GABAergic regulation of cerebellar NG2 cell development is altered in perinatal white matter injury

Marzieh Zonouzi1,2, Joseph Scafidi1,3, Peijun Li1, Brian McEllin1, Jorge Edwards1, Jeffrey L Dupree4, Lloyd Harvey5, Dandan Sun5, Christian A Hübner6, Stuart G Cull-Candy2, Mark Farrant2 & Vittorio Gallo1

Diffuse white matter injury (DWMI), a leading cause of neurodevelopmental disabilities in preterm infants, is characterized by reduced oligodendrocyte formation. NG2-expressing oligodendrocyte precursor cells (NG2 cells) are exposed to various extrinsic regulatory signals, including the neurotransmitter GABA. We investigated GABAergic signaling to cerebellar white matter NG2 cells in a mouse model of DWMI (chronic neonatal hypoxia). We found that hypoxia caused a loss of GABA_A receptor-mediated synaptic input to NG2 cells, extensive proliferation of these cells and delayed oligodendrocyte maturation, leading to dysmyelination. Treatment of control mice with a GABA_A receptor antagonist or deletion of the chloride-accumulating transporter NKCC1 mimicked the effects of hypoxia. Conversely, blockade of GABA catabolism or GABA uptake reduced NG2 cell numbers and increased the formation of mature oligodendrocytes both in control and hypoxic mice. Our results indicate that GABAergic signaling regulates NG2 cell differentiation and proliferation in vivo, and suggest that its perturbation is a key factor in DWMI.

Infants born prematurely (23–32 weeks gestation) are at high risk of developing DWMI, which is often linked to chronic hypoxia1,2. DWMI, also known as bilateral periventricular leukomalacia, is a leading cause of long-term neurological damage, which manifests as behavioral, cognitive or motor defects3,4. DWMI is associated with severely disrupted development of the subcortical white matter5 and has been linked to the loss of late NG2 cells6,7.

DWMI in preterm infants is associated with reduced expression of GABAergic markers in the cortex, subplate and white matter8, and recent studies have identified a reduction in cortical GABA in a clinically relevant mouse model of DWMI9. GABA has been recognized as a critical regulator of neuronal development and restricts the proliferation of embryonic and adult neuronal precursor cells10,11 and glial fibrillary acidic protein–positive (GFAP+) subventricular zone stem cells12,13.

In recent studies, neonatal hypoxia has been shown to enhance Notch signaling and downregulate the cell cycle arrest protein p27(Kip1) in NG2 cells, contributing to their disrupted developmental progression and the dysmyelination of subcortical white matter14,15. Enhancing NG2 cell proliferation and maturation results in improved functional outcomes16,17. Thus, GABAergic signaling during cell cycle progression could provide a mechanism for controlling the proliferation and differentiation of NG2 cells into mature oligodendrocytes in an activity-dependent manner.

We examined GABAergic regulation of NG2 cell development in cerebellar white matter. Several studies have identified disrupted cerebellar development as a common feature of brain injury in preterm infants18–21, yet the underlying mechanisms are relatively unexplored. The cerebellum is important not only for motor coordination and motor learning, but also for cognitive function22,23, suggesting that cerebellar abnormalities in infants with DWMI might contribute to the development of cognitive and affective disturbances24.

In an established mouse model of chronic hypoxia that reproduces key features of DWMI25,26, we observed delayed Purkinje cell maturation and disrupted cerebellar development. These changes were associated with dysmyelination, extensive proliferation of NG2 cells and a loss of mature oligodendrocytes. We also observed a loss of GABA_A receptor–mediated synaptic input to NG2 cells from local white matter interneurons. The effects of hypoxia on oligodendrocyte lineage cells were mimicked by blockade of GABA_A receptors or deletion of the chloride-accumulating transporter NKCC1 and were reversed by inhibition of GABA catabolism or uptake. Together, these findings suggest that GABA, acting through GABA_A receptors, regulates cerebellar NG2 cell development and that this is altered in a model of diffuse white matter injury.

RESULTS
Neonatal hypoxia disrupts myelination in the cerebellum
To study the effect of hypoxia on the GABAergic regulation of NG2 cells and myelination in cerebellar white matter, we used a mouse model of DWMI25,26. Mice in which oligodendrocyte lineage cells expressed DsRed (NG2DsRed mice) were exposed to hypoxic conditions (10.5% O2) from postnatal days 3 to 11 (P3–11). Initially,
we examined cerebellar sections from mice at four time points: midway through the hypoxic treatment (P7), immediately following the treatment (P11) and at two ages following return to normoxic conditions (P15 and P30) (Fig. 1a). Neonatal hypoxia led to changes in cerebellar gross anatomy and cellular development. Specifically, following the hypoxic treatment (P11), there was a reduction in cerebellar size, coupled with structural changes, including a poorly developed intercrural fissure separating cerebellar lobules VI and VII (Supplementary Fig. 1). At P11, these changes were associated with a reduction in the thickness of the molecular layer and a thickening of the external germinal layer (Fig. 1b).

Purkinje cells are GABAergic neurons that provide the sole output of the cerebellar cortex. Immunofluorescence for a Purkinje cell marker, calbindin, revealed that hypoxic treatment led to marked changes in cerebellar myelination following neonatal hypoxia. (a) Timeline showing the experimental design. (b) Effect of hypoxia on thickness of cortical layers at P11. Note the lack of change in internal granule cell layer (IGL, n = 4 mice each, P = 0.21), but reduced thickness of the molecular layer (ML, n = 6 and 5 mice) and increased thickness of the external granule cell layer (EGL, n = 6 and 5 mice). For all panels, bar graph data are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired Welch two-sample t tests). (c) Representative confocal images of sagittal sections from P11 mice showing calbindin immunofluorescence (green). PCL, Purkinje cell layer. Scale bars represent 50 μm. (d) Effects of hypoxic treatment on Purkinje cell (PC) number. Two-way ANOVA showed a significant main effect of treatment, but no effect of age (P = 0.71) and no interaction between age and treatment (P = 0.98) (n = 4–7 normoxic mice and 5 hypoxic mice). There was no effect of hypoxia on Purkinje cell number at any age (P = 0.077, 0.28, 0.72 and 0.45 for P7, P11, P15 and P30, respectively). (e) Hypoxic treatment decreased Purkinje cell length at P7–15. Two-way ANOVA showed significant main effects of hypoxia and age and a significant interaction (n = 4–7 normoxic mice and 5 hypoxic mice at each age). (f) Representative confocal images showing NF200 (green) and MBP (red) immunofluorescence. WM, white matter. Scale bars (top row) represent 50 μm. Boxed areas are shown enlarged in lower row. Scale bars represent 25 μm. Arrowheads indicate myelination of presumptive Purkinje cell axons. Note the reduced myelination of these axons following hypoxia. (g) Hypoxic treatment decreased NF200 immunofluorescence in white matter. Two-way ANOVA showed a significant effect of hypoxia, no significant effect of age (P = 0.96) and no interaction (P = 0.98) (n = 6–10 normoxic mice and 5–12 hypoxic mice). (h) Hypoxic treatment decreased MBP immunofluorescence in white matter. Two-way ANOVA showed significant effects of hypoxia, no effect of age (P = 0.059) and no interaction (P = 0.40) (n = 5–10 normoxic mice and 4–9 hypoxic mice). (i) Electron microscopy images from P11 white matter. Scale bars represent 1 μm. (j) Left, scatter plot of g ratios of individual axons versus axon diameter; pooled data from three normoxic and three hypoxic mice (blue and red, respectively). Fitted lines are linear regressions and shaded areas indicate 95% confidence bands. Right, plot of normalized cumulative probability of g ratio for the three normoxic mice (blue dashed lines) and the three hypoxic mice (red dashed lines). Solid lines are the averaged probability plots and the shaded areas denote the s.e.m. Hypoxia increased the g ratio from 0.81 ± 0.02 to 0.91 ± 0.004. See Supplementary Methods Checklist for full details of statistical tests.
changes in dendritic extent and arborization at P7–15 without any effect on Purkinje cell number (Fig. 1c,d and Supplementary Fig. 2a). Purkinje cell dendritic length recovered to control values at P30 (Fig. 1e).

Immunolabeling with antibodies to markers for neurofilament (NF200) and myelin basic protein (MBP) suggested a reduction in the number of myelinated axons in the granule cell layer and white matter (Fig. 1f–h and Supplementary Fig. 2b). At P11 and P15, the loss of MBP labeling persisted when immunofluorescence was normalized to that of NF200, suggesting hypomyelination rather than axonal loss alone. Notably, hypoxia did not increase apoptosis or axonal damage (Supplementary Fig. 2c–e). At P30, the MBP/NF200 ratio was not reduced by hypoxia (data not shown). These observations were supported by western blot data: the MBP/NF200 ratio was reduced at P11 (from 0.80 ± 0.09 to 0.39 ± 0.09, t\textsubscript{19} = 3.25 P = 0.032), but not at P30 (1.06 ± 0.22 versus 0.73 ± 0.16, t\textsubscript{12} = 3.65, P = 0.029, both n = 3, Welch two-sample unpaired t test; Supplementary Fig. 3a,b). Although these data suggest partial recovery with age, electron microscopy provided direct evidence for hypomyelination, with increased g ratios at both P11 and P30 (Fig. 1i) and Supplementary Fig. 3c,d). Together, the results are indicative of disrupted cerebellar axonal myelination following neonatal hypoxia.

Neonatal hypoxia delays the development of NG2 cells

We next investigated whether the loss of axonal MBP following neonatal hypoxia was a result of abnormalities in the development of NG2 cells, as has been described previously for subcortical white matter\textsuperscript{14–16}. We examined the proliferation and maturation of NG2 cells in the cerebellar white matter by labeling cells for the oligodendroglia marker Olig2, the cell cycle marker Ki67 and the mature oligodendrocyte marker CC1 (Fig. 2a,b). Neonatal hypoxia led to an increase in the number of Olig2\textsuperscript{+} NG2 cells (oligodendrocyte progenitors), an increase in the number of Ki67\textsuperscript{+} NG2 cells (proliferating NG2 cells) and a decrease in the number of CC1\textsuperscript{+} cells (mature oligodendrocytes) at P7, 11, 15 and 30 (Fig. 2c).

Confirming the increase in NG2 cell proliferation following hypoxia, at P11 we observed an increase in the number of oligodendrocyte precursor cells expressing PDGFR\textalpha (platelet-derived growth factor-\textalpha receptor) that were positive for the cell cycle marker BrdU (bromodeoxyuridine) (from 13.2 ± 2.1 to 37.4 ± 3.6 cells per 10\textsuperscript{6} µm\textsuperscript{3}, t\textsubscript{5,95} = 4.96, P = 0.0019, n = 5 normoxic and 4 hypoxic mice; Welch two-sample unpaired t test). In addition, western blot analysis revealed a corresponding decrease at P7, 11, 15 and 30 in the level of the mature oligodendrocyte marker CNPase (2',3'-cyclic nucleotide-3'-phosphodiesterase; Supplementary Fig. 4). At P60, we identified a recovery in the number of CC1\textsuperscript{+} NG2 cells in the cerebellar white matter (Fig. 2c), suggesting a delay in maturation of NG2 cells following hypoxia rather than a persistent block of differentiation.

Decreased Purkinje cell firing following neonatal hypoxia

The proliferation of NG2 cells and their differentiation into myelinating oligodendrocytes has been shown to depend on the activity of neighboring axons\textsuperscript{27,28}. Indeed, NG2 cells in both gray and white matter receive glutamatergic and GABAergic synaptic input from neurons\textsuperscript{16,17,29–31}. Previous studies have reported a loss of GABAergic neurons in the cortical white matter of preterm infants with white matter damage\textsuperscript{8}, and delayed interneuron maturation and decreased GABA content in the cortex of mice following perinatal hypoxia\textsuperscript{9}. We therefore investigated whether alterations in the development of white-matter NG2 cells following neonatal hypoxia could reflect a reduced GABAergic ‘input’ from neurons.

First, given that Purkinje cell axons have been suggested as a potential source of inhibitory postsynaptic currents (IPSCs) recorded from NG2 cells in the white matter of the developing cerebellum\textsuperscript{12}, we asked whether the changes in the morphology of Purkinje cells following neonatal hypoxia were accompanied by changes in their activity. Using cell-attached recordings in acute slices (P10–11), we observed a clear shift in the pattern of Purkinje cell simple-spike firing (Supplementary Fig. 5). The mean frequency of firing was reduced to <50% of that seen in slices from normoxic mice, indicating a marked change in this potential source of GABAergic input to cerebellar NG2 cells.

Loss of cerebellar interneurons following neonatal hypoxia

Next, using GAD65-GFP mice, we examined the effect of neonatal hypoxia on the prevalence of interneurons in the cerebellar molecular layer and white matter. We found that GAD65\textsuperscript{+} cells were present in both

Figure 2 Altered development of NG2 cells following neonatal hypoxia. (a) Representative confocal images of sagittal sections of cerebellar white matter from NG2DsRed mice at P11. NG2 cells (red) were co-labeled with Olig2 (an oligodendrocyte lineage marker, white), Ki67 (a marker of proliferation, white), CC1 (a marker of mature oligodendrocytes, green) and DAPI (blue). Arrowheads indicate selected triple-labeled NG2 cells and double-labeled CC1\textsuperscript{+} cells. Scale bar represents 25 µm. (b) Representative images as shown in a, but from P60 mice. Scale bar represents 50 µm. (c) Pooled data showing the effect of hypoxia on the numbers of Olig2\textsuperscript{+} NG2\textsuperscript{+}, Ki67\textsuperscript{+} NG2\textsuperscript{+} and CC1\textsuperscript{+} cells. Graphs are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired Welch two-sample t tests; n = 5–10, 6–9, 8–6, 4–14 and 3 mice at P7, 11, 15, 30 and 60, respectively). See Supplementary Methods Checklist for full details of statistical tests.
Figure 3  Loss of cerebellar GABAergic neurons following neonatal hypoxia. (a) Representative confocal images of sagittal cerebellar sections from GAD65-GFP mice showing GAD65 expressing neurons (green) in the white matter of normoxic and hypoxic littermates at P11 and P15. Lower panels show merged images with DAPI (blue). Scale bar represents 200 μm. (b) Pooled data showing the effect of hypoxic treatment on the number of cerebellar white matter GAD65+ neurons (n = 5 and 4 mice at P11 and 5 mice each at P15). (c,d) Data are presented as in a and b for molecular layer (n = 5 mice each at P11 and 6 and 5 mice at P15). (e) Representative confocal images from P11 mice showing expression of the transcription factor Pax2 (white, middle) in GAD65 + neurons (green, upper). Lower panels show merged images with DAPI (blue). Scale bars represent 200 μm. Selected regions of white matter (boxed) are enlarged to the right. (f) Pooled data showing the effect of hypoxic treatment on the number of Pax2 + GAD65-GFP cells in the white matter at P11 (n = 11 and 12 mice). All graphs are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired Welch two-sample t test). See Supplementary Methods Checklist for full details of statistical tests.

Reduced GABAergic input to NG2 cells and interneurons

To determine the source of GABAergic input to cerebellar NG2 cells, we performed whole-cell recordings from white-matter NG2 cells at P7–8. In the presence of 1 μM strychnine hydrochloride, 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 20 μM d-(-)-2-amino-5-phosphopentanoic acid (d-AP5; to block AMPA, NMDA and glycine receptors), we observed spontaneous outward currents in 20% of cerebellar NG2 cells. We observed a marked reduction in the number of cerebellar white matter GAD65+ neurons (n = 5 and 4 mice at P11 and 5 mice each at P15). (c,d) Data are presented as in a and b for molecular layer (n = 5 mice each at P11 and 6 and 5 mice at P15). (e) Representative confocal images from P11 mice showing expression of the transcription factor Pax2 (white, middle) in GAD65 + neurons (green, upper). Lower panels show merged images with DAPI (blue). Scale bars represent 200 μm. Selected regions of white matter (boxed) are enlarged to the right. (f) Pooled data showing the effect of hypoxic treatment on the number of Pax2 + GAD65-GFP cells in the white matter at P11 (n = 11 and 12 mice). All graphs are presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 (unpaired Welch two-sample t test). See Supplementary Methods Checklist for full details of statistical tests.

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NG2 cells are innervated by local interneurons

Carbachol, a muscarinic acetylcholine receptor agonist, has been shown to potently activate hippocampal interneurons, resulting in an increase in sIPSC frequency and a 68% decrease in phasic charge transfer (Online Methods) was reduced by 75% (Fig. 4b). The formation of GABAergic synapses between molecular layer interneurons is known to precede their morphological and neurochemical differentiation35. Consistent with synaptogenesis being an early step in interneuron maturation, we observed sIPSCs in almost all of the recordings from GAD65+ neurons in the white matter at P7–8 (27 of 31 cells from 10 mice; Fig. 4c,d). In parallel with the disrupted GABAergic input to NG2 cells, we also observed a decrease in the prevalence of interneuron sIPSCs following hypoxia (8 of 18 cells from 7 mice). Although there was no change in sIPSC mean amplitude or decay (peak amplitude 22.9 ± 2.5 pA to 18.8 ± 4.2 pA, Δt50 = 0.70, P = 0.51; τw,decay 52.0 ± 5.5 ms to 51.0 ± 13.7 ms, Δt40 = 0.06, P = 0.95; n = 25 and 4 cells from 3 and 10 mice, respectively; Welch two-sample unpaired t test), there was a 49% decrease in the number of white-matter interneurons with sIPSCs, a 74% decrease in overall sIPSC frequency and a 68% decrease in phasic charge transfer (Fig. 4c,d).
increased frequency of sIPSCs in hippocampal NG2 cells\textsuperscript{16,36}. We found that carbachol (10 µM) increased the frequency of sIPSCs in white-matter NG2 cells from normoxic, but not hypoxic, mice (Fig. 5a,b), consistent with a loss of presynaptic input. GABA\textsubscript{A} receptors were present in NG2 cells following hypoxia, as infrequent synaptic events were still observed (Fig. 5a) and whole-cell currents could be evoked by bath application of GABA (data not shown). Recent studies have demonstrated that carbachol can directly activate muscarinic receptors in NG2 cells\textsuperscript{37}, raising the possibility that the increase in sIPSC frequency could be independent of neuronal input. However,
the loss of response following hypoxia makes this unlikely. Moreover, we found that the effect of carbacol was blocked in normoxic mice by prior treatment with TTX or reversed by its subsequent application (Supplementary Fig. 7).

The effect of carbacol on sIPSC frequency appeared to reflect an increase in the synaptic input from white-matter interneurons rather than from Purkinje cells, as carbachol markedly increased the firing of GAD65+ white-matter interneurons (Fig. 5b) while having no effect on the firing of Purkinje cells (Fig. 5c). In addition, electrical stimulation in the Purkinje cell layer failed to evoke IPSCs in the majority of white-matter NG2 cells, but did evoke IPSCs in the majority of white-matter interneurons (data not shown). Furthermore, application of the specific SK2 channel inhibitor apamin (100 nM), which produced a marked increase in Purkinje cell firing and bursting (Fig. 5d) without affecting the firing of white-matter interneurons (Fig. 5e), did not alter the frequency of sIPSCs in NG2 cells (0.034 ± 0.045 Hz to 0.053 ± 0.020 Hz, P = 0.30, n = 6 cells from 3 mice, Welch two-sample paired t-test). Together, these results suggest that cerebellar white matter NG2 cells are innervated by local interneurons.

**GABAergic signaling regulates NG2 cell development in vivo**

We next investigated whether manipulation of GABAergic transmission in vivo could alter the proliferation and differentiation of NG2 cells. To do this, we initially injected NG2-DeR mice daily from P5–11 with the GABA_A receptor antagonist bicuculline and examined immunolabeling for Olig2, Ki67, and CC1 in the cerebellar white matter. Treatment with bicuculline (1 mg per kg of body weight, intraperitoneal, i.p.) resulted in a more than twofold increase in the number of Olig2+ NG2 cells, a greater than threefold increase in the number of Ki67+ NG2 cells and a 40% decrease in the number of CC1+ cells (Fig. 6a and Supplementary Fig. 8a). We also examined the effect of increasing the availability of GABA by treating mice with the GABA transporter inhibitor tiagabine (50 mg per kg i.p., P5–11) or the GABA transaminase inhibitor vigabatrin (100 mg per kg i.p., P5–11). In marked contrast with the effects of bicuculline, both tiagabine and vigabatrin decreased NG2 cell proliferation and increased the number of mature oligodendrocytes compared with vehicle-injected mice (Fig. 6a and Supplementary Fig. 8a).

Do these changes in NG2 cell development reflect altered GABA_A receptor–mediated signaling to NG2 cells? To address this, we examined the effect of GABA_A receptor drugs on the proliferation of purified cortical NG2 cells in culture (Online Methods). Treatment with bicuculline (50 μM) did not affect NG2 cell proliferation. Conversely, the agonist muscimol (100 μM) produced a decrease in the number of BrdU+ NG2 cells (Supplementary Fig. 8b). Thus, GABA_A receptors on NG2 cells can directly influence their proliferation.

In both neuronal and glial precursors, GABA causes depolarization as a result of the expression of Na^+–K^+–Cl^- co-transporter 1 (NKCC1), which maintains an elevated intracellular chloride13,16,38,39. Consistent with a role for GABAergic signaling in the regulation of NG2 cells, cerebellar sections from mice deficient in NKCC1 displayed changes similar to those seen with bicuculline. Thus, at P7, 11 and 15, Nkcc1<sup>−/−</sup> mice showed increased proliferation of white-matter NG2 cells and decreased numbers of mature CC1<sup>+</sup> oligodendrocytes compared with littermate controls (Fig. 6b and Supplementary Fig. 9).
To determine the role of GABA-mediated depolarization in NG2 cells specifically, we crossed Nkcc1loxP/loxP mice (in which the Nkcc1 gene was flanked with loxP sites) with Rosa26-YFP reporter mice. The offspring were crossed with Pdgfra-creERT2 mice, allowing the selective deletion of Nkcc1 from NG2 cells and the persistent identification of oligodendrocyte lineage cells (Online Methods). We compared Pdgfra-creERT2, Nkcc1loxP/loxP, Rosa26-YFP with littermate controls (Pdgfra-creERT2, Nkcc1loxP, Rosa26-YFP) at P15 and determined the percentage of GFP+ oligodendrocyte lineage cells that expressed NG2, Ki67 or CC1, and the intensity of MBP labeling in the white matter. The selective deletion of Nkcc1 from NG2 cells increased the proportion of oligodendrocyte lineage cells that expressed NG2, increased the proportion of cells that expressed Ki67 and decreased MBP labeling in the white matter (Fig. 6c,d). Of note, the number of CC1+ GFP+ cells in the littermate controls was not different from that seen in Pdgfra-creERT2, Rosa26-YFP mice (data not shown). Together, these data are consistent with the idea that GABA_A receptor-mediated depolarization promotes the development of cerebellar NG2 cells in vivo.

**Vigabatrin and tiagabine ameliorate the effects of hypoxia**

Next, we asked whether increasing the availability of GABA in vivo after hypoxia could influence the proliferation and differentiation of NG2 cells. Following hypoxic treatment (P3–11), we injected mice daily for 5 d (P11–15) with tiagabine (50 mg per kg i.p.), vigabatrin (100 mg per kg i.p.) or saline. We prepared cerebellar sections at P15 from hypoxic and normoxic drug-treated and control animals and examined immunolabeling for Olig2, Ki67 and CC1 in the cerebellar white matter. Both treatments reduced the number of Olig2+ and Ki67+ NG2 cells, whereas tiagabine increased the number of CC1+ cells (Fig. 7a,b and Supplementary Fig. 10). Following vigabatrin or tiagabine, the number of mature oligodendrocytes was not different from that in untreated normoxic animals.

To determine whether the restoration of mature oligodendrocyte numbers was associated with recovery of myelination, we examined MBP immunolabeling. Following tiagabine treatment, MBP expression, as determined by fluorescence intensity, was indeed increased compared to the hypoxic control group. This effect was associated with disruption of GABAergic signaling from white-matter interneurons to NG2 cells. In addition, we found that treatment of mice with drugs known to modify GABA levels and/or GABAergic signaling, such as vigabatrin, ameliorated the effects of hypoxia on NG2 cells and promoted oligodendrogenesis and myelination.

**DISCUSSION**

Using a clinical relevant in vivo mouse model of DWMI, we found that chronic neonatal hypoxia results in dysregulated development of cerebellar white-matter NG2 cells, leading to hypomyelination. This effect was associated with disruption of GABAergic signaling from white-matter interneurons to NG2 cells. In addition, we found that treatment of mice with drugs known to modify GABA levels and/or GABA_A receptor–mediated signaling altered the developmental progression of NG2 cells into mature oligodendrocytes. Equivalent results were also obtained by selectively abating the Cl− transporter Nkcc1 in NG2 cells. Together, our findings suggest that reducing GABAergic synaptic transmission contributes to the cerebellar hypomyelination seen in DWMI. Our findings are relevant not only to the understanding of cerebellar maturation and the etiology of DWMI, but also to the development of therapies to promote repopulation of damaged brain regions with myelinating oligodendrocytes.

**Neonatal hypoxia disrupts cerebellar myelination**

Our observation of reduced myelin formation following hypoxia echoes the decreases in cerebellar white-matter volume detected in premature neonates with DWMI. We found that neonatal...
hypoxia resulted in increased proliferation of NG2 cells in the cerebellar white matter and a reduction in the number of mature CC1+ oligodendrocytes. In premature infants, the highest risk for DWMI is around 23–32 weeks of gestation. This time period corresponds to the onset of premyelinating oligodendrocyte production and early white-matter myelination. Motor impairment and the development of cognitive defects have been linked to cerebellar damage in premature infants21,24, and Purkinje cell abnormalities have been seen in post mortem studies of patients with autism spectrum disorders (ASDs) and in a mouse model of ASD23. Thus, it seems likely that the cerebellar dysmyelination seen in our model of DWMI may be clinically relevant.

Neonatal hypoxia disrupts GABAergic signaling in the cerebellar white matter

Neonatal hypoxia, in addition to disrupting the development of GABAergic Purkinje cells, resulted in a loss of proliferating GAD65+ interneuron progenitors in the cerebellar white matter. A similar loss has been reported in mice deficient for the DNA repair factor XRCC1, an effect linked to an increase in the expression of the cell cycle arrest protein p53 (ref. 41). Moreover, loss of the cell cycle protein cyclin D2 has also been shown to lead to a loss of cerebellar molecular arrest protein p53 (ref. 41). Moreover, loss of the cell cycle protein has been reported in mice deficient for the DNA repair factor XRCC1, suggesting that control of proliferation is critical for interneuron development. Notably, mice lacking XRCC1 also have reduced hippocampal CNPase44, suggesting that a reduction in GABAergic signaling could also alter the generation of mature oligodendrocytes in the hippocampus.

NG2 cells express GABA A receptors16,39 and, in the cortex31, hippocampus6,17,30 and cerebellum52, these cells receive GABAergic synaptic input during the first postnatal week. Purkinje cells have been suggested to provide GABAergic synaptic input to cerebellar NG2 cells32. In contrast, our recordings from cerebellar white-matter NG2 cells at P7–8 suggest that they receive GABAergic synaptic input solely from local GAD65+ interneurons. The sIPSCs in NG2 cells had varied rise times, suggesting that they may result from both direct synaptic contact and GABA spillover. Following neonatal hypoxia, we observed a marked loss of GABAergic currents in cerebellar white-matter NG2 cells, and disrupted differentiation of NG2 cells into mature oligodendrocytes, suggesting that GABAergic activity may regulate this process. Of note, a loss of mature oligodendrocytes has also been detected in an animal model of Rett syndrome43, in which GABAergic synaptic transmission is reduced44.

GABAergic regulation of NG2 cell development

GABA is known to be important in the regulation of neurogenesis, as GABAergic synaptic input to neuronal precursor cells promotes the survival and maturation of neuronal progenitors11,45. In recent studies, GABA has been shown to reduce proliferation of neuronal stem cells via activation of two PIKK family members, the ataxia-telangiectasia mutated (ATM) and ATM Rad 3-related (ATR) kinases, leading to the phosphorylation of the histone variant H2AX (γH2AX)12,46. An ability of GABA to regulate the cell cycle progression and development could also be involved in NG2 cell development.

In hippocampal NG2 cells from young rats, GABA is depolarizing, as a result of the expression of NKCC1 (ref. 39). We found that mice lacking NKCC1, either globally or specifically in NG2 cells, exhibited increased proliferation of cerebellar NG2 cells and a reduction in the number of mature oligodendrocytes. We previously linked proliferation of NG2 cells and dysmyelination of the subcortical white matter following chronic hypoxia with loss of the cell cycle arrest protein p27KIP1 (ref. 14). In this regard, it is particularly interesting to note that, in retinal progenitor cells, where GABA maintains proliferation, p27KIP1 is downregulated by GABA A receptor–mediated depolarization47. These results suggest that GABA-mediated depolarization in NG2 cells could provide a regulatory pathway for controlling NG2 cell development.

Our results suggest potential therapeutic avenues for promoting the repopulation of myelinating oligodendrocytes in DWMI. Following hypoxia, we found that treatment with the established anti-epileptic drugs tiagabine or vigabatrin decreased the proliferation of NG2 cells and increased the number of mature oligodendrocytes to control levels. Although treatment of neonates with anti-epileptics may be beneficial in terms of DWMI-induced dysmyelination, our findings raise the possibility that the use of such drugs to treat infants with perinatal seizures may itself perturb myelination. This deserves investigation.

Activation of Notch signaling inhibits differentiation of oligodendrocyte precursors48. Recently, we have shown that intranasal epidural growth factor (EGF) treatment or enhancement of EGF receptor signaling in NG2 cells by inhibiting Notch signaling have the capacity to rescue white-matter loss in DWMI15. Other studies have shown that over-activity in the Wnt signaling pathway following hypoxia also causes arrest in the maturation of oligodendrocyte precursors49. While our manuscript was in revision, a study was published that identified a role for HIP1/2a activity and autocrine Wnt7a/7b signaling in regulating OPC maturation and the response to hypoxia50. How these different levels of regulation combine to determine NG2 cell proliferation and differentiation remain unknown. Nevertheless, our findings on the GABAergic regulation of oligodendrocyte lineage progression suggest that modulation of GABAergic signaling in DWMI may offer a complementary approach to ameliorate the devastating effects of this currently untreatable condition.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.Z., J.S., S.G.C.-C., M.F. and V.G. designed the experiments. M.Z. and P.L. performed the electrophysiology. M.Z. and J.S. carried out the immunocytochemistry and drug treatment studies. J.S. and V.G. generated the Pdgfra-creERT2; Nkcc1loxP/loxP, Rosa26-yfp mouse. M.Z., J.S. and J.L.D. performed electron microscopy. B.M. performed culture experiments. J.E. performed genotyping. L.H. and D.S. provided the Nkcc1−/− mouse brain tissue. C.A.H. provided the Nkcc1−/− mouse brain tissue. C.A.H. and M.F. analyzed the experimental data. M.Z. and M.F. prepared the figures and wrote the manuscript, with contributions from S.G.C.-C., J.S. and V.G.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
ONLINE METHODS

Mice. We used NG2DsRed mice expressing DsRed-T1 under the control of the NG2 promoter31, GAD65-GFP mice (also known as Gad2-egfp) expressing GFP under the control of the Gad2 promoter35, Nkcc1loxPloxP mice (also known as Slc12a2loxPloxP), in which the Nkcc1 (Slc12a2) sequence is loxP-flanked83, Rosα26-ylp reporter mice (Jackson Laboratories, stock number 006148) and Pdgfra-creERT2 mice that express tamoxifen-inducible Cre under the control of the Pdgfra promoter54, Nkcc1loxPloxP mice were crossed with Rosα26-ylp mice and their offspring crossed with Pdgfra-creERT2 mice. Cre recombination produced the selective knockout of Nkcc1 from NG2 cells and deletion of the loxP-flanked stop sequence ahead of the yellow fluorescent protein sequence enabled the persistent identification of oligodendrocyte lineage. To induce Cre recombination, tamoxifen was administered daily (P8–11) at a dosage of 75 mg per kg body weight15. Genotyping was performed as previously reported53,54. Tissue from Nkcc1−/− mice (mixed 129/Sv and Black Swiss background) carrying a null mutation in the Nkcc1−/− mice (mixed 129/Sv and Black Swiss background) were examined from P60 animals. Mice were anesthetized with isoflurane and cerebellar slices from mice at P7, 11, 15 and 30. In some cases, additional slices were used as follows: FITC-conjugated goat anti-mouse IgG (H+L) (Jackson, 115-096-003, 1:200), Cy5-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, 111-175-144, 1:200) and Cys3-conjugated Cy5 (Jackson ImmunoResearch, 111-175-144, 1:200). Sections were incubated with secondary antibodies: FITC-conjugated goat anti-mouse IgG (H+L) (Jackson, 115-096-003, 1:200), Cy5-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, 111-175-144, 1:200) and Cys3-conjugated Cy5 (Jackson ImmunoResearch, 111-175-144, 1:200). Sections were incubated with secondary antibodies and were used as follows: FITC-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch, 115-096-003, 1:200), Cy5-conjugated goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, 111-175-144, 1:200) and Cys3 conjugated (Invitrogen, A10522, 1:200). Sections were incubated with secondary antibodies for 1 h at 23–26 °C followed by a 10-min incubation with DAPI (Invitrogen, D1006, 300 nM) and mounted using anti-Fade gold (Invitrogen P36930). Cultured NG2 cell cultures were labeled with NG2 (rabbit, Millipore, MAB5384, 1:400) and BrdU (rat, Accurate Chemical, YSRTMCA2060GA, 1:100) antibodies.

Cell counting and fluorescence measurements. Four different lasers were used to image the localization of FITC (488-nm laser line excitation; 522/35 emission filter), DsRed (558-nm excitation; 583 emission), Cy5 (647 excitation; 680/32 emission) and DAPI (excitation 345, 354/400 emission).

Cell counts were obtained using 40-µm-thick sagittal cerebellar sections from P7–60 mice. Images were captured using a confocal microscope (Olympus FV1000). We consistently imaged cerebellar white matter located between the borders of the internal granule cell layers at the junction of folium VIII and IX. The white matter was first identified using a 10× objective, with DAPI stain to highlight the internal granule cell layers. The slice was then viewed using a 40× objective and images acquired with a z step size of 1 µm. The z-stack was viewed using NIH ImageJ61 (http://imagej.nih.gov/ij/) and the white matter delineated using the freehand selection tool (~50,000 µm²). Double- or triple-labeled cells within this area were manually counted in each optical section using the Image ‘Cell Counter’ plugin (ImageJ, http://rsb.info.nih.gov/ij/plugins/Cell-counter.html). For each marker, any cells displaying immunoreactivity visually judged to be above background were scored as positive49. The results were expressed as cells per 10⁵ µm² (ref. 15). For each condition (and littermate or vehicle controls), data were obtained from 4–14 mice. For each mouse, data were pooled from 4 adjacent near-midline slices.

BrdU®. NG2 cells in culture were quantified using the Stereo Investigator system (http://www.mnbbioscience.com/stereo-investigator) allowing random sampling of cell counts using the optical fractionator method. Cells were counted using a 40× objective and a three-dimensional counting frame in a sampling grid as previously described4. In each condition a minimum of 100 NG2 cells were counted.

Western blot analysis. Cerebellar tissue was homogenized in RIPA lysis buffer with protease inhibitors (Millipore 20-188). Protein extracts were boiled for 5 min before loading onto 4–20% gradient gels (GeneMate; 15 µl of protein per lane). Gels were electrophoresed to a 0.2-µm nitrocellulose membrane (Millipore). Blots were blocked in 5% milk (wt/vol) in TBST for 1 h, then incubated at 4 °C overnight with one of the following antibodies: anti-MBP (mouse, Covance SMI-94R, 1:1,000), anti-CNPase (mouse, Abcam 6319, 1:1,000), anti-casp-act-1 (rabbit, Abcam ab3280, 1:10,00), NF200 (rabbit, Sigma 4142, 1:1,000). Transferred proteins were detected with appropriate horseradish peroxidase-conjugated (HRP) secondary antibodies: goat anti-mouse IgG-HRP (Santa Cruz sc-2005, 1:1,000), or goat anti-rabbit IgG-HRP (Santa Cruz sc-2030, 1:1,000), reacted with chemiluminescent ECL substrate (GE Healthcare), and visualized by X-ray exposure. Band intensity was measured using a calibrated densitometer (Bio-Rad GS-800). Western blots were obtained from three animals in each group. All antibodies used in immunocytochemical or western blot analysis have been validated for use in the respective assays and species (see Antibodypedia (http://www.antibodypedia.com/)), CiteAb (http://www.citeab.com/), suppliers’ literature or refs. 15,51).

Electron microscopy. P11 and P30 mice were perfused with 4% paraformaldehyde containing 10% picric acid (vol/vol) and 5% glutaraldehyde (vol/vol), and the brains were removed and post-fixed for 2 weeks15. Cerebellar tissue was sectioned (200 µm) using a slicing microtome (Leica RM2255), and postfixed in 1% osmium tetroxide (vol/vol) in PBS for 2 h (ref. 62). Ultrathin sagittal sections (0.1 µm) of the cerebellar white matter were examined using a Jeol transmission electron microscope (JEM 1010 or 1400). Images were acquired using a Gatan ORIUS SC1000 camera and measurements made using ImageJ. The myelin thickness was calculated from the mean of four measurements per sheath. The axon diameter was calculated from the measured circumference. The extent of myelination was compared by determining g ratios (the ratio of the inner axonal diameter to the total outer diameter). Measurements were performed by JS, who was blind to the origin of the tissue. At least 100 axons were measured for each brain.

Cerebellar slice preparation. Mice (P5–11) were anesthetized with isoflurane and decapitated. After brain dissection, 250-µm-thick sagittal slices were cut in an ice-cold oxygenated solution (87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 7 mM MgCl2, 0.5 mM CaCl2, 25 mM NaHCO3, 25 mM glucose, 75 mM sucrose;
Electrophysiology. Slices were placed in a submerged chamber on the stage of an upright microscope (Olympus BX51) and perfused at ~2 ml min⁻¹ with recording solution (above). DrsRed NG2 cells and GFP+ neurons were visualized using epifluorescence illumination and infrared differential interference contrast or infrared oblique illumination. Recording pipettes were pulled from thick-walled borosilicate glass tubing (1.5-mm outer diameter, 0.86-mm inner diameter, Harvard Apparatus) and coated with Syglard (Dow Corning 184). For whole-cell recording, pipettes were filled with internal solution (130 mM CsCH₃SO₃H, 20 mM HEPES, 10 mM EGTA, 2 mM Na-ATP and 0.2 mM Na-GTP; pH 7.3) and had a resistance of 5–10 MΩ. Currents were recorded at 22–26 °C using an Axopatch-200A or 700B amplifier (Molecular Devices), filtered at 5 kHz (low-pass 8-pole Bessel filter) and sampled at 10 kHz.

Recordings were made from NG2 cells and GFP+ neurons in the white matter. NG2 cells were identified by their lack of obvious contact with neighboring fluorescent cells or blood vessels, characteristic rounded soma with soma processes, and the expression of voltage-gated Na+ channels. Series resistance compensation of ~60% was applied in all whole-cell voltage-clamp recordings. For NG2 cells, the final series resistance was 16.8 ± 2.1 MΩ and 21.5 ± 2.3 MΩ for normoxic and hypoxic cells (n = 31 and 29, respectively). For GFP+ neurons, the final series resistance was 18.8 ± 0.8 MΩ and 16.8 ± 1.4 MΩ for normoxic and hypoxic cells (n = 31 and 18, respectively). sIPSCs were recorded at +30 mV in the presence of 1 mM strychnine hydrochloride, 20 µM DNBX and 20 µM n-AP5 (Tocris Bioscience) to block glycine, AMPA and NMDA receptors, respectively. 5 µM QX314 (Abcam) was included in the internal solution for interneuron recordings. In some recordings, extracellular stimulation (20–100 V, 20–100-µs duration at 0.1 Hz; DS2 stimulator, Digitimer) was delivered to the Purkinje cell layer using a second glass micropipette containing external solution. In all cases when tested, evoked IPSCs were fully blocked by 20 µM SR-95531 (Tocris Bioscience). mIPSCs were recorded in the additional presence of 1 µM TTX. Focal application of hyperosmotic sucrose (500 mM) was achieved using gravity-fed perfusion via a patch electrode (~1–1.5 MΩ resistance).

IPSC analysis. Records were analyzed using Igor Pro 6.10 (Wavemetrics). Spontaneous currents were detected using a scaled template algorithm based on rising and decaying exponentials (NeuroMat 2.6, http://www.neuromatic.thinkrandom.com/). Measurements of mean amplitude, decay and 10–90% rise time from the fitted curves were performed using an internal solution containing 150 mM potassium glutonate, 3 mM MgCl₂, 0.5 mM EGTA, 2 mM MgATP, 0.3 mM Na2GTP and 10 mM HEPES; pH 7.4 with KOH. Whole-cell recording, rather than cell-attached recording, was used for interneurons, as they were not always active; this allowed us to judge the quality of the recording by first checking that the cell fired in response to current injection. Subsequently, we also made cell-attached recordings form some interneurons to test the effect of apamin. In this condition, as spontaneous firing was rare, at the end of each recording we applied carbachol to induce activity and thus verify the quality of the recording.

Drug injections. NG2DrsRed mice received daily i.p. injections (from P5–11 or from P11–15) of either 1 mg per kg bicuculline (Abcam) in sunflower seed oil (Sigma 47123), 100 mg per kg vigabatrin in saline or 50 mg per kg tiagabine in saline (Tocris Bioscience). Control mice were injected with vehicle alone. Injection volume was 10 µl per g body weight, and solutions were warmed to 37 °C. The dose of bicuculline used was roughly half the CD₅₀ reported for P7 rats, two mice exhibited seizures and were excluded from the study.

Data presentation and statistical analysis. Summary data are presented in the text as mean ± s.e.m. from n cells or animals. Comparisons involving two data sets only were performed using a two-sided Welch two-sample t test that does not assume equal variance (normality was not tested statistically, but gauged from Q–Q plots and/or density histograms). For Purkinje cell firing data, where we did not assume a normal distribution (Supplementary Fig. 5), a non-parametric Wilcoxon Mann–Whitney rank sum test was used. Analyses involving data from three or more groups were performed using one-way or two-way analysis of variance (Welch heteroscedastic F test) followed by pairwise comparisons using two-sided Welch two-sample t tests (with Holm’s sequential Bonferroni correction for multiple comparisons). For analysis of contingency tables we used Fisher’s exact test. Differences were considered significant at P < 0.05. Statistical tests were performed using Prism version 6 (GraphPad Software) or R (version 3.0.2, the R Foundation for Statistical Computing, http://www.r-project.org/) and R Studio (version 0.98.477, RStudio). The following packages were used: biocLite, cat2, coin2 andphia. No statistical test was used to predetermine sample sizes; these were based on standards of the field. No randomization was used, except in quantification of purified NG2 cells in culture (Stereo Investigator system). Blinding of investigators was used only in the electron microscopy analysis.

A Supplementary Methods Checklist is available.
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