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Clemastine rescues myelination defects and promotes functional recovery in hypoxic brain injury

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Hypoxia can injure brain white matter tracts, comprised of axons and myelinating oligodendrocytes, leading to cerebral palsy in neonates and delayed post-hypoxic leukoencephalopathy (DPHL) in adults. In these conditions, white matter injury can be followed by myelin regeneration, but myelination often fails and is a significant contributor to fixed demyelinated lesions, with ensuing permanent neurological injury. Non-myelinating oligodendrocyte precursor cells are often found in lesions in plentiful numbers, but fail to mature, suggesting oligodendrocyte precursor cell differentiation arrest as a critical contributor to failed myelination in hypoxia. We report a case of an adult patient who developed the rare condition DPHL and made a nearly complete recovery in the setting of treatment with clemastine, a widely available antihistamine that in preclinical models promotes oligodendrocyte precursor cell differentiation. This suggested possible therapeutic benefit in the more clinically prevalent hypoxic injury of newborns, and we demonstrate in murine neonatal hypoxic injury that clemastine dramatically promotes oligodendrocyte precursor cell differentiation, myelination, and improves functional recovery. We show that its effect in hypoxia is oligodendroglial specific via an effect on the M1 muscarinic receptor on oligodendrocyte precursor cells. We propose clemastine as a potential therapy for hypoxic brain injuries associated with white matter injury and oligodendrocyte precursor cell maturation arrest.

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Abbreviations: CAP = compound action potential; cKO = conditional knockout; DPHL = delayed post-hypoxic leukoencephalopathy; OPC = oligodendrocyte precursor cell

Introduction

Approximately 63 000 very low birth weight (⩽1500 g) (Martin *et al.*, 2008) infants are born in the USA each year, representing 1.5% of all live births. The significance of brain injury in this large group is highlighted by the occurrence of cognitive or behavioural deficits in 25–50%, with major motor deficits (cerebral palsy) occurring in 5–10% (Woodward *et al.*, 2005; Bayless and Stevenson, 2007; Platt *et al.*, 2007; Allin *et al.*, 2008; Kobaly *et al.*, 2008; Larroque *et al.*, 2008; Volpe *et al.*, 2009). Recent improvements in neonatal critical care led to a marked increase in survival (50–70%) of extremely low birth weight (⩽1000 g) premature infants; however, permanent disability exceeds 50% in these children contributing significantly to their burden of care (Marlow *et al.*, 2005, 2007; Wood *et al.*, 2005; Wolke *et al.*, 2008; Volpe *et al.*, 2009). Post-hypoxic white matter injury is commonly encountered in premature infants. In humans, premyelinating oligodendrocytes are most abundant at the gestational age of 23–32 weeks and are thought to be selectively vulnerable to hypoxic injury in the neonatal brain (Back *et al.*, 2002). White matter injury is the most common ischaemic brain injury in premature infants, and comprises focal injury to white matter tracts as well as diffuse gliotic lesions (Khwaja and Volpe, 2008). Such diffuse and focal injuries, collectively known as white matter injury in the newborn brain, are the most reliable prognostic indicators of development of severe cerebral palsy and cognitive disability in premature infants (Woodward *et al.*, 2006).

In these neonatal injuries, oligodendrocyte precursor cells (OPCs) are recruited to areas of injury and can regenerate damaged myelin sheaths in a process termed remyelination. However, failure of remyelination often occurs and contributes significantly to ongoing neurological dysfunction, axonal loss and disease progression. Although oligodendrocytes are thought to be cellular targets of excitotoxic damage in newborn brain injuries (Káradóttir *et al.*, 2005; Khwaja and Volpe, 2008), evidence indicates that failure of myelination is a significant contributor to fixed demyelinated lesions (Billiards *et al.*, 2008; Segovia *et al.*, 2008; Fancy *et al.*, 2011, 2014; Buser *et al.*, 2012; Verney *et al.*, 2012). Non-myelinating OPCs are often found in neonatal white matter injury lesions in plentiful numbers, but fail to differentiate (Billiards *et al.*, 2008; Segovia *et al.*, 2008; Fancy *et al.*, 2011, 2014; Buser *et al.*, 2012). Thus, failure in myelin generation seems to be due to both early premyelinating oligodendrocyte degeneration and a later phase of persistent premyelinating oligodendrocyte maturation arrest. This maturation block has profound implications for our approach to white matter injury pathogenesis, raising questions about the nature of the differentiation block, and suggesting that promotion of OPC differentiation through therapeutic manipulation might yield improved clinical outcomes.

In an effort to identify small molecules that promote OPC differentiation and myelination, we reported a novel *in vitro* high-throughput screening platform using micropillar arrays (Mei *et al.*, 2014). Upon screening 1000 bioactive molecules, we identified a cluster of antimuscarinic compounds that significantly enhanced oligodendrocyte differentiation. The most efficacious of these was clemastine, a widely available first-generation antihistamine with a favourable safety profile, used primarily for symptomatic treatment of seasonal allergies. Clemastine significantly promoted differentiation of purified OPCs cultured in isolation as well as myelination when OPCs were co-cultured with purified dorsal root ganglion neurons. Clemastine also promoted remyelination in an *in vivo* focal gliotoxic model of demyelination (Mei *et al.*, 2014), as well as in cuprizone-induced demyelination (Li *et al.*, 2015), and has been shown to enhance myelination in the prefrontal cortex of socially isolated mice (Liu *et al.*, 2016). Here we report a new application for clemastine in the treatment of hypoxic brain injury.

Our interest in clemastine for the treatment of hypoxia stemmed from a case of an adult patient who developed delayed post hypoxic leukoencephalopathy (DPHL), a rare adult condition (with no murine model) that occurs following a period of prolonged cerebral hypoxia. Most DPHL patients experience irreversible neurological injury due to demyelination with subsequent failed remyelination. In contrast, our patient made a nearly complete recovery in the setting of treatment with clemastine. The remarkable clinical and radiographic recovery following DPHL in the setting of treatment with clemastine, and its benign side effect profile, suggested potential therapeutic benefit in the much more clinically prevalent hypoxic white matter injury seen in premature newborns. Using a murine model of neonatal hypoxia, we show that clemastine significantly promotes OPC differentiation and myelination, ultrastructural myelin production, and improves functional recovery, via an oligodendroglial specific effect on the M1 muscarinic receptor on OPCs.

Materials and methods

DPHL patient

The clemastine-treated DPHL patient provided written informed consent to describe our observations. The hospital ethics committee approved off-label treatment with clemastine.

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Murine neonatal hypoxia and clemastine treatment

Mice pups with their parents were subjected to chronic sublethal hypoxia starting at postnatal Day 3, receiving chronic neonatal hypoxaemia [10% fraction of inspired oxygen (FiO_2)]. Mice pups exposed to hypoxia were either treated daily with clemastine by oral gavage (10 mg per kg body weight) or vehicle saline control. The clemastine dose used in this study was the same as previous studies (Mei *et al.*, 2014, Li *et al.*, 2015, Liu *et al.*, 2016). Hypoxic mice pups were compared to normoxic mice and clemastine-treated littermates at postnatal Days 10, 13 or 14. At least four mice per group (for each of the three groups normoxia/hypoxia/hypoxia with clemastine) per time point were used for all experiments. Saline and clemastine-treated animals exposed to hypoxia were from the same litters. Multiple CNS regions at these times, including optic nerve, corpus callosum, striatal white matter, cerebellar white matter, and spinal cord white matter were assessed. All animal husbandry and procedures were performed according to UCSF guidelines under IACUC approved protocols.

In situ hybridization

The technique has been described previously (Fancy *et al.*, 2011). Briefly, DIG-labelled antisense probes, including *Plp* and *Mag* were used to target mature oligodendrocytes, and *Pdgfra* was used to target OPCs. The targeted mRNA-expressing cells were visualized as a dark purple deposition with NBT/BCIP–alkaline phosphatase combination. Cell counting was performed under a 10× objective lens for each sample using an Image-Pro Plus software.

Immunohistochemistry

The method has been described previously (Fancy *et al.*, 2011). Primary antibodies were to the following proteins: PDGFRα (1: 200, rat anti-mouse, 558774, BD Biosciences), MBP (1: 500, rat anti-mouse, MCA409S, Serotec), Olig2 (1: 2000, rabbit anti-mouse, from C.D. Stiles, Harvard), MOG (1: 500, mouse, MAB5680, Chemicon), NF (1: 1000, mouse anti-mouse, MAB5266, Chemicon), NF (1: 1000, rabbit anti-mouse, N4142, Sigma), Caspr (1: 400, rabbit anti-mouse, from E. Peles, Weizmann Institute), AnkG (1: 150, mouse anti-mouse, 75-146, Antibodies Inc), SMI-32 (1: 1000, mouse, NE1023, Millipore), Ki67 (1:200, rabbit, ab16667, Abcam). MBP-positive areas and total MBP fluorescent signal intensities were measured in the fixed area of striatum using Image-Pro Plus software, and relative expression changes among the three groups were normalized to the normoxia control group.

Western blot

Western blot was used to measure the quantity of MBP protein in the striatum of hypoxic versus clemastine-treated hypoxic animals (four animals in each group) at postnatal Day 10. To dissect striatum tissue from whole brain, striatum was rapidly sliced and picked out using coronal brain matrices at intervals of 1 mm. Protein samples were homogenized and extracted using RIPA lysis buffer, and the lysates containing 60 µg protein separated on 12.5% SDS-PAGE gels, transferred to nitrocellulose membranes and probed with antibody against MBP (1: 2000, rat anti-mouse, MCA409S, Serotec). β-actin (1: 2000, mouse anti-mouse, NB600-501, Novus Biologicals) was used as the loading control. Quantification of band intensity was analysed using Image-Pro Plus software.

Electron microscopy and g-ratio analysis

As previously described (Fancy *et al.*, 2011), mice were anaesthetized and perfused transcardially with 4% glutaraldehyde and brain samples were post-fixed overnight. Samples were stained with osmium tetroxide overnight and dehydrated in a series of ethanol dehydration treatments. Embedding was performed in TAAB resin. Sections were cut at 1-µm intervals and stained with toluidine blue for light microscopy analysis. Axons were then examined using electron microscopy, and g-ratios were calculated as the diameter of the axon divided by the diameter of the axon and the surrounding myelin sheath. G-ratio statistics included myelinated and unmyelinated axons, and combined all observations from each animal, comparing means between animals.

Compound action potential measurement

Compound action potential (CAP) recordings were performed as previously described (Shen *et al.*, 2014). In brief, optic nerves were dissected and placed in oxygenated recording solution at room temperature. Each end of the nerve was drawn into a custom made suction electrode. A stimulus was applied at one end of the nerve and the CAP was recorded at the opposite end. The latency of the peak of the CAP and the length of the nerve was used to calculate conduction velocity.

Statistical analyses

Statistical significance between groups was determined with GraphPad Prism 5 software. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. A probability of $P < 0.05$ was considered statistically significant.

Results

Clemastine treatment in human DPHL case

White matter injury and demyelination following necrosis of vulnerable oligodendrocytes is thought to underlie the pathophysiology of DPHL, a rare adult condition occurring following a period of prolonged cerebral hypo-oxygenation. DPHL is characterized by a hypoxic insult leading to coma with complete initial recovery followed by progressive neurologic deficits days to weeks after the initial event (Shillito *et al.*, 1936; Plum *et al.*, 1962; Choi, 1983). There are no recognized therapies for DPHL, no animal model of the condition, and most patients experience irreversible

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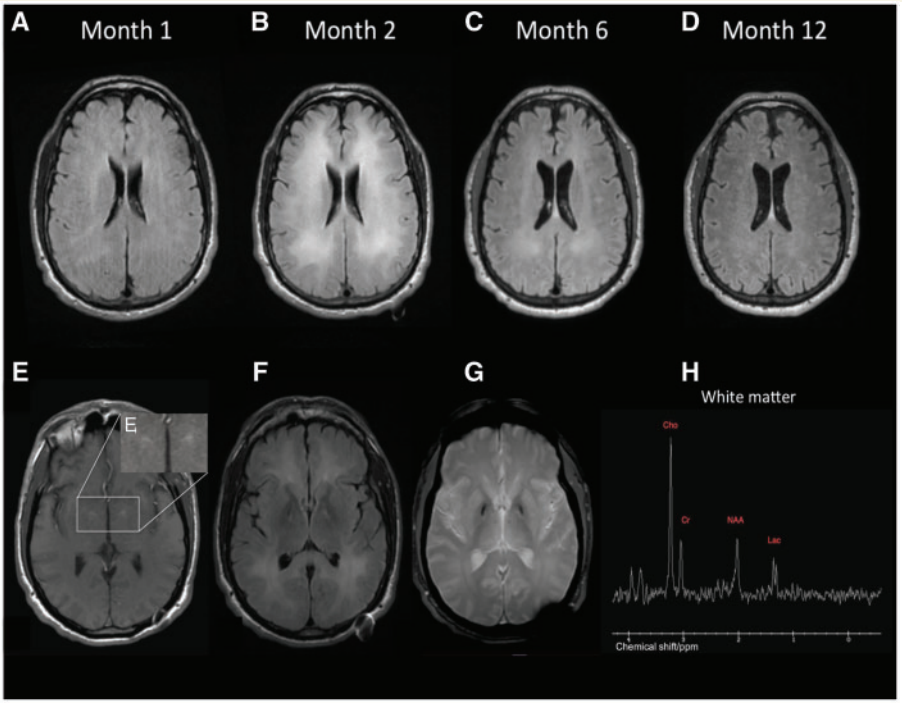

Figure 1 Findings in human DPHL case treated with clemastine. Axial FLAIR (fluid attenuated inversion recovery) at 1 (**A**), 2 (**B**), 6 (**C**), and 12 (**D**) months after injury in a human case of DPHL, showing MRI white matter abnormalities on FLAIR and diffusion weighted images, subtle at 1 month and striking at 2 months, parallel to patient's worsening clinical course; white matter signal changes showed a periventricular/deep white matter distribution with involvement of the corpus callosum (particularly splenium), with sparing of U-fibres. The patient was started on clemastine treatment at 2 months. At 6 months, MRI abnormalities in the white matter were partially normalized, showing fuzzy signal changes, unchanged at 12 months follow-up. The long-term follow-up (6 and 12 months) also showed a progressive global volume loss with consequent prominent sulci and lateral ventricles. (**E–G**) Globus pallidi MRI findings in DPHL. Brain MRI showed **E** and *inset* symmetrical T_1 shortening, (**F**) T_2/FLAIR hypointensity with (**G**) corresponding areas of susceptibility artefacts on T_2*-weighted images within the globi pallidi. CT scan was unremarkable for calcification within the basal ganglia (not depicted). The findings remained unchanged from presentation throughout the course and—in the context of the patient's history—likely represent chronic haemorrhage and necrosis secondary to the initial hypoxic damage. (**H**) Single voxel spectroscopy, acquired 2 months after the hypoxic event showed elevated peaks of choline (Cho) and lactate (Lac) and low *N*-acetylaspartate (NAA) within the deep white matter, consistent with myelinopathy. Spectroscopy of white matter was acquired with short (not shown) and long echo times each (echo time 27 ms and 288 ms, respectively, repetition time 1500 ms, field strength 3 T).

neurological injury. Most cases are due to carbon monoxide poisoning (Shillito *et al.*, 1936; Plum *et al.*, 1962; Choi, 1983) or drug overdose (Weinberger *et al.*, 1994; Gottfried *et al.*, 1997) leading to respiratory arrest, although DPHL was also reported following cardiac arrest, surgery, anaesthesia, asphyxia, gas poisoning and shock (Hori *et al.*, 1991; Heckmann *et al.*, 1998; Shprecher and Mehta, 2010). A sustained deterioration phase following the relapse reflects widespread demyelination and necrosis of oligodendrocytes in arterial border zones from the hypoxic-ischaemic insult with subsequent failure of remyelination (Grinker 1926; Plum *et al.*, 1962; Ginsberg, 1979).

We report a case of a 45-year-old male with a history of obstructive sleep apnoea, major depression and anabolic steroid use for bodybuilding who was found unresponsive and cyanotic at home. An extensive workup revealed non-ischaemic biventricular dilated cardiomyopathy. The acute respiratory failure and cardiogenic shock were thought to be secondary to anabolic steroid use and obstructive sleep apnoea. His cardiac function began to improve with supportive care, and he was discharged after 8 days with intact cognitive status. Three weeks later the patient developed rapidly progressive cognitive decline with profound short-term memory loss, impaired executive function, and paucity of speech, psychomotor retardation, urinary incontinence and gait impairment. His exam showed a flat affect, orientation only to self, perseveration, apraxia, impaired verbal recall, left-right confusion and bilateral frontal release signs. His Montreal Cognitive Assessment (MoCA) score was 6/30. Gait was wide-based, ataxic, and he was able to walk only a few steps requiring one- or two-person assistance. An extensive diagnostic evaluation for infectious, autoimmune, and toxic-metabolic aetiology was unrevealing. Brain MRI performed 1 month after the initial hypoxic event was normal (Fig. 1A). A repeat brain MRI 2 months after the hypoxic event showed extensive, diffuse increased signal throughout the hemispheres (Fig. 1B). The globus pallidus showed changes consistent with sequelae of global

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ischaemia (Fig. 1E–G) and magnetic resonance spectroscopy showed elevated choline, decreased *N*-acetyl aspartate and a lactic acid peak—changes consistent with diffuse myelinopathy (Fig. 1H). DPHL was diagnosed based on the history of hypoxic coma with initial complete recovery followed by rapidly progressive cognitive decline as well as the characteristic changes on MRI (Lee and Lyketsos, 2001; Molloy *et al.*, 2006; Zamora *et al.*, 2015) and magnetic resonance spectroscopy (Gottfried *et al.*, 1997). Based on our preclinical experience with clemastine, we reasoned that this patient's recovery might be favourably influenced through inducing oligodendrocyte maturation with this putative remyelinating medication. Given the severity of the patient's neurological deficits and likely poor recovery, clemastine fumarate (5.36 mg twice daily, a dose based on preclinical studies) (Mei *et al.*, 2014) was started as an innovative, empiric, off-label therapy. Treatment began 2 months after the hypoxic event and was continued for 10 months.

The patient was seen in follow-up 5 months after his hypoxic event. His cognitive function had markedly improved and he was able to return to work (in information technology), although he endorsed persistent impairment in short-term memory and attention, as well as lack of motivation and depression. His performance on the MoCA improved to 30/30 and the remainder of his neurological exam was normal. Follow-up brain MRI at 6 and 12 months following the hypoxic event showed improvement and nearly complete resolution of the diffuse white matter hyperintensity (Fig. 1C and D). Taken together these findings are highly consistent with global hypoxia, followed by DPHL with striking radiographic recovery by 12 months after the acute injury. The clinical and radiographic recovery following DPHL in the setting of treatment with clemastine suggested that this therapy might promote white matter repair in hypoxic brain injuries. Moreover, the benign side-effect profile of this medication suggested possible application to the more common white matter injury seen in premature newborns.

Clemastine promotes oligodendrocyte precursor cell differentiation in neonatal hypoxia

To investigate whether clemastine might be a useful treatment following hypoxic brain injury in neonates we made use of a model where mice are subjected to chronic sublethal hypoxia during the early postnatal period (10% FiO_2 between postnatal Days 3–10). This early postnatal period in mice is a period of rapid brain development, where OPCs and premyelinating oligodendrocytes are most abundant, mimicking human gestational ages of 23–32 weeks when premature infants are exposed to hypoxia in the extrauterine environment. Moreover, this murine model replicates the significant OPC maturation delay that is a hallmark of human neonatal injury, without leading to widespread oligodendroglial death, making it a useful model for testing OPC differentiation-promoting therapies.

Non-myelinating OPCs are often found in human neonatal white matter injury lesions in plentiful numbers, but show a persistent maturation arrest with subsequent deficits in myelination (Billiards *et al.*, 2008; Segovia *et al.*, 2008; Fancy *et al.*, 2011, 2014; Buser *et al.*, 2012). Similarly, we found significant delays in expression of myelin proteins such as MBP in corpus callosum (Fig. 2A and B) and striatal white matter (Fig. 2C and D) of neonatal mice exposed to chronic hypoxia when compared to normoxic controls. Neurofilament (NF) staining (Fig. 2E and F) (and lack of SMI-32 + axonal spheroids, Supplementary Fig. 1) suggests that reductions in MBP expression in hypoxic mice are not due to loss of axonal targets for myelin wrapping. Treatment of neonatal mice during hypoxia with 10 mg/kg clemastine once daily by oral gavage between postnatal Days 3–10 leads to a significant increase in MBP ($P = 1.75 \times 10^{-4}$) (Fig. 2A, B and Supplementary Fig. 2A) and myelin oligodendrocyte glycoprotein (MOG) (Supplementary Fig. 2B) protein expression detected by immunohistochemistry in corpus callosum, and in MBP protein detected by immunohistochemistry ($P = 7.79 \times 10^{-4}$) (Fig. 2C and D) and western blot analysis (Supplementary Fig. 2C and D) in striatal white matter compared to untreated hypoxic littermates. Post-treatment levels of MBP in hypoxic mice were not significantly different from normoxic controls in corpus callosum and striatum (Fig. 2B and D), suggesting a rescue of MBP expression defects by clemastine during chronic hypoxia. Neonatal hypoxia leads to similar effects on cerebellar white matter, with significant (~5-fold) (Fig. 2G and H) reductions in expression of MBP protein. As in corpus callosum and striatum (Fig. 2E and F), neurofilament staining suggests that the reduced MBP is not due to loss of axonal targets for myelination (Fig. 2I and J, and Supplementary Fig. 1). Daily treatment with oral clemastine during hypoxia leads to significant (~3-fold) increases in MBP in the cerebellar foliae compared to untreated hypoxic littermates (Fig. 2G and H).

OPCs in neonatal white matter injury lesions demonstrate a persistent maturation arrest, appearing blocked in their ability to differentiate into mature oligodendrocytes (Billiards *et al.*, 2008; Segovia *et al.*, 2008; Buser *et al.*, 2012). We found that oral administration of clemastine in murine neonatal hypoxia leads to significant increases in the numbers of differentiating OPCs expressing the markers proteolipid protein (*Plp*) and myelin associated glycoprotein (*Mag*) mRNA in corpus callosum (Fig. 3A and B) and striatum (Fig. 3C and D) compared to untreated hypoxic littermates. These alterations in differentiation kinetics occur in the absence of changes in OPC numbers, which appear unaltered with either hypoxia or clemastine treatment compared to normoxic controls (Fig. 3E, F and Supplementary Fig. 3). Additionally, we observe no appreciable Caspase 6 + or Tunel + cell death in either hypoxic animals or normoxic controls (Supplementary Fig. 4), suggesting that the increases in numbers of *Plp* + and *Mag* + cells are the result of clemastine promoting OPC

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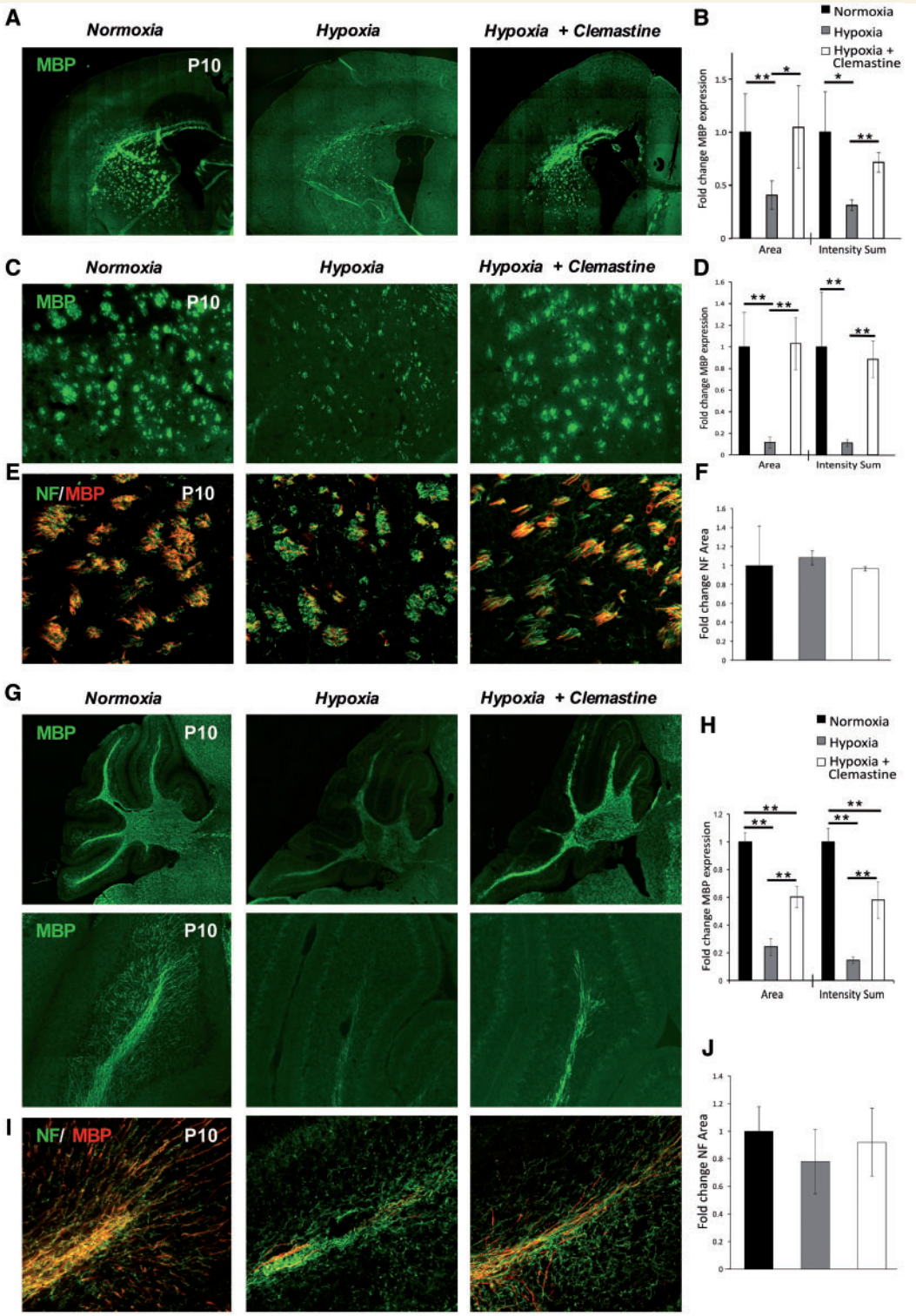

Figure 2 Clemastine promotes myelin protein expression in neonatal hypoxia. (**A**) MBP protein expression in the (**A**) forebrain, (**C**) striatum white matter and (**G**) cerebellum of postnatal Day 10 (P10) normoxic mice ('Normoxia'), versus those exposed to neonatal hypoxia ('Hypoxia'), versus hypoxic littermates treated with clemastine ('Hypoxia + Clemastine'), quantified, in (**B**) corpus callosum, (**D**) striatum, and (**H**) cerebellum as total MBP positive area ('Area') and total MBP fluorescent signal intensities ('Intensity Sum') measured using Image-Pro Plus software (with relative expression among the three groups normalized to the normoxia group). (**E** and **I**) Neurofilament (NF) and MBP staining in the three groups in (**E**) postnatal Day 10 striatum and (**I**) cerebellum suggests no axonal loss under chronic hypoxic conditions, with quantification in (**F**) striatum and (**J**) cerebellum as total neurofilament area (fold change relative to normoxic group). For all graphs, $*P < 0.05$, $**P < 0.001$.

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differentiation rather than being cytoprotective for premyelinating oligodendrocytes. Assessment of *Enpp6*+ cell numbers in the corpus callosum of normoxic, hypoxic, and clemastine-treated hypoxic mice shows that hypoxia leads to a block in OPC differentiation at an early stage, with significant ($P = 0.003$) reductions in *Enpp6*+ cells, which are significantly ($P = 0.0001$) normalized by the administration of clemastine (Fig. 3G and H).

Clemastine promotes ultrastructural myelination in neonatal hypoxia

We made use of the cerebellum to assess whether the effect of clemastine on OPC differentiation in neonatal hypoxia leads to improved ultrastructural myelin membrane wrapping of axons. The cerebellum is responsible for co-ordination of movement and higher order relay functions in the brain, and has large myelinated axonal tracts for rapidly coordinating these neuronal inputs and outputs. It is susceptible to hypoxic damage in premature infants, with damage to cerebellar white matter structures contributing to ataxic cerebral palsy where infants experience problems in arm, leg, and trunk coordination, decreased muscle tone, intention tremor and nystagmus. We assessed myelin wrapping ultrastructurally in the white matter of cerebellar foliae following neonatal murine hypoxia from postnatal Days 3–14 using resin embedded sections for light microscopy and electron microscopy. White matter tracts in the foliae of untreated hypoxic mice were difficult to observe with light microscopy, compared to normoxic mice (Fig. 4A). G-ratio analysis on representative electron micrographs showed that chronic neonatal hypoxia leads to a markedly greater proportion of umyelinated axons (44% in hypoxic mice, compared to 6% in normoxic mice) at postnatal Day 14, and that the myelinated axons have significantly thinner myelin sheaths (Fig. 4B and C). Clemastine treatment during neonatal hypoxia led to significant improvements in myelin thickness and extent (12% axons unmyelinated in clemastine treated, compared to 44% in untreated hypoxic littermates), and partial normalization towards normoxic levels (Fig. 4B and C). Ultrastructural assessment of myelin wrapping in the corpus callosum following neonatal murine hypoxaemia from postnatal Days 3–14 showed similar findings (Supplementary Fig. 5). Evidence of myelin wrapping on electron microscopy is almost completely absent in hypoxic postnatal Day 14 corpus callosum, but a recovery towards normoxic levels is seen in hypoxic mice treated with clemastine (Supplementary Fig. 5).

Additionally, we assessed extent of myelination in brain white matter using node of Ranvier counts. Nodes and paranodes form as a result of myelination, and are therefore an indirect measure of the extent of functioning myelin. We found that neonatal hypoxia leads to significant reductions in the number of nodes (marked by nodal and paranodal markers Ankyrin-G and Caspr, respectively) in cerebellar white matter ($P = 5.3 \times 10^{-4}$) (Fig. 5A–C) and corpus callosum ($P = 8.5 \times 10^{-7}$) (Fig. 5D and E). Clemastine treatment results in a significant sparing of the number of nodes reduced by hypoxic injury in both cerebellar white matter ($P = 6.0 \times 10^{-4}$) (Fig. 5B and C) and corpus callosum ($P = 8.1 \times 10^{-4}$) (Fig. 5D and E).

Clemastine improves axonal functional recovery in neonatal hypoxia

We made use of the optic nerve to assess whether clemastine improves axonal function and restores conduction velocities in CNS white matter tracts following hypoxic injury. As in other CNS white matter tracts, chronic neonatal hypoxia in mouse optic nerve leads to significant reductions ($P = 1.3 \times 10^{-14}$) in MBP expression (Fig. 6A and B) (with axons being spared) (Fig. 6A and C, and Supplementary Fig. 1), while OPC numbers seem unchanged (Fig. 6D and E), and clemastine treatment leads to significant recovery ($P = 1.2 \times 10^{-5}$) in extent of MBP expression (Fig. 6A and B) (but not fully to normoxic levels). CAP recordings are a sum of all action potentials in a given nerve and are regularly used as a functional assay of CNS myelination and axon integrity (Stys *et al.*, 1991; Shen *et al.*, 2014). The latency between stimulation and the peak of the CAP, along with nerve length, are used to determine conduction velocity (Stys *et al.*, 1991). Optic nerves from mice treated with clemastine during neonatal hypoxia showed a reduced latency to the peak of CAP compared to hypoxic untreated littermates (Fig. 6F). Consistent with the recovery of myelination in other CNS regions, optic nerve conduction velocities were significantly faster ($P = 0.013$) in hypoxic mice treated with clemastine (165 mm/s ± 10) than those treated with vehicle (120 mm/s ± 5) indicating a partial recovery in velocities compared to normoxic mice (220 mm/s ± 5) (Fig. 6G).

To verify that this increase in conduction velocity results from increased myelination, we assessed extent of myelination using node of Ranvier counts and electron microscopy. Neonatal hypoxia led to reductions in myelination of the optic nerve on electron microscopy compared to normoxic mice (Supplementary Fig. 6), and clemastine treatment during hypoxia led to markedly improved myelination on electron microscopy compared to untreated hypoxic littermates (Fig. 6H), and significantly more nodes of Ranvier ($P = 8.3 \times 10^{-3}$) in the optic nerve (Fig. 6I and J), with partial normalization to normoxic levels in accordance with the conduction velocities.

Clemastine effects in hypoxia are oligodendroglial specific via an effect on the M1 muscarinic receptor

To investigate the mechanism of action of clemastine on myelination in hypoxic brain injury, we sought to identify whether its effects were specific to oligodendroglia, or indirect via an action on other cell types. Whilst clemastine is US FDA approved for its use for symptomatic treatment of

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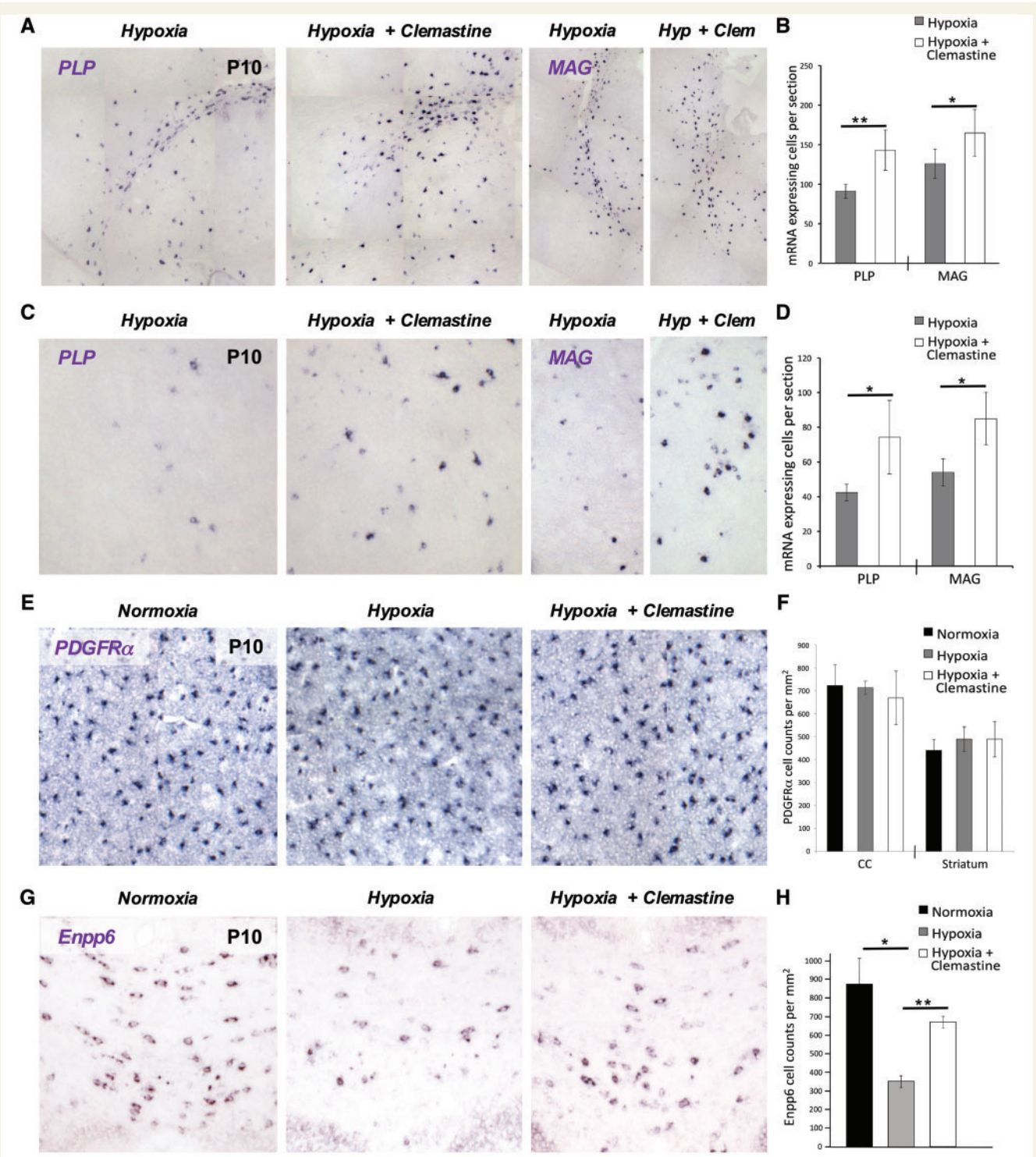

Figure 3 Clemastine promotes OPC differentiation in neonatal hypoxia. (**A**) Numbers of differentiating OPCs expressing mRNA by *in situ* hybridization for the markers *Plp* (PLP) and *Mag* (MAG) in corpus callosum (**A** and **B**) and striatum (**C** and **D**) of hypoxic neonatal mice at postnatal Day 10 compared to hypoxic littermates treated with clemastine ('Hypoxia + Clemastine'). (**E** and **F**) Numbers of undifferentiated OPCs expressing mRNA by *in situ* hybridization for the marker *Pdgfra* (PDGFRα) in striatum white matter (**E**) and counts (**F**) in striatum and corpus callosum (CC) of postnatal Day 10 normoxic mice versus hypoxic and hypoxic treated with clemastine. (**G**) Early differentiating OPCs expressing mRNA by *in situ* hybridization for the marker *Enpp6* in corpus callosum (**G**) and counts (**H**) in postnatal Day 10 normoxic mice versus hypoxic and hypoxic treated with clemastine. For all graphs, $*P < 0.05$, $**P < 0.001$.

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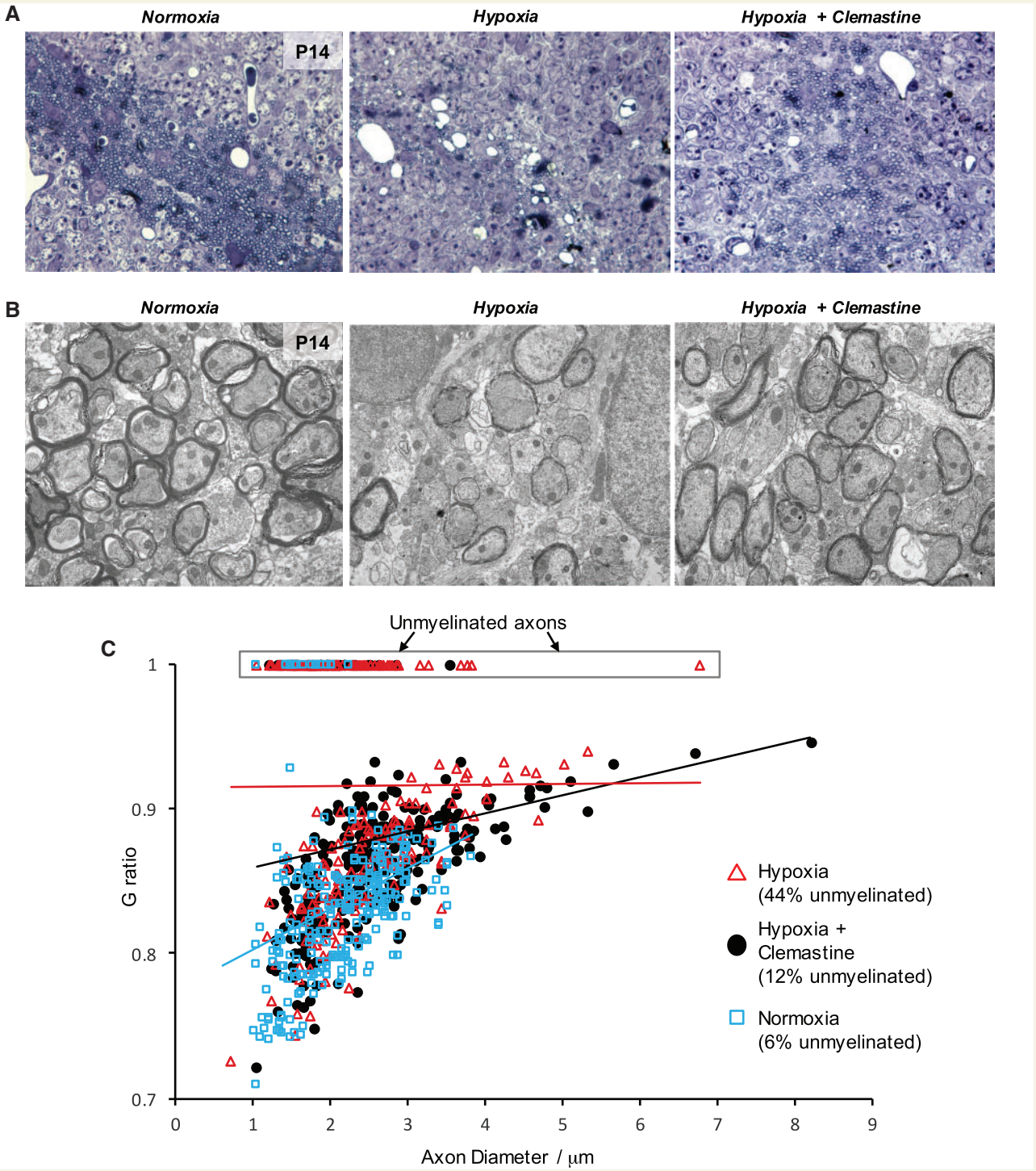

Figure 4 Clemastine promotes ultrastructural myelination on electron microscopy in neonatal hypoxia. (**A**) Light microscopy of the white matter of cerebellar foliae at postnatal Day 14 in resin embedded sections stained with toluidine blue from normoxic mice, hypoxic mice (Hypoxia P3-P14), and hypoxic littermates treated with clemastine (Hypoxia and Clemastine treatment P3-P14). (**B**) Representative electron microscopy images from cerebellar fimbriae of the three groups at postnatal Day 14. (**C**) Quantification of myelin sheath thickness and the proportion of myelinated and unmyelinated axons in normoxic (blue square), hypoxic (red triangle) and clemastine-treated hypoxic (black filled circle) mice at postnatal Day 14 by g-ratio analysis. The scatterplot displays g-ratios of individual axons as a function of axonal diameter. All g-ratios were analysed from transmission electron microscopy images.

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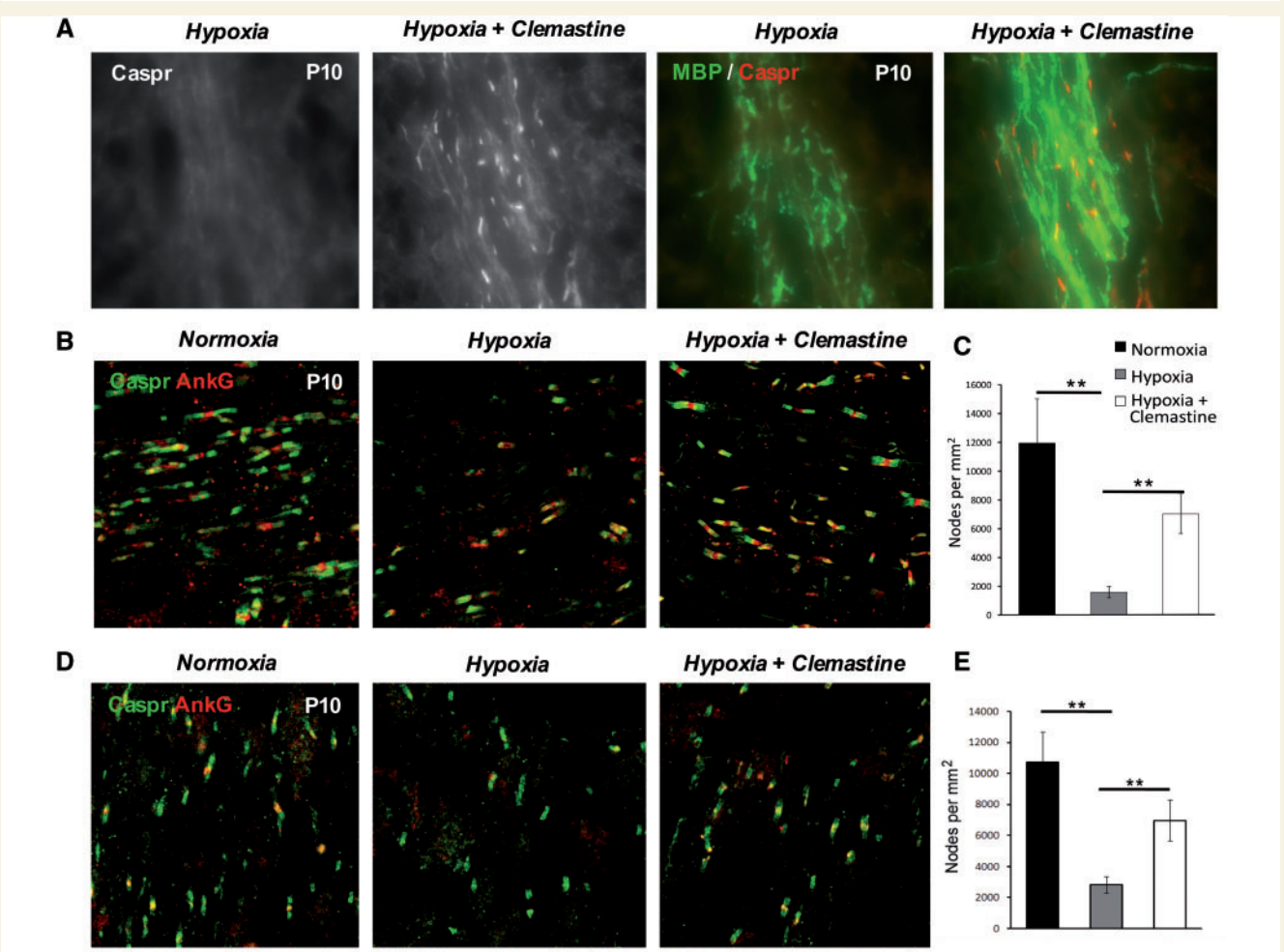

Figure 5 Clemastine increases node of Ranvier formation in neonatal hypoxia. (**A**) Magnified region of a cerebellar fimbria at postnatal Day 10 from neonatal hypoxic mice and hypoxic littermates treated with clemastine, showing expression of MBP and the paranodal marker Caspr, indicating nodes of Ranvier. (**B–E**) Node of Ranvier formation in the three groups in postnatal Day 10 cerebellar white matter (**B**) and corpus callosum (**D**) using markers Ankyrin-G (nodal marker) and Caspr (paranodal marker), with quantification in cerebellum (**C**) and corpus callosum (**E**) showing density of nodes (nodes were counted only if Ank-G/Caspr double positive). 'Normoxia' and 'Hypoxia + Clemastine' groups were not significantly different. For all graphs, $*P < 0.05$, $**P < 0.001$.

seasonal allergies as an antihistamine through the histamine receptor 1 (Hrh1), its effect on OPC maturation is mediated by antimuscarinic action at the M1 muscarinic acetylcholine receptor (Chrm1) (Mei *et al.*, 2016). We generated *Chrm1* conditional knockout (cKO) mice, which have the M1 muscarinic receptor specifically deleted from OPCs, by crossing the *Chrm1* floxed line with a *Cnp-cre* line. The resulting Chrm1-cKO (*Cnp-cre*; *Chrm1 fl/fl*) mice were exposed to chronic neonatal hypoxia between postnatal Days 3–10 as above, and compared to wild-type littermates. Treatment of wild-type mice during hypoxia with 10 mg/kg clemastine once daily by oral gavage between postnatal Days 3–10 leads to significant increases in MBP ($P = 0.006$) (Fig. 7A and E) protein and *Mag* mRNA ($P = 1.06 \times 10^{-9}$) (Fig. 7C and G) expression in striatal white matter compared to untreated wild-type hypoxic littermates. However, the effect of clemastine on both MBP protein expression and *Mag* mRNA expression was lost in Chrm1-cKO mice (Fig. 7A, C, E and G), with no significant difference found between clemastine-treated Chrm1-cKO and untreated wild-type or Chrm1-cKO mice (Fig. 7A, C, E and G). Similar results were found in the corpus callosum (Supplementary Fig. 7). Whilst hypoxic Chrm1-cKOs do show an ~30% increase in MBP and MAG expression in the corpus callosum compared to hypoxic wild-type mice (Supplementary Fig. 7D and E), this is not significantly different, and suggests that Chrm1-cKOs do not phenocopy the protective effects of the drug to the same extent in the setting of hypoxia as has been previously shown (Mei *et al.*, 2016). No significant differences were found in neurofilament staining (Fig. 7B and F) or OPC numbers (Fig. 7D and H) between any of the groups. Together these results indicate that the effects of clemastine on myelination and OPC differentiation in hypoxic brain injury are specific to

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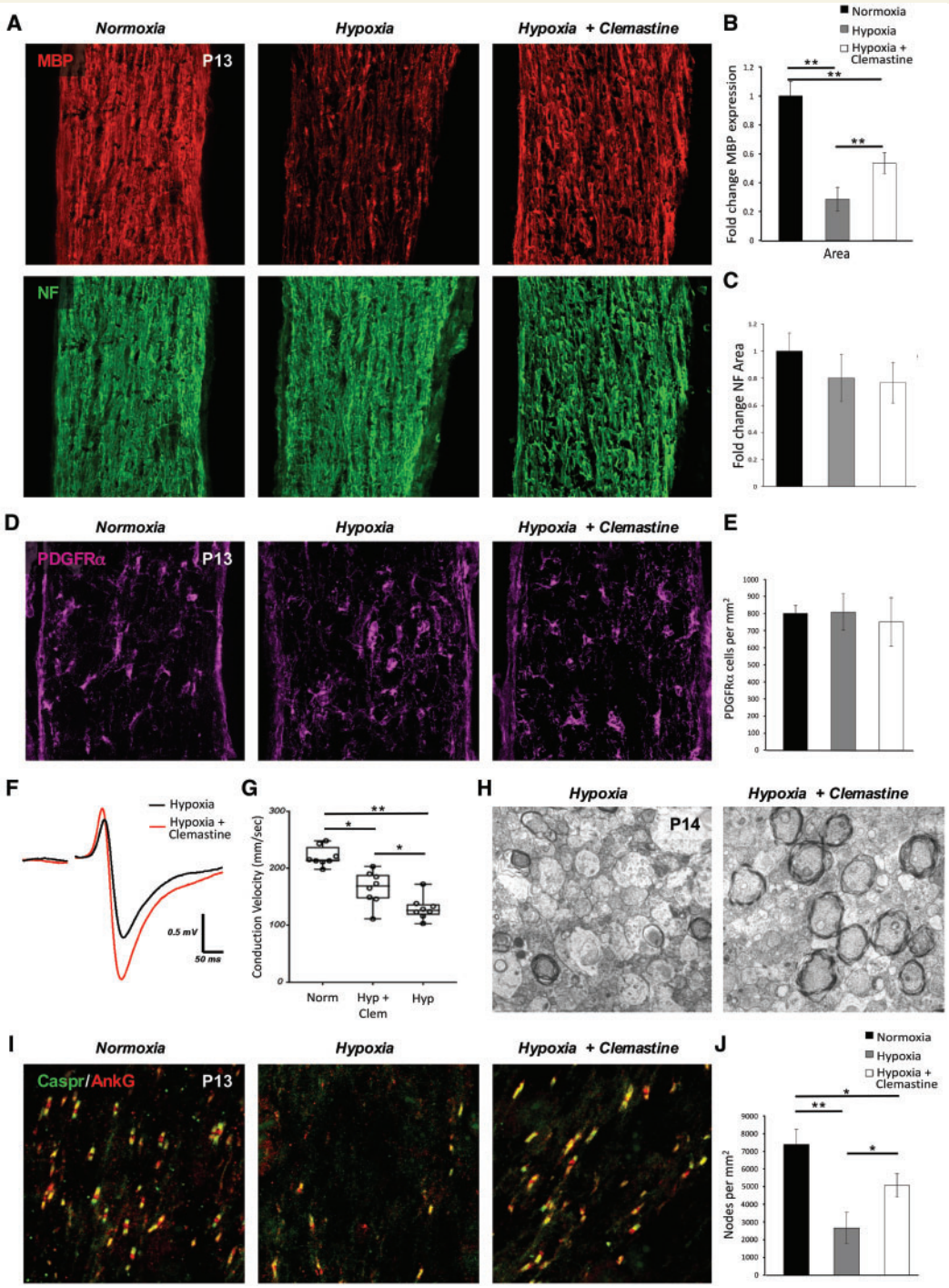

Figure 6 Clemastine improves myelination and CNS conduction velocities in neonatal hypoxic optic nerve. (**A**) MBP expression (*top*) and neurofilament (NF) staining (*bottom*) in the optic nerve of postnatal Day 13 normoxic mice, versus those exposed to neonatal hypoxia (postnatal Days 3–13), versus hypoxic littermates treated with clemastine, with quantification of (**B**) total MBP positive area and (**C**) neurofilament area measured using Image-Pro Plus software (relative expression among the three groups normalized to the normoxia group). (**D**) Undifferentiated OPCs in the optic nerves stained for PDGFRα with quantification of OPC density in (**E**). (**F**) Representative traces from postnatal Day 13 optic nerve CAP recordings from nerves of equal length of hypoxic (black) and clemastine-treated hypoxic (red) mice. Traces from normoxic mice were excluded because they were longer than nerves from hypoxic mice. (**G**) Conduction velocities of postnatal Day 13 optic nerves as measured by suction electrodes ($n = 8$ nerves per group, one-way ANOVA followed by Bonferroni's correction). Data are shown in box-and-whisker plots (maximum, 75th percentile, median, 25th percentile and minimum). (**H**) Representative electron microscopy images from optic nerves of the three groups at postnatal Day 14. (**I**) Node of Ranvier formation in the three groups in postnatal Day 13 optic nerve using markers Ankyrin-G (nodal marker) and Caspr (paranodal marker), with quantification in **J** showing density of nodes (nodes counted only if Ank-G/Caspr double positive). For all graphs, $*P < 0.05$, $**P < 0.001$.

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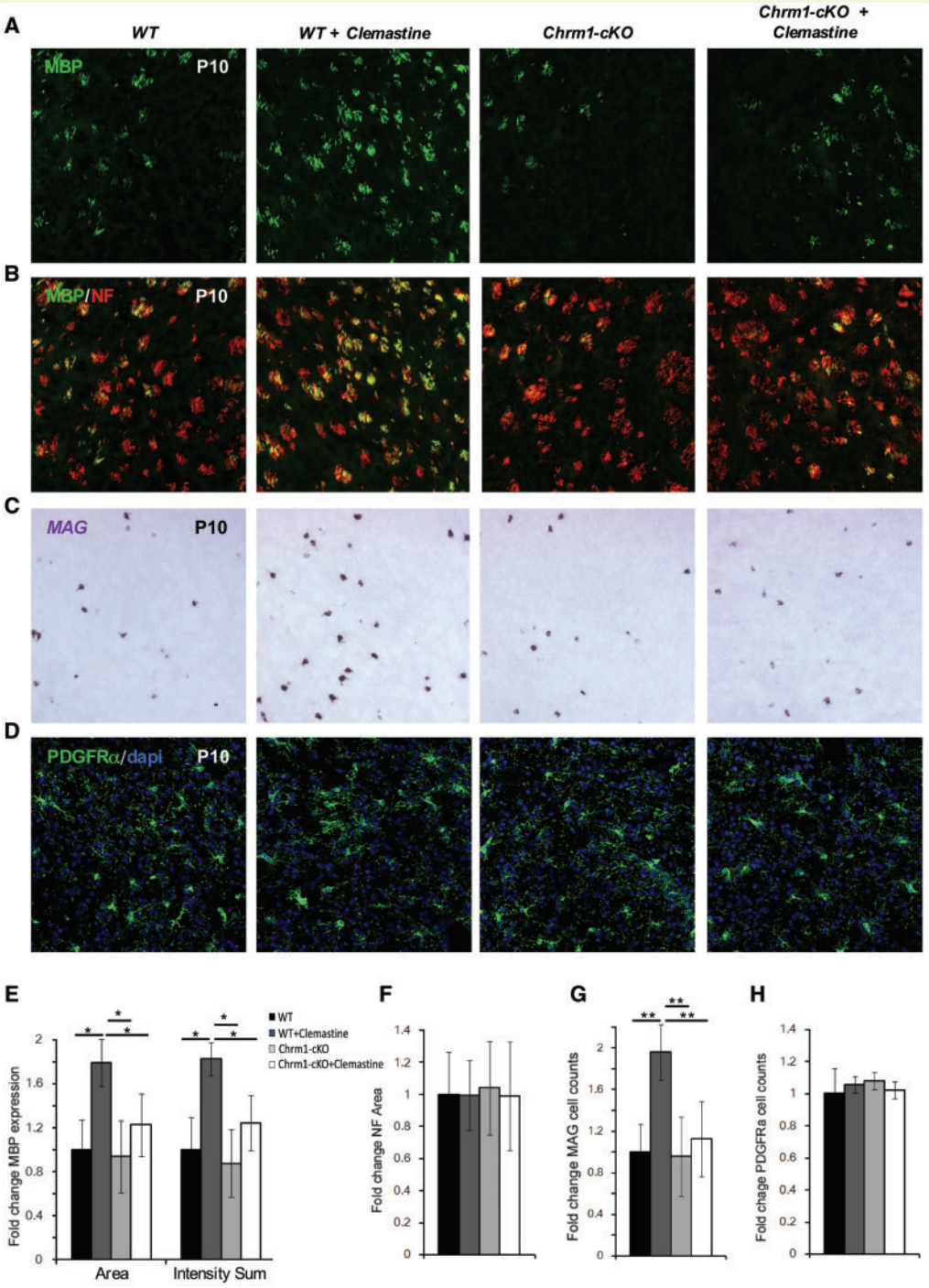

Figure 7 Clemastine function in hypoxia is oligodendroglial specific via an effect on the M1 muscarinic receptor. Chronic neonatal hypoxemia (10% FiO_2) between postnatal Days 3–10 was performed in conditional *Chrm1* knock-out mice (Chrm1-cKO mice are *Cnp-cre*; *Chrm1 fl/fl*) and their wild-type littermates [cre negative mice were considered as wild-type (WT)]. (**A**) MBP protein expression in the brain striatum white matter of postnatal Day 10 hypoxic wild-type mice, versus hypoxic wild-type mice treated with clemastine (WT + Clemastine), versus hypoxic *Chrm1* knock-out littermates (Chrm1-cKO), versus hypoxic *Chrm1* knock-out mice treated with clemastine (Chrm1-cKO + Clemastine). (**B**) Neurofilament (NF) and MBP staining in these groups. (**C**) Differentiation marker *Mag* mRNA and (**D**) OPC marker PDGFRα protein expression in striatum in the same groups. (**E**) Quantification in striatum of total MBP positive area ('Area') and total MBP fluorescent signal intensities ('Intensity Sum') measured using Image-Pro Plus software (with relative expression among the four groups normalized to the wild-type hypoxia group). (**F**) Total neurofilament area fold change in striatum in the four groups normalized to wild-type hypoxia group. (**G**) Numbers of *Mag* mRNA-positive cells in striatum among the four groups (normalized to the wild-type hypoxia group). (**H**) Numbers of PDGFRα positive OPCs in striatum in the four groups (normalized to the wild-type hypoxia group). For all graphs, $*P < 0.05$, $**P < 0.001$.

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oligodendroglial lineage via an effect on the M1 muscarinic receptor in OPCs.

Discussion

Other than supportive care, there are currently no proven treatments for hypoxic white matter injury in either the neonatal or adult setting. Evidence suggests that OPC differentiation arrest is a critical contributor to failed myelin generation during hypoxia, leading to progressive neurological injury, and suggesting that promotion of OPC differentiation through therapeutic manipulation could yield improved clinical outcomes. Evidence suggests that inhibiting the muscarinic pathway in oligodendrocyte precursors promotes their differentiation (Abiraman *et al.*, 2015; Mei *et al.*, 2016), and that Chrm1 on OPCs may be the specific target for this activity (Mei *et al.*, 2016), prompting us to assess anti-muscarinic therapy for hypoxic brain injury involving white matter injury and OPC maturation arrest.

The mechanisms of disease in DPHL are far less well understood than in neonatal hypoxic injury, due to the very rare nature of the condition in humans and lack of reflective animal models, but failure of myelin generation following a demyelinating insult is thought to underlie the pathophysiology. We present a case of severe DPHL who markedly improved in the setting of off-label use of clemastine. Because spontaneous recovery has been reported in some cases of DPHL (Min, 1986; Lee and Lyketsos, 2001) it is unclear how this patient would have improved without clemastine treatment. Nevertheless, because of the profound neurological deficits he experienced 2 months after the hypoxic event, his prognosis at that time was considered to be very poor based on a prior report of patients with delayed akinetic-mutism following carbon monoxide poisoning (Lee and Marsden, 1994). That the patient recovered to return to intellectually demanding work was sufficiently unusual to promote our interest in studying clemastine treatment for hypoxic injury. Using a murine neonatal model, we present data showing that clemastine dramatically promotes myelin generation during global hypoxic injury. These experimental studies provide biological plausibility that improvement in our case of adult DPHL could be clemastine-mediated. Proof that clemastine is beneficial in DPHL will require study through randomized controlled trials, which will be challenging to accomplish given the rarity of this disorder.

Our murine studies in neonatal hypoxia have broader implication: 1.5% of all live births in the USA each year are very low birth weight infants and recent improvements in neonatal critical care have led to a marked increase in survival (50–70%) of extremely low birth weight ($\leqslant$1000 g) premature infants. Other than therapeutic hypothermia there are no treatments for neonatal hypoxic injury and there is a significant healthcare burden from disability related to cerebral palsy (Glass and Rowitch, 2016). White matter injury is the most reliable prognostic indicator of development of severe cerebral palsy and cognitive disability in premature infants (Woodward *et al.*, 2006). A significant contributor to failure of myelin generation in these infants is persistent OPC maturation arrest. Our murine model replicates the significant OPC maturation delay that is a hallmark of the human hypoxic neonatal injury, making it an ideal model for testing OPC differentiation promoting therapies. We found that clemastine dramatically promotes OPC differentiation, myelination, and improves functional recovery in neonatal hypoxic injury. Moreover, we show that its effects in hypoxia are oligodendroglial-specific via an effect on the M1 muscarinic receptor on OPCs. Further work will be required to assess clemastine effects in human neonatal injury. Given the excellent safety profile of clemastine in adults, and the results from our animal studies, we propose clemastine as a potential treatment to promote myelin recovery in premature infants.

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Supplementary material

Supplementary material is available at *Brain* online.

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