Rats were randomly assigned to treatment (co-ontoxin MVIIC, 11 µM; NBQX, 250 µM; TTX, 50 nM) or control groups (0.9% saline infusion). Samples were included in only if the cannula was at least 0.1 mm of the lesion. For remyelination studies, animals were perfused fixed under terminal anesthesia (pentobarbitone sodium) 3 weeks after lesion induction.

Acute brain slices for electrophysiology and Ca2+ imaging.

For the majority of the experiments, rat sagittal slices (225 µm for patch clamping and 300 µm for compound action potential) including the CCP containing the lesion were made, either between 5 and 10 dpl, when OPC numbers within the lesion peak, or at 21 dpl, when remyelination is near completion. For the recordings of synaptic input in normal adult white matter at P5–P7 and P98–P105 (3 months old), slices were obtained from NG2-EYFP knock-in male and female mice67 with the same procedure. Slices were made in an ice-cold bicarbonate-buffered (5% CO2/95% O2, pH 7.4) slicing solution containing (mM): 120 NaCl, 26 NaHCO3, 2.5 KCl, 2 MgCl2, 10 glucose, 1.25 CaCl2, 1.25 MgCl2, 1.25 NaH2PO4, 2.5 kynurenic acid (a broad-spectrum glutamate receptor antagonist). For all experiments, slices were superfused at 22 ± 1°C with HEPES-buffered solution containing (mM): 144 NaCl, 2.5 KCl, 10 HEPES, 1.25 NaH2PO4, 2.5 CaCl2, 10 glucose, 0.1 glycine (to co-activate NMDA receptors), 0.005 strychnine (to block glycine receptors), pH adjusted to 7.4 with NaOH and bubbled with 100% O2.

Whole-cell patch clamp recordings.

Whole-cell patch clamp recordings were conducted using Multiclamp 700B (Molecular Devices) connected to a 16-bit analogue-to-digital converter (Digidata 1440A, Molecular Devices). The pClamp 10 software package was used for data acquisition (sampling rate 50 kHz, filter 10 kHz) and analysis. Electrodes contained solution comprising (mM): 130 Cs-glucuronate, 4 NaCl, 0.5 CaCl2, 10 HEPES, 1 BAPTA, 4 MgATP, 0.5 Na2GTP, 2 K- Lucifer yellow, pH set to 7.3 with CsOH. Series resistance was 5–25 MΩ (if higher, cells were excluded from the analysis) and electrode junction potentials (−14 mV) were corrected for. Cells were identified (Fig. 2a,b and Supplementary

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Intracellular Ca$$^{2+}$$ imaging. Slices were incubated for 1 h at 22 °C in slicing solution with 10μM Fluo4-AM, 25μM FuraRed-AM and 0.05% pluronic F-127 (Life Technologies: A17498, 1:500). Live cells (as confirmed by fluorescence microscope at 488 nm by scanning laser (Leica Sp5) and FuraRed. The ratio of the emission intensities of Fluo4 (maximum emission when bound to Ca$$^{2+}$$) and FuraRed (maximum emission when Ca$$^{2+}$$ is free) was used to measure the increase in Ca$$^{2+}$$, and only ratios that reflected both an increase of fluorescence and a decrease in Fluo4 emission when bound to Ca$$^{2+}$$ were analysed. The increased emission was normalised to the baseline value to produce the ratio of fluorescence emission when bound to Ca$$^{2+}$$ and free of Ca$$^{2+}$$.

**Histological analysis of demyelination and remyelination.** Animals were perfused with 4% glutaraldehyde (in phosphate buffer with 0.72 mM CaCl$_2$) and the brains left to post fix for 4–7 days. Tissue blocks encompassing the CCP were cut. Blocks were processed through 2% osmium tetroxide (Oxkem Ltd), dehydrated in increasing concentrations of ethanol and embedded in resin (TAAB Laboratories). One-micrometre sections were cut using a Leica microtome (RM 2065) and stained with methylene blue. In these sections, remyelinated axons were distinguished from normally myelinated axons outside the lesion by the thinness of the myelin sheath and remyelinated axons can be distinguished from demyelinated axons (Supplementary Fig. 1). Remyelination was independently ranked blindly by three persons.

**Electron microscopy.** Samples used for histological analysis were further processed for electron microscopy. Resin blocks were cut in 90-nm sections on an ultra microtome (Reichert Ultracut E) with a diamond knife (Diatome) and visualised using a transmission electron microscope. Sections were counterstained with 0.1% uranyl acetate and 0.3% lead citrate. The number of axons remyelinated and their g-ratios (the ratio of axon diameter to the outside diameter of the myelin) were analysed and compared using ImageJ software (version 1.45s) by blinded analysis. A total of 302 (TTX) and 312 (saline) axons were analysed.

**Statistics.** Numbers of experiments are indicated on bars. Data are presented as mean±s.e.m., unless stated otherwise. When relevant, normality of data was assessed using the Shapiro–Wilks test. Non-parametric tests that do not assume data follow a normal distribution gave the same conclusions for significant and non-significant differences in all cases. One-way ANOVA followed by Holm–Sidak post hoc was used to compare multiple samples. Other P-values for comparison of means are from Student’s two-tailed t-tests, with Welch’s correction where variances were unequal. Rank was analysed with Mann–Whitney ranking tests. Analysis of covariance was used to analyse regression lines. Cumulative frequency was analysed with Kolmogorov–Smirnov tests.

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Author contributions
H.O.B.G., R.T.K. and R.J.M.F. designed the lesion experiments and interpreted the results. H.O.B.G., K.E., K.V., R.J., S.S., I.L., F.J., C.L.P. and R.T.K. performed the experiments and data analysis. R.T.K. wrote the paper with input from all of the authors, and H.O.B.G., K.V., K.E. and R.T.K. made all the data figures.

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