Molecular Neurobiology (2018) 55:8051–8058
https://doi.org/10.1007/s12035-018-0971-6

Erythropoietin Upregulates Brain Hemoglobin Expression and Supports Neuronal Mitochondrial Activity

N. K. Singhal[1] · K. Alkhayer[1] · J. Shelestak[1] · R. Clements[1] · E. Freeman[1] · J. McDonough[1]

Received: 15 August 2017 / Accepted: 19 February 2018 / Published online: 1 March 2018

Abstract

Multiple sclerosis (MS) is a neuro-inflammatory and demyelinating disease. Downregulation of neuronal mitochondrial gene expression and activity have been reported in several studies of MS. We have previously shown that hemoglobin-β (Hbb) signals to the nucleus of neurons and upregulates H3K4me3, a histone mark involved in regulating cellular metabolism and differentiation. The present study was undertaken to evaluate the effect of erythropoietin (EPO) on the upregulation of hemoglobin and mitochondrial-associated neuroprotection. We found that administering EPO (5000 IU/kg intraperitoneally) to mice upregulated brain Hbb expression, levels of H3K4me3, expression of mitochondrial complex III, complex V, and mitochondrial respiration. We also found that the neuronal mitochondrial metabolite N-acetylaspartate (NAA), a marker of neuronal mitochondrial activity, was increased with EPO treatment. Further, we measured the effects of EPO on preventing mitochondrial deficits in the cuprizone toxic demyelinating mouse model of MS. We found that EPO prevented cuprizone-mediated decreases in Hbb, complex III, and NAA. Our data suggest that EPO mediated regulation of Hbb supports neuronal energetics and may provide neuroprotection in MS and other neurodegenerative diseases where a dysfunction of mitochondria contributes to disease.

Keywords Erythropoietin · N-acetylaspartate · Multiple sclerosis · H3K4me3 · Hemoglobin · Mitochondria

Introduction

In multiple sclerosis (MS), demyelination, axonal, and neuronal degeneration accumulate over time and result in progressive neurological disability [1, 2]. In early relapse remitting stages of disease (RRMS), inflammatory demyelination results in less efficient conduction of nerve impulses and neurological impairment. These impairments generally resolve over a period of weeks due to redistribution of sodium channels and remyelination [3]. Neurons are high respiring cells with a large metabolic demand, and in MS, they are particularly vulnerable to changes in energy supply due to demyelination and redistribution of Na^+ channels along axons. Decreases in neuronal energetics in MS lead to defects in ion homeostasis, conduction, and axonal transport and can result in cell death [4, 5]. In RRMS, this pathology is resolved and conduction is restored. However, neurons and axons are damaged during inflammatory demyelinating events. This damage accumulates with successive relapses ultimately leading to permanent neurological disability. Over time, many patients advance to secondary progressive stages of disease (SPMS) where accumulation of damage and loss of neurons leads to permanent disability underscoring the need for neuroprotective therapies for MS [6].

Mitochondrial involvement in neurodegeneration and disability in MS is supported by previous studies showing decreased expression of nuclear encoded mitochondrial electron transport chain genes in neurons and reduced mitochondrial respiration in MS cortex [7–11]. Consistent with these data, in vivo magnetic resonance imaging (MRI) and spectroscopy (MRS) studies have shown that the neuronal respiratory marker N-acetylaspartate (NAA) is decreased in MS cortex prior to brain atrophy suggesting that defects in neuronal respiration precede neurodegeneration [12, 13]. Reductions in mitochondrial activity contribute to axonal pathology in MS by impairing signal conduction and axonal transport [5]. These changes, including mitochondrial dysfunction and decreased levels of NAA, have also been reported in the cuprizone mouse model for MS [14]. In the cuprizone mouse model of MS, demyelination and activation of microglia are

✉ N. K. Singhal
nsingha1@kent.edu

✉ J. McDonough
jmcdonou@kent.edu

[1] Department of Biological Sciences and School of Biomedical Sciences, Kent State University, Kent, OH 44242, USA

experimentally induced by cuprizone which is incorporated into the diet. After cuprizone feeding oligodendrocyte apoptosis, activation of microglia, axonal damage, and motor impairment are apparent [15, 16].

Erythropoietin (EPO) is a well-known hematopoietic growth factor generally produced in the kidney, liver, and brain [17]. During brain development, EPO plays an important role in production and differentiation of neuronal precursor cells [18–20]. EPO also causes differentiation of oligodendrocyte progenitor cells into mature oligodendrocytes [21]. EPO also possess anti-oxidative, anti-inflammatory, and anti-apoptotic properties [22, 23] and has demonstrated neuroprotective properties under hypoxic, oxidative, and ischemic conditions [24–26]. Increased endogenous EPO expression or EPO injection induces neuronal hemoglobin expression and has been linked to increased neuronal survival under hypoxic conditions [27]. We have previously reported that over expression of hemoglobin-β (Hbb) signals to the nucleus in neurons and upregulates H3K4me3, a histone mark involved in regulating cellular metabolism and differentiation [28]. In the present study, we have investigated the effect of EPO in maintaining hemoglobin expression, levels of H3K4me3, and neuronal mitochondrial activity in the cuprizone toxin-induced demyelinating mouse model of MS.

Materials and Methods

Animal Treatment

Wild-type male C57BL6J mice (6 weeks) were obtained from Jackson laboratory. Mice were injected intraperitoneally with EPO (EMD Millipore) (5000 U/Kg weight of mice) [16] or phosphate buffer saline (PBS). After 24 h, mice were sacrificed via cervical dislocation. The brain was dissected out and cortical gray matter was isolated from the brain for all experiments. For the cuprizone model, mice were fed a diet containing 0.3% cuprizone diet for 6 weeks (Teklad, Madison, WI). To determine the effect of EPO on preventing pathology resulting from cuprizone treatment, a control group and cuprizone-treated mice were also injected intraperitoneally with EPO every other day for the last 3 weeks of cuprizone feeding.

Western Blotting

Western blots for H3K4Me3, mitochondrial respiratory complexes [ubiquinol-cytochrome c reductase core protein II (Uqcrc2 or complex III) and ATP synthase (Atp5a1 or complex V)] and Hbb were performed from the nuclear, mitochondrial, and cytoplasmic fraction isolated from cortical gray matter of mice. Mitochondria were isolated by a modified method of Schnaitman and Greenawalt, 1968 [29]. In brief, 10% homogenates of cortical gray matter tissue were made in MSHE+BSA (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA, and 0.5% BSA) with a glass homogenizer at 4 °C. Homogenates were centrifuged at 1000×*g* for 10 min at 4 °C. The pellet contains unbroken cells, nuclei, plasma membranes, and debris and the supernatant contains the mitochondrial and cytoplasmic fraction. The supernatant was centrifuged again at 8000×*g* for 10 min at 4 °C. The pellet (mitochondrial fraction) obtained was re-suspended in a minimal volume of MSHE+BSA. The supernatant contains the cytoplasmic fraction. Protein concentration (mg/ml) was determined using Bradford Assay reagent (Bio-Rad). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were incubated with their respective primary antibodies and then secondary antibodies conjugated with HRP. Blots were then developed by using luminol (Santa Cruz Biotechnologies, Dallas, TX). Primary antibodies were procured for H3K4me3 and H3 (Abcam, Cambridge, MA), Hbb (Santa Cruz Biotechnologies, Dallas, TX), complex III and complex V (Mitosciences), GAPDH (Millipore, Temecula, CA), and Aralar (Transduction laboratories).

Respirometry

A Seahorse Bioscience XF-24 Extracellular Analyzer (Seahorse Bioscience, Billerica, MA) was used to measure oxygen consumption rate (OCR) in mitochondria isolated from cortical gray matter of control, and EPO-treated mice. Mitochondria were isolated by the same method described for western blot. For the Seahorse XF-24 assay, mitochondria were diluted 10 times in cold 1X MAS + substrate (70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, and 0.2% bovine serum albumin +10 mM succinate and 2 μM rotenone), then plated on the XF assay plate in equal concentration (5 μg). Fifty microliter of mitochondrial suspension was delivered to each well (except for background correction wells). The XF cell culture micro-plate was centrifuged at 2000×*g* for 20 min at 4 °C to adhere the mitochondria. The plate was then transferred to the XF analyzer and the experiment initiated. Injection of ADP (4 mM final), oligomycin (2.5 μg/ml final), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (4 mM final), and antimycin A (4 mM final) were added sequentially and the OCR was measured in picomole per minute. Spare respiratory capacity was calculated by substracting basal respiration from maximal respiration (respiration after FCCP treatment). Statistical significance was measured by two-tailed Student's *t* test. *P* value ≤ 0.05 was considered statistically significant.

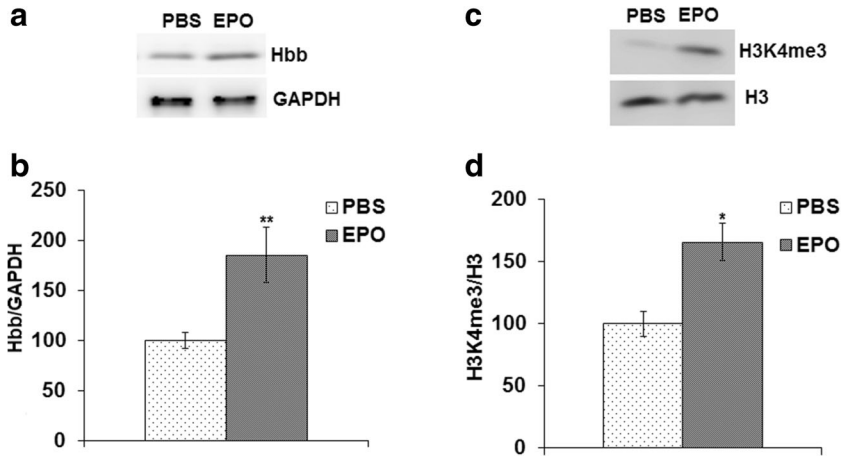

Fig. 1 Levels of Hbb and H3K4me3 are increased in mouse brains (cortical gray matter) treated with a single injection of EPO. **a** Representative western blot for Hbb/GAPDH from mice treated with EPO. **b** Densitometry of western blots show that Hbb is increased in brains of EPO-treated mice compared to controls. **c** Representative western blot for H3K4me3/H3 from brain nuclear fractions of mice treated with EPO. **d** Densitometry shows that H3K4me3 is increased in brains of EPO-treated mice as compared to controls ($n = 3$). Data are expressed as a percentage of control with the highest control set at 100%. Error bars indicate SEM. *$p < 0.05$ versus control, **$p < 0.01$ versus control

Measurement of NAA by HPLC

NAA was measured in cortical gray matter by high performance liquid chromatography (HPLC) as described elsewhere [30]. A 10% tissue homogenate was made in 90% methanol followed by vacuum drying. The dry powder was dissolved in 1 ml distilled water and purified by ion exchange column (Biorad). The eluate was lyophilized and dissolve in 300–400 μl of distilled water. Samples were run with mobile phase (0.1 M KH_2PO_4, 0.025 M KCl, pH 4.5) in a Whatman Partisil 10 SAX anion-exchange column (4.6 mm × 250 mm) in an Agilent 1100 series HPLC value system. The NAA peak was detected with an Agilent 1100 series UV detector at 214 nm at a flow-rate of 1.5 ml/min. NAA peak areas were acquired with Agilent Chemstation software. NAA concentrations were determined in triplicate and statistical significance of differences in NAA concentration was determined with a two-tailed Student's *t* test, with *p* values ≤0.05 considered statistically significant.

Black-Gold Staining

Mice were sacrificed by cervical dislocation and brains were removed and post-fixed in 4% paraformaldehyde overnight. Brains were treated with increasing concentration of 10, 20, and 30% sucrose for cryoprotection. Brains were sliced into 30-μm thick sections using a cryotome. Black-gold staining was performed as previously described [31]. Brain sections were washed with saline then incubated in 0.3% black-gold solution for 10–12 min at 60 °C. After this, sections were washed with saline twice followed by fixation in 1% sodium thiosulfate for 3 min. Again sections were washed with saline three times, and mounted on glass slides using glycerol. Images were taken using bright field microscopy.

Myelin Basic Protein Immunostaining

Brain sections were washed with PBS three times then blocked with 5% normal donkey serum made in PBS for 2 h at 4 °C. Sections were then washed with PBS three times followed by overnight incubation with primary antibody (Rabbit polyclonal anti-MBP, Cell signaling) for myelin basic

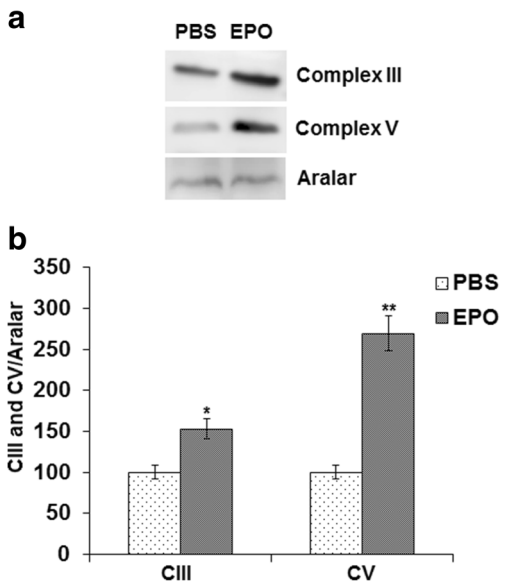

Fig. 2 Protein expression of complex III and complex V are increased in mouse brains treated with a single injection of EPO. **a** Representative western blots for complex III, complex V, and Aralar from brain mitochondrial fractions of mice treated with EPO. **b** Densitometry of western blots for complex III and complex V show that they are increased in brains of EPO-treated mice compared with controls ($n = 3$). Data are expressed as a percentage of control with the highest control set at 100%. Error bars indicate SEM. *$p < 0.05$ versus control, **$p < 0.01$ versus control

protein (MBP) at 4 °C. Again sections were washed with PBS three times followed by incubation with secondary antibody (Donkey anti-rabbit Alexa-488, Abcam) for 3 h at 4 °C. Sections were washed again with PBS and mounted on glass slides using EverBrite mounting medium. Images were taken using an Olympus Fv1000 confocal microscope.

Results

Changes in Levels of Hbb and H3K4me3 in EPO-Treated Mice

To elucidate mechanisms involved in EPO-mediated neuroprotection [32] we treated mice with EPO and measured its effects on levels of Hbb and H3K4me3, a histone methyl mark that has previously been shown to regulate expression of genes involved in oxidative phosphorylation [33]. We performed western blotting and found that EPO increased levels of Hbb in cortical gray matter by 1.8 fold as compared to mice treated with PBS as expected (Fig. 1a, b). We also found that treating mice with EPO increased levels of H3K4me3 by 1.7 fold in cortical gray matter compared to mice treated with PBS (Fig. 1c, d).

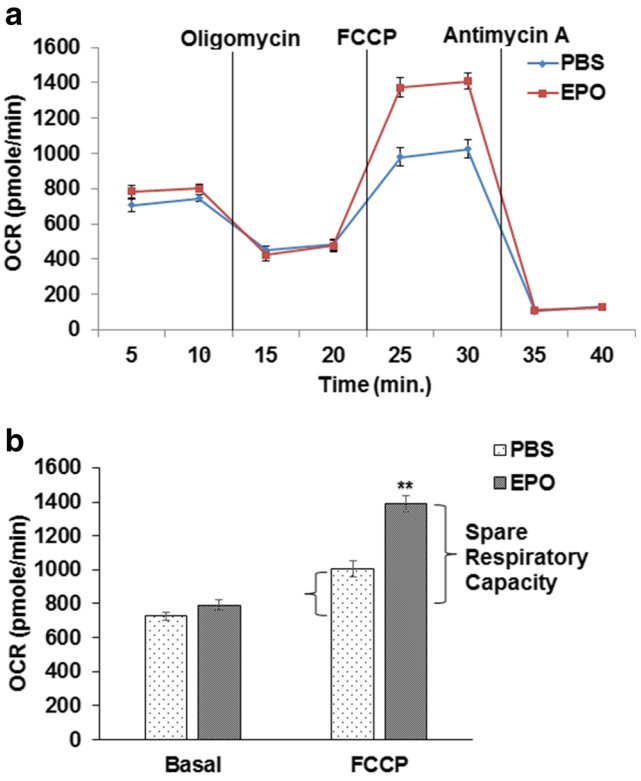

Fig. 3 Neuronal mitochondrial respiration measurements in mouse brains treated with a single injection of EPO. **a** Graph shows OCR of mitochondria isolated from cortical gray matter of control and EPO treated mice . **b** Quantitation of basal respiration and maximal respiratory capacity (after addition of the uncoupler FCCP) were determined for mitochondria isolated from brains of control and EPO treated mice ($n = 3$). Brackets represent spare respiratory capacity for EPO-treated mitochondria. $^{**}p < 0.01$ versus control

Mitochondrial Activity Is Altered in EPO-Treated Mice

We then measured levels of mitochondrial electron transport chain subunits by western blotting and found that expression of subunits from mitochondrial complex III and complex V were increased in brains of EPO-treated mice by 1.5-fold and 2.7-fold respectively compared to mice treated with PBS (Fig. 2). To determine the effects of EPO on mitochondrial respiration, we performed respirometry with a Seahorse XF-

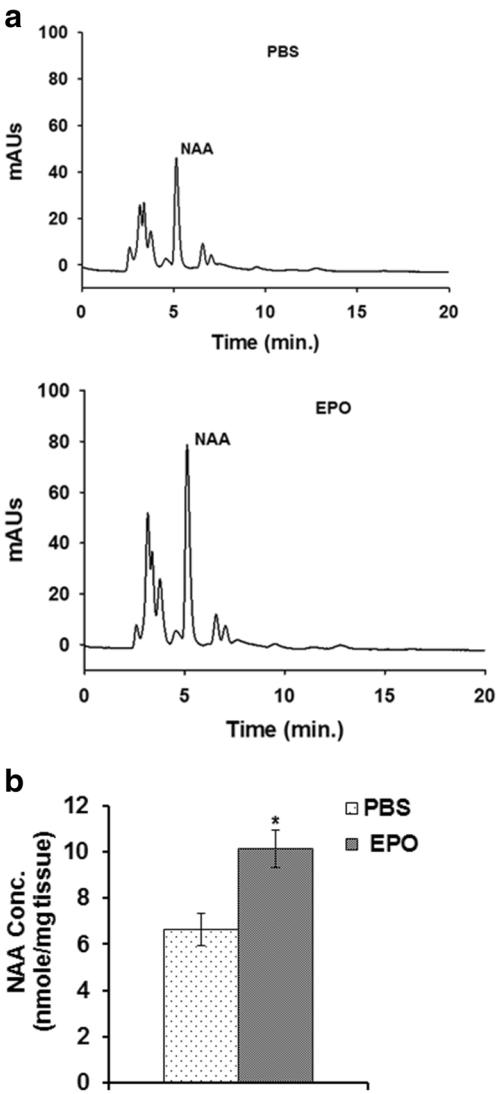

Fig. 4 NAA concentration was measured by HPLC in mouse brains treated with a single injection of EPO. **a** Representative HPLC chromatograms for quantification of NAA from EPO-treated and control mouse brain tissue. The NAA peak eluted at 5.1 min. **b** Quantitation of average NAA concentrations were measured in duplicate from EPO-treated and control brains ($n = 3$). Error bars indicate SEM. $^{*}p < 0.05$ versus control

24 respirometer. We measured OCR in mitochondria isolated from cortical gray matter of control (PBS treated) and EPO-treated mice. We found that EPO increased spare respiratory capacity by 2.2-fold compared to PBS-treated mice (Fig. 3). To measure the activity of neuronal mitochondria, we measured the concentration of NAA in cortical gray matter of control and EPO-treated mice by HPLC. We found a significant increase in NAA concentration in EPO-treated mice as compared to PBS-treated mice (Fig. 4).

EPO Restores Hbb, Complex III Expression, and NAA Levels in the Cuprizone Mouse Model of MS

To investigate whether EPO can support neuronal mitochondria under pathological conditions, we tested its effects on preventing reduced Hbb, complex III, and NAA levels in the cuprizone mouse model of MS. We performed western blots for Hbb in nuclear fractions and complex III in mitochondrial fractions (Fig. 5a–d). We found that Hbb and complex III expression was maintained in EPO-treated mice. We also analyzed NAA levels and found that NAA was reduced in cuprizone-treated mice by 1.5 fold compared to controls and this decrease in NAA concentration was restored by EPO. Restoration of NAA indicates that EPO increased neuronal mitochondrial activity (Fig. 5e).

EPO Restores MBP Levels but Not Myelin in the Cuprizone Mouse Model of MS

To confirm the loss of myelin induced by cuprizone, we performed black-gold and MBP staining which shows clear demyelination of cortical neurons in cuprizone-treated mice compared to control (Fig. 6). Interestingly, we did not find any recovery in black-gold staining for myelin in the cuprizone-EPO treated group as compared to cuprizone alone (Fig. 6a, b) but there is significant recovery in MBP staining in the cuprizone-EPO treated group as compared to cuprizone alone (Fig. 6c, d).

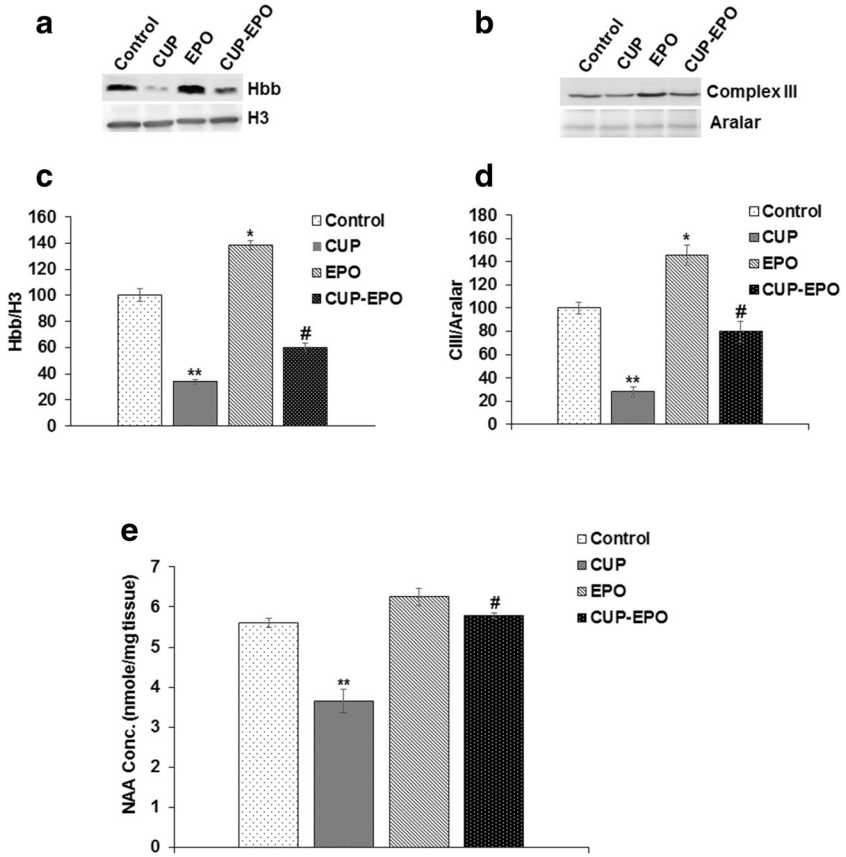

Fig. 5 EPO reverses the effects of cuprizone on Hbb, complex III, and NAA levels. **a** Representative western blots for Hbb/H3 from cortical nuclear fractions of mice treated with cuprizone and EPO. **b** Densitometry of western blots show that Hbb levels decrease in brains of cuprizone-treated mice which were rescued by EPO-treatment ($n = 3$). **c** Representative western blots for complex III/Aralar from cortical mitochondrial fractions of mice treated with cuprizone and EPO. **d** Densitometry of western blots show that complex III levels decrease in brains of cuprizone-treated mice which were rescued by EPO-treatment ($n = 3$). **e** NAA concentration was measured by HPLC. Bar graph shows average NAA concentrations were decreased in cuprizone fed mice. All experiments were performed in duplicate with three animals per group. NAA concentrations were preserved in cuprizone fed mice treated with EPO. Error bars indicate SEM. $^{*}p < 0.05$ versus control, $^{**}p < 0.01$ versus control. $^{\#}p < 0.05$ versus cuprizone

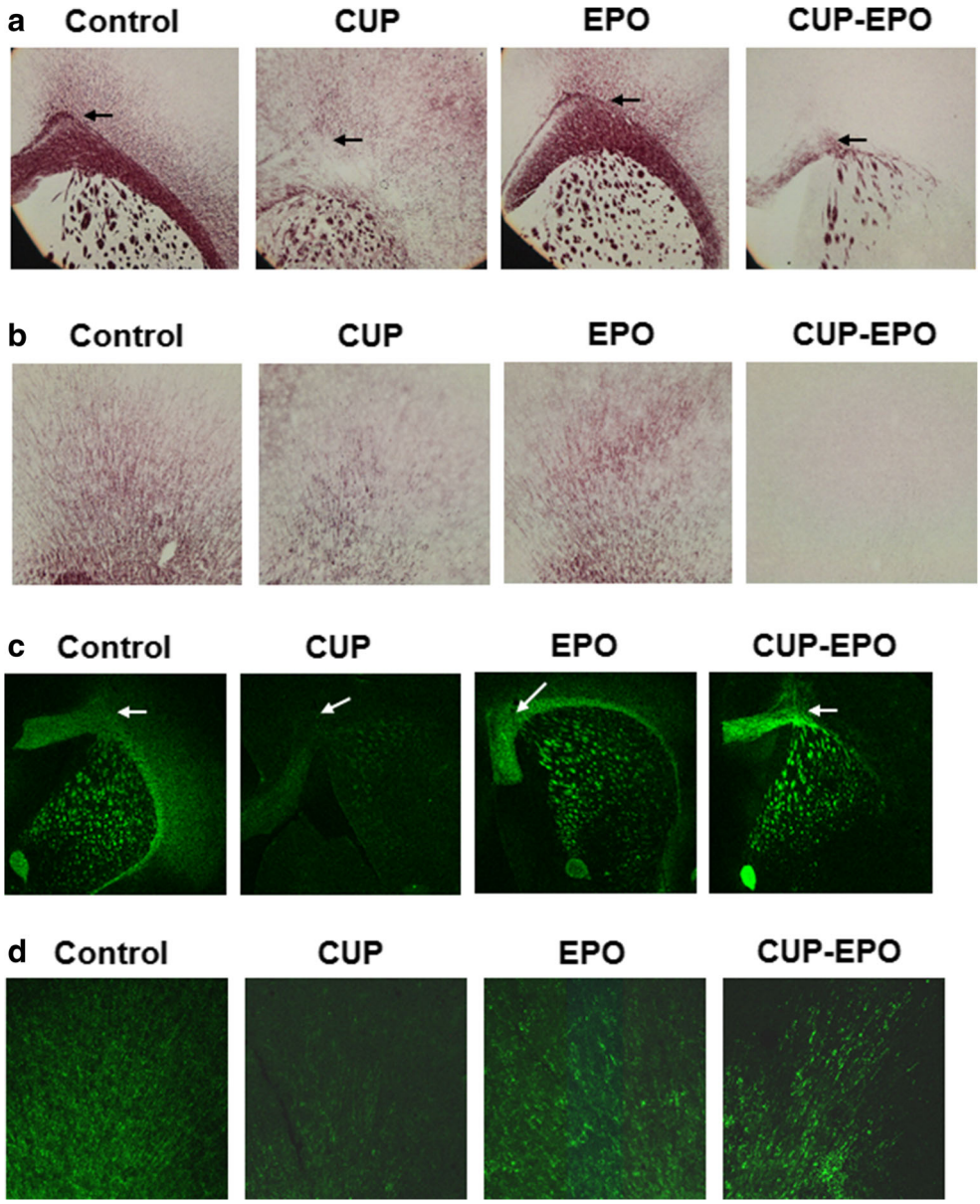

Fig. 6 Effect of EPO on myelin and MBP expression in the cuprizone mouse model. **a** Black-gold staining shows clear demyelination in corpus callosum in cuprizone-treated mice (× 4 magnification). **b** Black-gold staining of cortex (× 10 magnification) in cuprizone treated mice shows that EPO had no effect on myelination in cuprizone fed mice. **c** MBP staining shows decreased MBP expression in corpus callosum (× 4 magnification) and **d** cortex (× 20 magnification) in cuprizone-treated mice. MBP expression was enhanced with EPO treatment in both corpus callosum and cortex. Arrows denotes corpus callosum

Discussion

In the current study, we focused on identifying interventions that have the potential to prevent axonal damage during relapse remitting phases of MS and slow or prevent permanent disability. We utilized the cuprizone mouse model since it mimics many of the pathological features of RRMS [15]. Previous reports have shown that EPO has neuroprotective effects in animal models of MS and reduces axonal damage in the cuprizone model [16, 32, 34]. EPO has been shown to activate NF-kB and transcription of anti-oxidative and anti-excitotoxic gene expression programs [35]. Our data suggest that additional mechanisms are involved in this neuroprotection. Injection of EPO or EPO agonists have been shown to increase hemoglobin expression in both erythrocytes and in the brain of mice [36]. Alterations in Hbb expression in neurons affects the expression of genes involved in mitochondrial respiration [37] suggesting that enhanced mitochondrial respiration contributes to the mechanism of EPO mediated neuroprotection. It has been established that the neuronal mitochondrial metabolite NAA is decreased in MS brains and in the cuprizone mouse model of MS [14, 30]. Levels of NAA are dependent on oxidative phosphorylation and ATP production in neurons [30, 38, 39]. Our data confirm that cuprizone-induced demyelination caused a reduction in neuronal NAA production. EPO treatment increased Hbb levels and restored NAA levels in cuprizone-treated mice indicating that EPO improves neuronal mitochondrial activity (Fig. 7).

Our group and others have shown that hemoglobin is expressed in neurons in the rodent and human brain [10, 27,

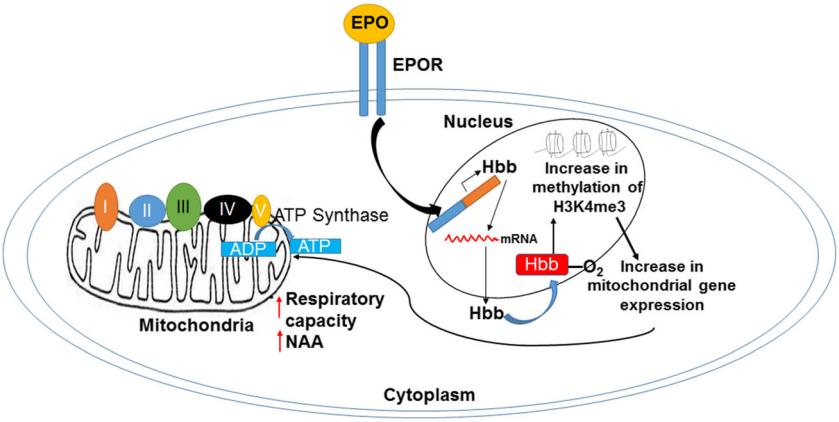

Fig. 7 Schematic diagram of EPO mechanism of action in neurons. EPO binds to its receptor EPOR and transmits the signal to the nucleus to increase expression of Hbb mRNA. Hbb increases H3K4me3 methylation levels in the nucleus which results in increased mitochondrial gene expression and respiratory capacity

28, 37, 40]. Hemoglobin α and β subunit mRNAs as well as proteins have been found to be expressed in neurons in the cortex, substantia nigra, and hippocampus. Changes in neuronal hemoglobin expression or subcellular localization have been linked to MS, Alzheimer's disease, and Parkinson's disease [28, 41, 42]. It has been shown that Hbb is localized to both mitochondria and the nucleus in neurons [14, 28]. We previously performed co-IP followed by LC-MS/MS to identify Hbb-interacting proteins. Consistent with its cytoplasmic and nuclear localization, we found that Hbb interacts with both mitochondrial and nuclear proteins including histones in the human brain and in rat primary neurons [28]. We also found that overexpression of the Hbb subunit in a neuroblastoma cell line increases H3K4me3 levels [28]. Consistent with these findings we found an increase in Hbb expression and H3K4me3 levels in EPO-treated mice compared to controls. Our data suggest that transport of Hbb to the nucleus is important for its effects on mitochondrial function. H3K4me3 is a histone mark which is present in actively transcribed regions of chromatin and regulates expression of oxidative phosphorylation genes [33]. Increased expression of the mitochondrial respiratory genes complex III and complex V, in EPO-treated mice suggests that EPO is acting to increase H3K4me3 methylation and gene expression through a Hbb-mediated pathway (Fig. 7).

While EPO appears to protect neuronal mitochondria, it also impacts oligodendrocytes. These myelinating cells are most sensitive to the toxic effects of cuprizone which leads to oligodendrocyte cell death and demyelination. EPO has also been shown to cause differentiation of oligodendrocyte progenitor cells [21]. The expression of MBP increases when oligodendrocyte progenitors differentiate in to mature myelinating oligodendrocytes. The enhanced MBP expression observed with EPO treatment of cuprizone-treated mice suggests that EPO caused existing oligodendrocyte progenitor cells to differentiate. EPO did not have any effect on preventing demyelination resulting from cuprizone treatment however. In conclusion, treatments that activate neuronal Hbb expression may be therapeutic in neurodegenerative diseases such as MS where a dysfunction of mitochondria contribute to axonal damage and neurodegeneration.

Acknowledgements This research was funded by the College of Arts and Sciences at Kent State University.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Bjartmar C, Kidd G, Mörk S, Rudick R, Trapp BD (2000) Neurological disability correlates with spinal cord axonal loss and reduced N-acetylaspartate in chronic multiple sclerosis patients. Ann Neurol 48:893–901
2. De Stefano N, Narayanan S, Francis GS, Arnaoutelis R, Tartaglia MC, Antel JP, Matthews PM, Arnold DL (2001) Evidence of axonal damage in the early stages of multiple sclerosis and its relevance to disability. Arch Neurol 58:65–70
3. England JD, Gamboni F, Levinson SR, Finger TE (1990) Changed distribution of sodium channels along demyelinated axons. Proc Natl Acad Sci U S A 87:6777–6780
4. Stys PK (2005) General mechanisms of axonal damage and its prevention. J Neurol Sci 233:3–13
5. Waxman SG (2006) Axonal conduction and injury in multiple sclerosis: the role of sodium channels. Nat Rev Neurosci 7:932–941
6. Confavreux C, Vukusic S (2006) Natural history of multiple sclerosis: a unifying concept. Brain 129:606–616
7. Dutta R, McDonough J, Yin X, Peterson J, Chang A, Torres T, Gudz T, Macklin WB et al (2006) Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients. Ann Neurol 59:478–489
8. Pandit A, Vadnal J, Houston S, Freeman E, McDonough J (2009) Impaired regulation of electron transport chain subunit genes by nuclear respiratory factor 2 in multiple sclerosis. J Neurol Sci 279:14–20
9. Campbell GR, Ziabreva I, Reeve AK, Krishnan KJ, Reynolds R, Howell O, Lassmann H, Turnbull DM et al (2011) Mitochondrial DNA deletions and neurodegeneration in multiple sclerosis. Ann Neurol 69:481–492

10. Broadwater L, Pandit A, Azzam S, Clements R, Vadnal J, Sulak M, Yong VW, Freeman EJ et al (2011) Analysis of the mitochondrial proteome in multiple sclerosis cortex. Biochim Biophys Acta 1812: 630–641
11. Witte ME, Nijland PG, Drexhage JA, Gerritsen W, Geerts D, van Het Hof B, Reijerkerk A, de Vries HE et al (2013) Reduced expression of PGC-1partly underlies mitochondrial changes and correlates with neuronal loss in multiple sclerosis cortex. Acta Neuropathol 125:231–243
12. Ge Y, Gonen O, Inglese M, Babb JS, Markowitz CE, Grossman RI (2004) Neuronal cell injury precedes brain atrophy in multiple sclerosis. Neurology 62:624–627
13. Cader S, Johansen-Berg H, Wylezinska M, Palace J, Behrens TE, Smith S, Matthews PM (2007) Discordant white matter N-acetylasparate and diffusion MRI measures suggest that chronic metabolic dysfunction contributes to axonal pathology in multiple sclerosis. NeuroImage 36:19–27
14. Xuan Y, Yan G, Wu R, Huang Q, Li X, Xu H (2015) The cuprizone-induced changes in (1)H-MRS metabolites and oxidative parameters in C57BL/6 mouse brain: Effects of quetiapine. Neurochem Int 90:185–192
15. Praet J, Guglielmetti C, Berneman Z, Van der Linden A, Ponsaerts P (2014) Cellular and molecular neuropathology of the cuprizone mouse model: Clinical relevance for multiple sclerosis. Neurosci Biobehav Rev 47:485–505
16. Hagemeyer N, Boretius S, Ott C, Von Streitberg A, Welpinghus H, Sperling S et al (2012) Erythropoietin attenuates neurological and histological consequences of toxic demyelination in mice. Mol Med 18:628–635
17. Masuda S, Okano M, Yamagishi K, Nagao M, Ueda M, Sasaki R (1994) A novel site of erythropoietin production. Oxygen-dependent production in cultured rat astrocytes. J Biol Chem 269: 19488–19493
18. Knabe W, Sirén AL, Ehrenreich H, Kuhn HJ (2005) Expression patterns of erythropoietin and its receptor in the developing spinal cord and dorsal root ganglia. Anat Embryol (Berl) 210:209–219
19. Knabe W, Knerlich F, Washausen S, Kietzmann T, Sirén AL, Brunnett G et al (2004) Expression patterns of erythropoietin and its receptor in the developing midbrain. Anat Embryol (Berl) 207:503–512
20. Juul SE, Anderson DK, Li Y, Christensen RD (1998) Erythropoietin and erythropoietin receptor in the developing human central nervous system. Pediatr Res 43:40–49
21. Hassouna I, Ott C, Wüstefeld L, Offen N, Neher RA, Mitkovski M, Winkler D, Sperling S et al (2016) Revisiting adult neurogenesis and the role of erythropoietin for neuronal and oligodendroglial differentiation in the hippocampus. Mol Psychiatry 21:1752–1767
22. Sargin D, Friedrichs H, El-Kordi A, Ehrenreich H (2010) Erythropoietin as neuroprotective and neuroregenerative treatment strategy: comprehensive overview of 12 years of preclinical and clinical research. Best Pract Res Clin Anaesthesiol 24:573–594
23. Siren AL, Fasshauer T, Bartels C, Ehrenreich H (2009) Therapeutic potential of erythropoietin and its structural or functional variants in the nervous system. Neurotherapeutics 6:108–127
24. Kumral A, Ozer E, Yilmaz O, Akhisaroglu M, Gokmen N, Duman N, Ulukus C, Genc S et al (2003) Neuroprotective effect of erythropoietin on hypoxic-ischemic brain injury in neonatal rats. Biol Neonate 83:224–228
25. Chattopadhyay A, Choudhury TD, Bandyopadhyay D, Datta AG (2000) Protective effect of erythropoietin on the oxidative damage of erythrocyte membrane by hydroxyl radical. Biochem Pharmacol 59:419–425
26. Bernaudin M, Marti HH, Roussel S, Divoux D, Nouvelot A, MacKenzie ET, Petit E (1999) A potential role for erythropoietin in focal permanent cerebral ischemia in mice. J Cereb Blood Flow Metab 19:643–651
27. Schelshorn DW, Schneider A, Kuschinsky W, Weber D, Krüger C, Dittgen T, Bürgers HF, Sabouri F et al (2009) Expression of hemoglobin in rodent neurons. J Cereb Blood Flow Metab 29:585–595
28. Brown N, Alkhayer K, Clements R, Singhal N, Gregory R, Azzam S, Li S, Freeman E et al (2016) Neuronal hemoglobin expression and its relevance to multiple sclerosis neuropathology. J Mol Neurosci 59:1–17
29. Schnaitman C, Greenawalt JW (1968) Enzymatic properties of the inner and outer membranes of rat liver mitochondria. J Cell Biol 38: 158–175
30. Li S, Clements R, Sulak M, Gregory R, Freeman E, McDonough J (2013) Decreased NAA in gray matter is correlated with decreased availability of acetate in white matter in postmortem multiple sclerosis cortex. Neurochem Res 38:2385–2396
31. Schmued L, Bowyer J, Cozart M, Heard D, Binienda Z, Paule M (2008) Introducing Black-Gold II, a highly soluble gold phosphate complex with several unique advantages for the histochemical localization of myelin. Brain Res 1229:210–217
32. Cervellini I, Ghezzi P, Mengozzi M (2013) Therapeutic efficacy of erythropoietin in experimental autoimmune encephalomyelitis in mice, a model of multiple sclerosis. Methods Mol Biol 982:163–173
33. Singhal NK, Li S, Arning E, Alkhayer K, Clements R, Sarcyk Z, Dassanayake RS, Brasch NE et al (2015) Changes in methionine metabolism and histone H3 trimethylation are linked to mitochondrial defects in multiple sclerosis. J Neurosci 35:15170–15186
34. Sättler MB, Merkler D, Maier K, Stadelmann C, Ehrenreich H, Bähr M et al (2004) Neuroprotective effects and intracellular signaling pathways of erythropoietin in a rat model of multiple sclerosis. Cell Death Differ 11(Suppl 2):S181–S192
35. Digicaylioglu M, Lipton SA (2001) Erythropoietin-mediated neuroprotection involves cross-talk between Jak2 and NF-kappaB signalling cascades. Nature 412:641–647
36. Horng LY, Hsu PL, Chen LW, Tseng WZ, Hsu KT, Wu CL, Wu RT (2015) Activating mitochondrial function and haemoglobin expression with EH-201, an inducer of erythropoietin in neuronal cells, reverses memory impairment. Br J Pharmacol 172:4741–4756
37. Biagioli M, Pinto M, Cesselli D, Zaninello M, Lazarevic D, Roncaglia P et al (2009) Unexpected expression of α- and β-globin in mesencephalic dopaminergic neurons and glial cells. Proc Natl Acad Sci U S A 106:15454–15459
38. Clark JB (1998) N-acetyl aspartate: a marker for neuronal loss or mitochondrial dysfunction. Dev Neurosci 20:271–276
39. Signoretti S, Marmarou A, Tavazzi B, Lazzarino G, Beaumont A, Vagnozzi R (2001) N-Acetylaspartate reduction as a measure of injury severity and mitochondrial dysfunction following diffuse traumatic brain injury. J Neurotrauma 18:977–991
40. Richter F, Meurers BH, Zhu C, Medvedeva VP, Chesselet MF (2009) Neurons express hemoglobin alpha- and beta-chains in rat and human brains. J Comp Neurol 515:538–547
41. Ferrer I, Gómez A, Carmona M, Huesa G, Porta S, Riera-Codina M et al (2011) Neuronal hemoglobin is reduced in Alzheimer's disease, argyrophilic grain disease, Parkinson's disease, and dementia with Lewy bodies. J Alzheimers Dis 23:537–550
42. Freed J, Chakrabarti L (2016) Defining a role for hemoglobin in Parkinson's disease. NPJ Parkinsons Dis 2:16021