Intranasal epidermal growth factor treatment rescues neonatal brain injury

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There are no clinically relevant treatments available that improve function in the growing population of very preterm infants (less than 32 weeks’ gestation) with neonatal brain injury. Diffuse white matter injury (DWMI) is a common finding in children and results in chronic neurodevelopmental impairments6,7. As shown recently, failure in oligodendrocyte progenitor cell maturation contributes to DWMI7. We demonstrated previously that the epidermal growth factor receptor (EGFR) has an important role in oligodendrocyte development7. Here we examine whether enhanced EGFR signalling stimulates the endogenous response of EGFR-expressing progenitor cells during a critical period after brain injury, and promotes cellular and behavioural recovery in the developing brain. Using an established mouse model of very preterm brain injury, we demonstrate that selective overexpression of human EGFR in oligodendrocyte lineage cells or the administration of intranasal heparin-binding EGF immediately after injury decreases oligodendroglia death, enhances generation of new oligodendrocytes from progenitor cells and promotes functional recovery. Furthermore, these interventions diminish ultrastructural abnormalities and alleviate behavioural deficits on white-matter-specific paradigms. Inhibition of EGFR signalling with a molecularly targeted agent used for cancer therapy demonstrates that EGFR activation is an important contributor to oligodendrocyte regeneration and functional recovery after DWMI7. Thus, our study provides direct evidence that targeting EGFR in oligodendrocyte progenitor cells at a specific time after injury is clinically feasible and potentially applicable to the treatment of premature children with white matter injury.

Chronic neonatal hypoxia is a clinically relevant model of premature brain injury caused by insufficient gas exchange from poor lung development7. This ‘hypoxic’ state is a major contributor to DWMI— a common finding in infants born very preterm (VPT)—resulting in sensorimotor deficits that persist throughout their lifetime7,8,9. We used a mouse model of chronic hypoxia, which replicates DWMI and other neuropathological hallmarks of brain injury resulting from premature birth7,9,10.

The cellular and molecular mechanisms underlying DWMI in VPT children—and in chronic hypoxia—are unknown. It has been previously demonstrated that enhanced EGFR signalling in white matter oligodendrocyte lineage cells promotes their proliferation, migration, myelination and remyelination in the adult11,12. We observed a significant increase in endogenous EGF levels in the white matter after chronic hypoxia (Extended Data Fig. 1). Therefore, we compared oligodendrocyte development in white matter injury and recovery in 2,3-cyclic nucleotide 3'-phosphodiesterase (CNP) enhanced green fluorescent protein (eGFP) mice (Rep mice) and Rep mice in which human (h)EGFR was overexpressed in the oligodendrocyte lineage under the CNP promoter (Rep-hEGFR mice)13,14. Hypoxia decreased myelin basic protein (MBP) expression in the white matter of Rep mice, but not in Rep-hEGFR mice (Fig. 1a–e). At postnatal day (P)60, MBP expression recovered in the hypoxia-treated Rep group (Fig. 1e). At P11, chronic hypoxia did not cause any change in the number of Rep Olig2+ cells and mature (Rep CC1+) oligodendrocytes (Fig. 1f). At P18, we observed a decrease in Rep Olig2+ and Rep CC1+ oligodendrocytes in the white matter of hypoxia-treated Rep mice (Fig. 1g), but no change in the Rep-hEGFR mice. Oligodendrocyte recovery was evident by P60 in the hypoxia-treated Rep group (Fig. 1h).

There was an increase in apoptosis of oligodendrocyte lineage cells in Rep mice after hypoxia treatment at P11 and P18, but no change at P60 (Extended Data Fig. 2c, e). No significant apoptosis was observed in Rep-hEGFR mice (Extended Data Fig. 2e). Hypoxia caused an increase in the number of propidium iodide (PI)+ cells (data not shown) and Rep PI+ cells (Extended Data Fig. 2a, d, f). This increase was not observed in Rep-hEGFR mice. These results indicate that enhanced EGFR expression prevents oligodendrocyte loss by decreasing cell death after exposure to chronic hypoxia.

We next assessed the effects of hypoxia on oligodendrocyte progenitor cell numbers and MBP expression rescued these abnormalities. Correlation between hypoxia-induced OPC proliferation and the g ratio and hEGFR expression prevented this increase (Fig. 1c, o). Next, we investigated behavioural deficits resulting from DWMI after perinatal chronic hypoxia by using subcortical white-matter-dependent sensorimotor behavioural tests (complex wheel and inclined beam-walking task)14,15,16. In the complex wheel task, there was no difference in training maximum velocity (Vmax) between all four groups (Fig. 1p). On day 15, all four groups had a decline in Vmax; however, the largest decline was in the hypoxia-treated Rep group (Fig. 1p). The hypoxia-treated group performed poorly on the complex wheel (days 15–21),
as compared to the other three groups (Fig. 1p), suggesting altered subcortical white matter integrity.

The inclined beam-walking task requires subcortical white matter integrity (Extended Data Fig. 3a–c) and no difference was observed between Rep mice and C57BL/6 mice (Extended Data Fig. 3d). At P30, the hypoxia-treated Rep group displayed more foot slips than the normoxia Rep group (Fig. 1q). Hypoxia-treated Rep-hEGFR mice showed no significant increase in the number of foot slips as compared with the normoxia Rep-hEGFR group. At P60, the hypoxia-treated Rep group continued to show an increase in number of foot slips, and no difference was seen in the hypoxia-treated Rep-hEGFR group (Fig. 1r). These behavioural studies confirm that exposure to chronic hypoxia during a critical period in myelin development results in poor performance on white-matter-specific behavioural tasks. Enhanced EGFR activity prevents the effects of hypoxia, strongly suggesting that EGFR signalling in oligodendrocyte lineage cells has a crucial role in white matter recovery after perinatal injury.

We directly tested the role of endogenous EGFR signalling in oligodendrocyte recovery after hypoxia by gefitinib administration from P12–P18 (Fig. 2a). Gefitinib, a specific EGFR antagonist, caused a small reduction in basal phosphorylated pEGFR levels in the white matter of normoxia mice at P18, but completely prevented the increase in pEGFR induced by hypoxia (Fig. 2b). Gefitinib caused a decrease of white matter Rep+ Olig2+ and Rep+ CC1+ oligodendrocyte lineage cells in normoxia mice (Fig. 2c). Hypoxia resulted in a decrease of these cell populations, which were further reduced by gefitinib (Fig. 2c). This decrease was attributed to a significant increase in oligodendrocyte apoptosis in both normoxia and hypoxia groups (Fig. 2d). Gefitinib also decreased Sox2- and Ascl1-expressing progenitors (Extended Data Fig. 4a–j). At P30, gefitinib still caused a decrease in oligodendrocyte lineage cells prevents oligodendrocyte and myelin loss, and ultrastructural and behavioural deficits caused by neonatal hypoxia. a–d, Confocal images of white matter immunostained for MBP. Hyp, hypoxia-treated group; Nx, normoxia group. Scale bar, 50 μm. e, Western blot of white matter tissue (n = 4 mice per group and per age except P18 normoxia and hypoxia-exposed mice, n = 5; one-way analysis of variance (ANOVA), Bonferroni post-hoc test for individual comparisons). f–h, Number of Rep+ Olig2+ and Rep+ CC1+ cells. i, Number of newly generated oligodendrocytes in white matter. f–i, n = 4 mice per group and per age; one-way ANOVA. Bonferroni post-hoc test for individual comparisons. j–m, Electron microscopy images from P60 white matter. Scale bar, 0.5 μm. n, o, Scatter plots of g ratios of individual axons relative to axon diameters (n = 3 mice per group; one-way ANOVA of all four groups with post-hoc unpaired t-tests). p, Vmax (m min−1) over time (days) on the complex wheel (linear regression comparison of slopes between all four groups; post-hoc comparison of individual days between groups: Nx Rep, n = 12; Nx Rep-hEGFR, n = 9; Hyp Rep, n = 12; Hyp Rep-hEGFR, n = 10).

q, r, Naive mice were tested on the 2-cm- and 1-cm-width inclined beam-walking task (Poisson multiple regression analysis; Nx Rep, n = 8; Nx Rep-hEGFR, n = 10; Hyp Rep, n = 10; Hyp Rep-hEGFR, n = 10). r, A separate group of naive mice was assessed (Poisson multiple regression analysis; Nx Rep, n = 9; Nx Rep-hEGFR, n = 8; Hyp Rep, n = 10; Hyp Rep-hEGFR, n = 9). Line graph and histograms are presented as means ± standard error of the mean (s.e.m.). *p = 0.05; **p < 0.05; ***p < 0.01.
Figure 2 | EGFR activity is crucial for white matter recovery after neonatal hypoxia. a, Protocol of gefitinib and BrdU administration. Different groups are indicated. Hyp, hypoxia treated; Nx, normoxia. b, Western blot of white matter shows that gefitinib decreased pEGFR in the normoxia group and prevented the increase in pEGFR after hypoxia (n = 5 mice per group; one-way ANOVA, Bonferroni post-hoc test for individual comparisons). c, Counts of Rep+Olig2+ and Rep+CC1+ cells in white matter. d, Gefitinib increased cell apoptosis in Nx and Hyp groups. e, f, Gefitinib decreased Rep+NG2+ OPCs (e) and oligodendrocyte-lineage cell proliferation (f) in normoxia mice and prevented hypoxia-induced increase in OPC and oligodendrocyte-lineage cell proliferation. g, Long-term effects of gefitinib on Rep+Olig2+ and Rep+CC1+ cells. h, Gefitinib decreased newly generated Rep+CC1+ oligodendrocytes in normoxia mice and prevented oligodendrogenesis after hypoxia. i, Gefitinib prevented recovery of CNPase and MBP expression after hypoxia. c–i, n = 4 mice per all groups and per age; one-way ANOVA, Bonferroni post-hoc test for individual comparisons. All histograms are presented as means ± s.e.m. *P = 0.05; **P < 0.01; ***P < 0.001.

We wanted to examine whether directly targeting EGFR with a selective ligand (recombinant heparin-binding EGF (HB-EGF)) delivered through the intranasal route promoted cellular recovery of white matter oligodendrocytes after hypoxia. The clinical relevance of targeting endogenous oligodendrocytes was demonstrated by the presence of EGFR-expressing oligodendrocyte lineage cells (Olig2+EGFR+) in neonatal preterm human white matter (Fig. 3a). The intranasal route allows rapid drug delivery directly to the brain from the nasal mucosa20–22. HB-EGF via the intranasal route enters the brain23–25 and acts on the white matter (Extended Data Fig. 5a–c), where Rep+EGFR+ cells could be identified (Extended Data Fig. 5d). FACS purification of hypoxia-exposed white matter Rep+ cells after HB-EGF treatment directly demonstrated activation of EGFR in oligodendrocyte lineage cells (Extended Data Fig. 5e). Treatment with seven doses of intranasal HB-EGF from P11–P14 (Fig. 3b)

Figure 3 | Intranasal HB-EGF accelerates oligodendrocyte regeneration and promotes cellular recovery in white matter after neonatal hypoxia. a, Confocal image of the corpus callosum (CC) in a preterm infant, highlighting an Olig2+EGFR+ cell (box). Scale bars, 50 μm. b, Protocol of intranasal HB-EGF/BrDU administration and tissue collection. Different groups are indicated. Hyp, hypoxia treated; Nx, normoxia. c, Number of white matter Rep+Olig2+ and Rep+CC1+ cells in HB-EGF-treated mice. d, HB-EGF attenuated or prevented the effects of hypoxia on oligodendrocyte apoptosis. e, HB-EGF had an additive effect on hypoxia-induced increase of Rep+NG2+ OPCs at P15, but not at P18. f, HB-EGF had an additive effect on hypoxia-induced increase of OPC proliferation. g, HB-EGF promoted oligodendrogenesis after hypoxia at P18. h, Fate mapping of OPCs (PGDFβR-CreER/Z/EG (GFP) mouse) demonstrated that oligodendrogensis occurred from PGDFβR+ cells. c–h, n = 4 mice per group and per age; one-way ANOVA, Bonferroni post-hoc test for individual comparisons. i, Removal of EGFR in PDGFRβ-CreER/Z/EG (GFP) mouse caused a decrease in NG2+OPCs after hypoxia and prevented the effects of HB-EGF (n = 4 mice per group except hypoxia HB-EGF PDGFRβ-CreER/Z/EG group, n = 3; one-way ANOVA of all four groups with post-hoc unpaired t-tests). j, HB-EGF promoted recovery in white matter MBP and PLP protein levels after hypoxia (western blot; n = 6 mice per group except Nx HB-EGF, n = 4; one-way ANOVA, Bonferroni post-hoc test for individual comparisons). All histograms are presented as means ± s.e.m. *P = 0.05; **P < 0.01; ***P < 0.001.
prevented white matter oligodendrocyte lineage cell loss after hypoxia (Fig. 3c). Rep+ cells were stained for caspase 3 in all groups. At all ages examined, HB-EGF treatment reduced (P15) or prevented (P18) the effects of hypoxia on oligodendrocyte cell death (Fig. 3d).

HB-EGF treatment also caused an increase in white matter Rep+ NG2+ OPCs in both normoxia and hypoxia groups, and enhanced OPC proliferation24 (Fig. 3e, f). To determine whether oligodendrocyte recovery was a result of increased oligodendrogenesis from this expanded pool of proliferative OPCs, we performed BrdU-pulse chase labelling of newly generated oligodendrocytes. HB-EGF increased the number of Rep+ CC1+ BrdU+ cells under normoxic and hypoxic conditions at P18 (Fig. 3g). At P30, the additive effect of hypoxia and HB-EGF was not as evident (Fig. 3g). Genetic lineage tracing of oligodendrocytes in vivo by using the platelet-derived growth factor-α receptor (PDGFRα-CreER; Z/EG) (GFP+) reporter mouse25 (Extended Data Fig. 6 and Fig. 3h) confirmed that hypoxia and HB-EGF treatment have an additive effect on the generation of Olig2-expressing cells (Extended data Fig. 6j). Conversely, PDGFRαR-driven EGFR deletion in OPCs prevented the cellular effects of HB-EGF on NG2+ OPCs (Fig. 3i). HB-EGF treatment after hypoxia also resulted in recovery of MBP and proteolipid protein (PLP) expression (Fig. 3j). These results indicate that, after hypoxia, HB-EGF promotes expansion of the OPC pool, and oligodendrocyte regeneration and maturation.

Electron microscopy analysis revealed that HB-EGF treatment rescued the increase in g ratio observed in hypoxia-treated mice and partially prevented the decrease in percentage of myelinated axons (Fig. 4a–f and Extended Data Fig. 7). Diffusion tensor imaging (DTI) demonstrated that, at P60, fractional anisotropy (FA) values are significantly decreased in the corpus callosum, cingulum and external capsule regions of hypoxia-treated mice (Fig. 4g). DTI analysis of all four groups with post-hoc unpaired t-tests (Fig. 4h) shows that HB-EGF attenuates hypoxia-induced reduction of FA values in the corpus callosum, cingulum and external capsule (arrows point to regions of interest; n = 5 for each group). k, CAP extracellular recordings. Representative waveforms and histograms show that the Hyp HB-EGF group had larger myelinated (M) amplitudes compared with the Hyp saline group (n = 7 per group per age except P60; Nx HB-EGF, n = 5; one-way ANOVA, Bonferroni post-hoc test for individual comparisons). l, Ex vivo measurements of white matter NAA using 1H-nuclear magnetic resonance (1H-NMR) spectroscopy. Representative 1H-NMR spectra showing the NAA peak. At P18 there was significantly less NAA in the Hyp saline group compared with the Hyp HB-EGF group (n = 5 per group per age; one-way ANOVA, Bonferroni post-hoc test for individual comparisons, except P11 unpaired t-test). m, On the complex wheel, Hyp HB-EGF mice performed similarly to the Nx saline group (linear regression comparison of slopes between all three groups and post-hoc comparison of individual days between groups; Nx saline, n = 8; Hyp saline, n = 7; Hyp HB-EGF, n = 10). n, o, On the inclined beam-walking task, HB-EGF treatment (P11–P14) decreased the number of foot slips observed in hypoxia-exposed mice (Poisson multiple regression analysis; all groups were n = 9, except for the P30 Hyp HB-EGF group, n = 9). p, Delayed HB-EGF administration at P18–P21 resulted in no difference in foot slips (Poisson multiple regression analysis; Nx saline and Nx HB-EGF n = 7; Hyp saline and Hyp HB-EGF, n = 8). All histograms are presented as means ± s.e.m. *P < 0.05; **P < 0.01.
amplitude of myelinated axons observed after hypoxia (Fig. 4k). Finally, analysis of N-acetylaspartate (NAA) in the white matter showed decreased levels at P18 and P30, which was prevented by HB-EGF (Fig. 4l and Extended Data Fig. 8).

On the complex wheel, hypoxia-exposed HB-EGF-treated mice showed a similar performance to the normoxia saline-treated group (Fig. 4m). Importantly, when HB-EGF treatment was performed at P18–P21, it had no effect on the hypoxia-induced behavioural phenotype (Fig. 4n) and behavioural recovery. Intranasal treatment is a plausible route to promote cellular, developmental and myelin structural improvement, a clinically feasible (intranasal) mode of entry during a critical window of opportunity.

Intranasal treatment is a plausible route to improve cellular and functional recovery after neonatal brain injury. Enhancing EGFR signalling through overexpression of the EGF receptor prevents DWMI, promotes the generation of new oligodendrocytes and prevents behavioural deficits in different white-matter-related tasks. Furthermore, a brief pharmacological treatment that targets endogenous EGFRs using a clinically feasible (intranasal) mode of entry during a critical window promotes cellular, developmental and myelin structural improvement, and behavioural recovery. Intranasal treatment is a plausible route to improve sufficient HB-EGF into the brain and white matter of critically ill VPT infants.

METHODS SUMMARY

Data presented are from male mice on a C57BL/6 background. Mice underwent chronic perinatal hypoxia from P3–P11, as previously described. In the first set of experiments, Rep mice that did not express HEGFR were used as littermate controls. The EGFR antagonist gefitinib (AstraZeneca) was administered intraperitoneally at a concentration of 100 ng g−1 body weight once daily for 7 days. Normoxia and hypoxia control groups received vehicle on the same days and times. HB-EGF was administered intranasally at a concentration of 100 ng g−1 body weight in 5 μl increments separated by 10 min. Equal volumes of saline were used as a vehicle control.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 26 October 2012; accepted 13 November 2013.

Published online 25 December 2013.

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METHODS

Animals. The CNP-EGFP (Rep) and CNP-EGFP-hEGFR (Rep-hEGFR) strains were generated as described previously and backcrossed to a C57BL/6 genetic background greater than nine generations. The Rep-hEGFR mice used for these experiments were crossed with heterozygote Rep mice to ensure that all pups were positive for GFP, but not all expressed hEGFR. This ensured littermate controls. For experiments that required only the Rep line of mice, a Rep adult mouse was crossed with a C57BL/6 adult mouse (Jackson Laboratories). Only mice that expressed eGFP (Rep) during screening on P2 with ultraviolet goggles were used. The PDGFαR-CreERT2 line (courtesy of D. Bergles; referred to in the text as PDGFαR-Cre) was crossed with Z/EG reporter mice (Jackson Laboratories; stock number 009320) and genotyped as previously reported. Z/EG reporter mice were crossed with EGFR+/− (ref. 29) followed by PDGFαR-CreERT2 transgenic mice. These mice were backcrossed to ensure homozygous EGFR−/− mice, so that all mice were PDGFαR-CreERT2Z/EG−/−. To induce Cre recombination, tamoxifen was administered at a dosage of 75 μg 1 body weight. The Ascl1GFP mice (Jackson Laboratories, stock 012881; also known as Mash1) were used to determine the effects of gefitinib on white matter Ascl1-expressing cells. Unless described later or in the figure legends, only male mice were used, owing to male preterm children showing more clinically relevant injury and neurological deficits compared with females. All animal procedures were performed according to the Institutional Animal Care and Use Committee of the Children’s National Medical Center and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health).

Hypoxic rearing. Mice were randomly chosen to either undergo hypoxic rearing or serve as normoxic controls. The hypoxic mice were placed in a sealed chamber maintaining O2 concentration at 10.5% by displacement with N2 as described previously. Hypoxia began at P3 for 8 days until P11. This time window was chosen for the following reasons: (i) the timing of the critical period for oligodendrocyte development in the developing corpus callosum is P0-P14 (15,16); (ii) hypoxia at P3 is well tolerated (17); (iii) P3-P11 provides sufficient time for a window of 8 days to observe oligodendrocyte development; and (iv) P11 is prior to the time of myelination (17).

BrdU administration. Mice were injected intraperitoneally (i.p.) at the same time of the day with BrdU (50 μg 1 body weight) daily for 4 days (P11–P14) in the morning. In studies using HB-EGF or gefitinib, BrdU was administered 1 h before vehicle or drug administration.

Gefitinib administration. Gefitinib (Iressa; Astra Zeneca (Tocris)) was prepared using 0.45% normal sterile saline solution at a concentration of 20 mg 1 saline. Male mice in each litter were randomly chosen to receive either drug or vehicle. The drug dose for this study was 75 mg kg−1 day−1 and was administered once daily. An equal amount of vehicle was administered to control animals. A total of seven doses of vehicle or drug were administered beginning at P12.

UBC-EGF administration. The intranasal route allows for small molecules to rapidly enter the cerebrospinal fluid from the nasal cavity, followed by subsequent distribution to the brain and spinal cord (18–20). Recombinant human hepatocellular growth factor constituent free (HB-EGF; R&D Biosciences) was prepared using 0.45% normal sterile saline solution at a concentration of 20 μg 1 saline and stored at −20 °C. Mice were randomized to the vehicle (saline) or HB-EGF group. Saline or HB-EGF was administered intranasally at no more than 5 μl increments 5–10 min apart for a total of 100 ng 1 dose. The mouse was held ventral-side up, and a small-modified 27–French catheter was inserted into each nare. Saline or drug was slowly administered and the mouse was held for 1–2 min to ensure absorption. Drug or saline was administered every 12 h beginning on the evening of P11.

Immunohistochemistry and antibodies. Freshly cut, free-floating brain sections (40 μm thick) from P11–P60 mice were prepared as described previously (21,22,32). Primary antibody dilutions were 1:500 for anti-BrdU (Accurate), anti-NG2 (Millipore), and anti-CD68 (Dako) (diluted 1:500); and anti-NEC1 (Covance), and the secondary antibodies being administered. The secondary antibodies (1:200) used were AlexaFlour 488, AlexaFlour 546 and AlexaFlour 633 conjugated goat anti-rabbit, anti-rat or anti-mouse IgG (Invitrogen). Sections were incubated with secondary antibodies for 1 h at room temperature, followed by three 1 PBS washes. Sections were treated with 4',6-diamidino-2-phenylindole (DAPI) (Sigma–Aldrich) for 10–15 min at room temperature and mounted with Mowiol. Human tissue was obtained from a deceased 3-day-old infant born at 36-week gestation with approval from the Children’s National Institutional Review Board. Sections of corpus callosum and periventricular region were removed after fixation of the brain for 2 weeks in formalin solution. Tissue was kept in PBS for 1 week with daily changes of PBS solution to remove excess formalin. The brain was then placed in 20% glycerol solution for 24 h. Freshly cut, free-floating sections (40 μm thick) were made on a sliding microtome. Sections were immunostained as described earlier.

Microscopy and cell counting. All fluorescent images were taken on a Zeiss LSM confocal laser-scanning microscope with sequential scanning mode using ×40 oil objectives. Microscopy and cell counting were performed as recently described (21,22). Z-stack images of 1-μm-thick single planes were captured throughout the entire thickness of the slice and each cell was analysed using a Zeiss LSM Image Browser (version 4.2) in its entire z-axis to exclude false double labelling due to overlay of signals from different cells. Four different laser lines were used to perform image localization of fluorescein isothiocyanate (FITC) (488 nm excitation; 522/35 emission filter), CY3 (560 nm excitation; 605/32 emission filter), Cy5 (647 nm excitation; 680/32 emission filter) and DAPI (400 nm excitation). Data acquisition and processing were controlled by modified LSM software. Analysis of immunofluorescence was performed on a confocal z-stack as previously described (21,22). Cells were counted in 225 × 225 × 10 μm (X, Y, Z planes) for cells per volume quantification. Data were obtained from an average of six tissue sections per mouse per immunofluorescence staining condition.

Assay procedure was performed according to the manufacturer’s instructions (R&D Systems, Mouse EGF Quantikine ELISA Kit). Experiments were performed in triplicates and averaged.

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FACS. White matter microdissected tissue from hypoxia-exposed P15 Rep^- (CNP-EGFP^+) vehicle- or HB-EGF-treated mice were FACS purified. Two-to-three male and female brains were pooled for each sample (individual n). Tissue was dissociated into single-cell suspensions as previously described^11,41, and analysed (Stoetling) and 2 artefact^42,43. Axon diameters were calculated from measurement of the axon circumference at four points per sheath, avoiding areas of tongue processes or fixation using ImageJ. Myelin thickness was calculated from the average of radial measurements from electron microscope (JEM-1400), and pictures were taken with a Gatan SC1000 ORIUS CCD camera. Measurements and image processing was performed using ImageJ. Myelin thickness was calculated from the average of radial measurements and 4,600 images were zero-filled to 256 time to traverse the beam^18. In pilot studies, we determined that hypoxia-exposed Rep mice less than 30 days of age—or on a beam that was inclined more than 30°—were unable to perform this task (data not shown). To confirm whether this sensorimotor task is dependent on subcortical white matter, bilateral LPC- or 0.9% saline-injected adult mice were tested on day 5 after surgery (described earlier). Bilateral demyelination was confirmed after testing by perfusing the brains as described earlier and immunohistochemical analysis of the corpus callosum was performed using anti-MBP. Only mice that displayed clear bilateral lesions on microscopic examination were included in the behavioural analysis. Three (n = 3) mice were excluded from the study because bilateral white matter demyelination was not clearly evident.

DTI analysis. Mice (P60) used for DTI were perfused and imaged as previously described. One hour before DTI scans the brains were soaked 3 times for 10 min each time in 10 ml PBS to remove the PFA solution. The brains were placed into a custom-built MRI-compatible tube filled with Fluorinert—an MRI susceptibility-clearing fluid (Sigma-Aldrich). The DTI data sets were obtained on a 9.4 T horizontal bore magnet (Bruker) with a custom-made 1H radio frequency coil. The DTI experiments were performed using the Stejskal–Tanner spin-echo diffusion-weighted sequence with a diffusion gradient of 5 ms and a delay between the two diffusion gradients of 15 ms. Twenty-four contiguous coronal slices of 0.5 mm thickness were acquired using a repetition time (TR) of 2 s and an echo time (TE) of 25.1 ms. Two Shimmar–Le Roux (SLR) pulses of 1 ms each were used for excitation and readout, respectively. Twenty average acquisitions were made on each slice and the 128 x 64 pixel resolution images were zero-filled to 256 x 256 pixel resolution, resulting in an in-plane spatial resolution of 100 μm x 100 μm. Sixteen different images were acquired for each slice, 15 corresponding to various non-collinear diffusion-weighting directions with b = 1,000 s mm^-2 and one with no diffusion weighting. DTI processing and analysis was performed blindly as described previously^44,45.

CAs. Compound action potential (CAP) recordings were performed in all four groups at P30 and P60 using methods previously described^29,30. Briefly, after mice were killed, coronal slices 400 μm in thickness were obtained using a VT1000S vibrotome (Leica) in ice-cold slicing solution (85 mM NaCl, 2.5 mM KCl, 25 mM NaHCO3, 1.25 mM NaH2PO4, 0.5 mM CaCl2, 7 mM MgCl2, 25 mM glucose, 75 mM sucrose). Slices were placed in recording solution (125 mM NaCl, 2.5 mM KCl, 26 mM NaHCO3, 1.25 mM NaH2PO4, 2.5 mM CaCl2, 1.3 mM MgCl2, 11 mM glucose, pH 7.4) bubbled in 95% O2 and 5% CO2, and maintained at 37 °C for 1 h then kept in the same solution at room temperature until recording. CAP recordings were performed on five slices corresponding to sections 13–18 (Bregma 1.32 to –0.94 mm) of the National Institutes of Mental Health’s mouse brain atlas (http://www.mbl.org/atlas100/atlas170_frame.html). Slices were placed in a recording chamber superfused with oxygenated recording solution at a flow rate of 2 ml min^-1 and viewed using the 10 x objective of an Olympus BX61WI microscope. CAP recordings were performed using an HCA concentric bipolar stimulating electrode and an extracellular recording electrode with a tip resistance of 1 MΩ. The stimulating and recording electrodes were placed ~2 mm apart (~1 mm on each side of the midline) in the corpus callosum of subcortical white matter, and a constant stimu-

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between the four groups for each individual day. For the beam-walking behavioural results, the number of foot slips, the time to traverse the beam and the size of the beam were analysed using a Poisson multiple regression analysis, which allowed us to overcome the lack of normality in count-type data and account for other variables to compare the rate of foot slips. The one-way ANOVA with post hoc comparisons was performed using GraphPad Prism 5.0 (for Mac). All histograms and scatter plots in this manuscript were created with GraphPad Prism.

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Extended Data Figure 1 | Hypoxia results in a significant increase in EGF levels in the white matter. The white matter was dissected out at P11, P15 and P18 in normoxia (Nx)- and hypoxia (Hyp)-exposed CNP-EGFP (Rep) and CNP-EGFP-hEGFR (Rep-hEGFR) mice. At P11, in both Hyp groups, there was a significant increase in EGF levels, as measured by ELISA. There was no significant difference between the two Hyp groups, indicating that overexpression of EGFR in oligodendrocyte lineage cells does not modify endogenous EGF levels. At P15 and P18, there was no difference between all four groups. All histograms are presented as mean absorption (optical density (OD)) relative to total protein concentration ± s.e.m. **P < 0.05; ***P < 0.01 (P11 and P15, n = 4 mice per group and per age; P18, n = 3 mice per group; one-way ANOVA, Bonferroni post-hoc test for individual comparisons).
Extended Data Figure 2 | Enhanced EGFR expression in oligodendrocyte lineage cells prevents oligodendrocyte death and promotes proliferation of OPCs in white matter. a–d, Representative ×40 confocal images of Rep PI cells from normoxia and hypoxia-exposed white matter at P11 in Rep and Rep-hEGFR mice. e, At P11 and P18, hypoxia resulted in a significant increase in the number of oligodendrocyte cells undergoing apoptosis (Rep Casp3−). Enhanced EGFR expression prevented this increase at all time points, except P60 where no difference was evident. Comparison of the Hyp Rep with the Hyp Rep-hEGFR groups demonstrates that hEGFR in oligodendrocyte lineage cells is protective against apoptosis induced by hypoxia (n = 4 mice per group and per age; one-way ANOVA, Bonferroni post-hoc test for individual comparisons). f, PI was injected intraperitoneally 1 h before mice were killed. A significant increase in the number of Rep PI− oligodendrocyte cells indicated membrane disruption contributing to cell death (n = 3 mice per group; one-way ANOVA, Bonferroni post-hoc test for individual comparisons). g–j, Representative ×40 confocal images of Rep NG2− OPCs from normoxia and hypoxia-exposed white matter at P18 in Rep and Rep-hEGFR mice. k, At P11 and P18, Rep-hEGFR Nx mice had more NG2+ OPCs compared with the Rep Nx group. Hypoxia resulted in a significant increase in the number of Rep NG2− OPCs in both Rep and Rep-hEGFR groups; however, overexpression of hEGFR did not have an additive effect. l, At P11 and P18, the Rep-hEGFR group had more Rep Ki67− cells; however, this did not reach significance (P > 0.05). Hypoxia resulted in enhanced oligodendrocyte-lineage proliferation at P11 and P18, but hEGFR overexpression did not have a significantly additive effect when compared with the Rep Hyp group. k, l, n = 4 mice per group and per age; one-way ANOVA, Bonferroni post-hoc test for individual comparisons. m, More OPCs were in a proliferative state in Rep-hEGFR Nx mice compared with Rep Nx. Hypoxia enhanced OPC proliferation, and overexpression of hEGFR resulted in a significantly additive increase compared to the Rep Hyp group (n = 3 mice per group; one-way ANOVA, Bonferroni post-hoc test for individual comparisons). All histograms are presented as means ± s.e.m. Scale bars, 50 μm (a–d, g–j). *P = 0.05; **P < 0.05; ***P < 0.01.
Extended Data Figure 3 | The inclined beam-walking task requires normal subcortical white matter. a–c, This study was performed to test the hypothesis that the inclined beam-walking task is a good assessment of subcortical white matter function. To test this hypothesis, we tested a well-established model of subcortical white matter demyelination induced by bilateral lysolecithin injections in 8-week-old C57BL/6J male and female mice. Animals were tested at 5 days after surgical intervention—which is a time period when demyelination is at its maximum—to determine whether subcortical white matter integrity is important in this behavioural task. Control mice received bilateral injections of normal saline using the same coordinates as the lysolecithin group. a, b, Bilateral demyelination was confirmed after testing by removal of brains and immunohistochemical analysis of corpus callosum. Only mice that had clear bilateral lesions on microscopic examination were included in the behavioural analysis (n = 3 mice were excluded). c, The lysolecithin-injected mice had a marginally significant or very significant increase in average number of foot slips on the 2-cm- and 1-cm-wide inclined beam, respectively (two-tailed Mann–Whitney test, n = 6 per group).

d, We wanted to determine whether the performance of Rep (CNP-eGFP) transgenic mice on the inclined beam-walking task was similar to that of C57BL/6J mice (wild type). No difference in performance was evident between the two different lines of male mice. We found that the Rep and wild-type mice performed similarly in either normoxic or hypoxic conditions (Poisson multiple regression analysis; Nx Rep, n = 7; Nx wild type, n = 11; Hyp Rep, n = 8; Hyp wild type, n = 11). All histograms are presented as means ± s.e.m. *P = 0.05; **P < 0.05; ***P < 0.01.
Extended Data Figure 4 | Inhibition of EGFR prevents expansion of progenitor cells in the developing white matter and after hypoxia.

a–d, Representative ×40 confocal images of subcortical white matter Sox2+ cells, a transcription factor expressed in proliferating multipotential neural progenitor cells. e, Gefitinib, a specific EGFR inhibitor, resulted in a significant decrease in the number of white matter Sox2-expressing cells compared with normoxia vehicle-treated mice. After hypoxia, there was a significant expansion of Sox2+ cells in the white matter compared with the Nx vehicle group; however, this expansion was prevented by gefitinib (Hyp vehicle versus Hyp gefitinib, P < 0.01) (n = 4 for each group; one-way ANOVA, Bonferroni post-hoc test for individual comparisons).

f–i, Representative ×40 confocal images of subcortical white matter Ascl1+ cells (Mash1) in the Ascl1–eGFP transgenic mice. Ascl1 is a proneural transcription factor expressed in proliferating multipotential neural progenitor cells. j, Similar to above, gefitinib resulted in a significant decrease in the total number of Ascl1–eGFP+ cells in the white matter compared with normoxia vehicle-treated mice. Hypoxia resulted in a significant expansion in the number of Ascl1–eGFP+ cells, which gefitinib prevented (Hyp vehicle versus Hyp gefitinib, P < 0.05) (n = 4 for each group; one-way ANOVA, Bonferroni post-hoc test for individual comparisons). All histograms are presented as means ± s.e.m. **P < 0.05; ***P < 0.01. a–d, f–i, Scale bars, 50 μm.
Extended Data Figure 5 | Intranasal HB-EGF does enter the brain and activates EGFRs in oligodendrocyte lineage cells. a–c, Saline or HB-EGF was administered intranasally once in P11 mice, which were then killed at 1, 5, 15 and 30 min after administration. a, Western blot analysis was performed on microdissected white matter probing for actin, HB-EGF and pEGFR (Tyr 1068 phosphorylation site). In the saline group, no HB-EGF was detected in the white matter and no change in pEGFR was detected. In the mice that received HB-EGF, the HB-EGF protein was detected at 5 min and increased up to 30 min. The pEGFR signal steadily increased at 5, 15 and 30 min after HB-EGF administration. b, c, The line graphs represent relative abundance of protein compared with actin (n = 3 for each time point and condition). Line graphs are presented as means ± s.e.m. d, A normoxia-treated P11 mouse was administered HB-EGF and killed 30 min later. Immunohistochemistry of pEGFR was performed. In the white matter, there were several Rep⁺pEGFR⁺DAPI⁺ cells, indicating that oligodendrocyte lineage cells express activated EGFR (pEGFR). Shown is a 3×40 representative image of the white matter demonstrating Rep⁺pEGFR⁺DAPI⁺ cells. e, In this set of experiments, Hyp Rep mice received either intranasal saline or HB-EGF from P11–P14 (Fig. 3b). The subcortical white matter was microdissected at P15 and CNP–eGFP⁺ cells were FACS purified. Western blot analysis was performed to probe for pEGFR. The western blot demonstrates that a more robust signal for pEGFR was present in the Hyp HB-EGF group, relative to actin (n = 4 for each group of 2–3 pooled brains).
Extended Data Figure 6 | Intranasal HB-EGF treatment increases the number of oligodendrocyte lineage cells derived from PDGFαR-expressing OPCs. a, PDGFαR-CreER,Z/EG transgenic mice were divided into four groups. Saline or HB-EGF was administered intranasally. Intraperitoneal injections of tamoxifen were administered at P12, P13 and P14 in the morning, 1 h before the morning dose of saline/HB-EGF. Mice were killed at P18. b–e, Representative ×40 confocal images of CC1+ cells (red) derived from PDGFαR-CreER,Z/EG (GFP+) (green) progenitors at P18. f–i, Cells derived from PDGFαR-expressing progenitors after hypoxia and after HB-EGF treatment belong to the oligodendrocyte lineage (Olig2+). Representative ×40 confocal images of the subcortical white matter in all four groups at P18. In serial sections from each PDGFαR-CreER,Z/EG mouse, all GFP+ cells in the subcortical white matter co-stained with anti-Olig2+ antibody in all four groups (n = 4 for each group). In all four groups, no GFP+ cells co-stained with anti-GFAP or anti-glutamate/aspartate transporter (GLAST) antibody (data not shown). j, Hypoxia results in a significant increase in the number of GFP+ cells in the white matter and HB-EGF has an additive effect (n = 4 mice per group; one-way ANOVA, Bonferroni post-hoc test for individual comparisons). Histograms are presented as means ± s.e.m. b–i, Scale bars, 50 μm. **p < 0.05; ***p < 0.01.
Extended Data Figure 7 | HB-EGF treatment prevents hypoxia-induced changes in white matter axonal g ratios at P30.  a, b, Scatter plots depicting g ratios versus axon diameter. The lines represent linear fits to pooled data from all mice for each genotype (n = 3 mice per group). a, The scatter plot demonstrates that the Nx saline and Nx HB-EGF groups were similar. b, The scatter plot demonstrates that the Nx HB-EGF and Hyp HB-EGF groups were similar. c, Histogram demonstrating that, at P30, the percentage of myelinated subcortical white matter fibres was significantly decreased in the Hyp saline group. No significant difference was found in the Hyp HB-EGF group. **P < 0.01; ***P < 0.01.
Extended Data Figure 8 | Intranasal HB-EGF prevents loss of NAA after hypoxia. a, $^1$H-NMR spectroscopy was performed on dissected white matter at P11, P18 and P30. A full-scale representative spectrum is shown where the peak for NAA is at 2.0 p.p.m. The spectrum shown on Fig. 4l is truncated. b, Western blots of aspartoacylase (ASPA), an enzyme found in oligodendrocytes and responsible for hydrolysis of NAA for myelin production in the developing brain. Hypoxia does not result in any significant change in the amount of ASPA present in the white matter at each of the time points listed (P11 and P30, \( n = 4 \) for each group and age; P18, \( n = 5 \) for each group; one-way ANOVA, Bonferroni post-hoc test for individual comparisons, except P11 unpaired \( t \)-test). Histograms are presented as means ± s.e.m.
Extended Data Figure 9 | Protocol for late HB-EGF administration used for this study. In this study, HB-EGF or saline was administered at a later time point. Beginning in the morning of P18, HB-EGF or saline was administered every 12 h until the morning of P21. Intraperitoneal BrdU was administered 1 h before HB-EGF or saline administration, from P18–P21. The inclined beam-walking task was performed at P35. Only Rep mice were used for this study. The histogram is presented in Fig. 4p.
Extended Data Figure 10 | Intranasal HB-EGF accelerates oligodendrocyte maturation in the white matter after hypoxia by preventing Notch activation. 

a, Microdissected white matter was probed for activated Notch intracellular domain (NICD) and its ligand Delta1. Western blot analysis obtained from microdissected white matter at P11, P14.5 and P18 with actin as a loading control. 

b, c, Histograms represent quantification of the density of NICD (b) and Delta1 (c) signal normalized to actin. 

b, At P11 and P14.5, there was a significant increase in the amount of NICD in the Hyp group. No significant difference was evident at P18. The Hyp HB-EGF group had no significant increase at P14.5 compared with the Nx HB-EGF group, and significantly less than the Hyp saline group (n = 4 mice for each group and age; one-way ANOVA, Bonferroni post-hoc test for individual comparisons). 

c, Delta1 was increased at P14.5 only in the Hyp saline group (P11: n = 4 for each group; P14.5 and P18: n = 5 for each group and age; one-way ANOVA, Bonferroni post-hoc test for individual comparisons). 

**P < 0.05; ***P < 0.01.

d–g, Representative ×40 confocal images of subcortical white matter in the transgenic Notch reporter (TNR) mice, where eGFP is expressed upon activation of Notch effector C-promoter binding factor 1 (CBF1), a downstream transcriptional target of Notch. 

h, Histogram represents the number of eGFP*Olig2* cells at P14.5 in the white matter. The Hyp saline group showed a significant increase in eGFP*Olig2* cells, corresponding to enhanced Notch activation in oligodendrocyte lineage cells. This contributes to delayed maturation of oligodendrocyte lineage cells observed after hypoxia (n = 4 mice per group; one-way ANOVA, Bonferroni post-hoc test for individual comparisons). 

Histogram is presented as means ± s.e.m. 

d–g. Scale bars, 50 µm. **P < 0.05; ***P < 0.01.