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Targeted Knockdown of Bone Morphogenetic Protein Signaling within Neural Progenitors Protects the Brain and Improves Motor Function following Postnatal Hypoxia-Ischemia

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Abstract

Hypoxic-ischemic injury (HI) to the neonatal human brain results in myelin loss that, in some children, can manifest as cerebral palsy. Previously, we had found that neuronal overexpression of the bone morphogenetic protein (BMP) inhibitor noggin during development increased oligodendroglia and improved motor function in an experimental model of HI utilizing unilateral common carotid artery ligation followed by hypoxia. As BMPs are known to negatively regulate oligodendroglial fate specification of neural stem cells and alter differentiation of committed oligodendroglia, BMP signaling is likely an important mechanism leading to myelin loss. Here, we showed that BMP signaling is upregulated within oligodendroglia of the neonatal brain. We tested the hypothesis that inhibition of BMP signaling specifically within neural progenitor cells (NPCs) is sufficient to protect oligodendroglia. We conditionally deleted the BMP receptor 2 subtype (BMPR2) in NG2-expressing cells after HI. We found that BMPR2 deletion globally protects the brain as assessed by MRI and protects motor function as assessed by digital gait analysis, and that conditional deletion of BMPR2 maintains oligodendrocyte marker expression by immunofluorescence and Western blot and prevents loss of oligodendroglia. Finally, BMPR2 deletion after HI results in an increase in noncompacted myelin. Thus, our data indicate that inhibition of BMP signaling specifically in NPCs may be a tractable strategy to protect the newborn brain from HI.

Keywords

Bone morphogenetic protein; Hypoxic-ischemic injury; Motor function; Neural progenitors; Oligodendroglia neuroprotection

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Disclosure Statement

The authors declare no competing financial interests.

Introduction

Hypoxia-ischemia (HI) commonly affects the approximately 63,000 preterm infants with very low birth weight (<1,500 g) born annually [1]. Oligodendroglial progenitor cells (OPCs) that are particularly vulnerable to HI are abundant within this patient population whose gestational age ranges between 26 and 32 weeks [2, 3]. As a result, these patients are susceptible to oligodendroglial loss and white matter injury [4]. Children with white matter injury during infancy have high rates of cognitive and behavioral deficits and are at risk for cerebral palsy, a nonprogressive dysfunction of motor control, for which no specific preventive medical therapies currently exist [5, 6].

Bone morphogenic protein (BMP) signaling is known to influence the cellular events that lead to myelination. It inhibits oligodendroglial fate choices by neural stem cells/progenitors [7, 8]. In committed oligodendrocytes (OLs), BMP signaling inhibits expression of myelin proteins and development of mature OLs [9, 10]. BMP signaling can thus affect the differentiation of cells that participate in myelination as well as the production of proteins that play key roles in white matter production.

Several studies have found that BMP signaling is increased in models of central nervous system (CNS) injury or disease [reviewed in 11]. We have shown that BMP4 expression is increased in a mouse model of postnatal HI, and that inhibition of BMP signaling in the brain by transgenic overexpression of the BMP inhibitor noggin increases both OPCs and mature OLs and improves motor function [12]. In those experiments, noggin was constitutively expressed by neurons, and thus BMP downregulation was present prior to and at the time of injury as well as during convalescence [12]. Furthermore, in those experiments, the cells affected by noggin could not be specified, nor was the timing of noggin exposure controlled.

Here, we show that canonical BMP signaling is increased in oligodendroglia after postnatal HI. We tested if inhibition of BMP signaling, specifically in neural progenitor cells (NPCs), after postnatal HI is sufficient to protect oligodendroglial lineage cells using a conditional Cre/LoxP approach to ablate the BMP receptor (BMPR) in NG2-expressing cells following postnatal HI. NG2 (nerve/glia antigen-2) is a type I transmembrane glycoprotein expressed by multipotent NPCs that are found throughout the postnatal CNS and give rise predominantly to OLs [13]. NG2 cells are not confined to the persistent generative zones of the brain and can be found proximate to injured areas, making them readily available for immediate responses. BMPR is a heterotetrameric receptor that includes 2 BMPR1 subunits (BMPR1a/BMPR1b) and 2 BMPR2 subunits [14]. Deletion of BMPR2 thus prevents any BMP signaling by preventing the formation of a functional receptor. We found that knockdown of BMP signaling within NPCs after HI decreases ventriculomegaly and improves motor function by increasing myelin proteins and noncompacted myelin. Our studies provide the first direct evidence that interruption of BMP signaling in NG2 cells may be a useful strategy for neuroprotection.

Materials and Methods

Animals

Experiments were done using the following mice: C57Bl/6J (Jackson Laboratories, Bar Harbor, ME, USA), NG2CreERTM [13], ROSA^{EYFP/EYFP} and ROSA^{mTmG} floxed stop Cre reporters (Jackson Laboratories), and BMPR2^{fl/fl} [15]. The NG2CreERTM mouse was crossed with the BMPR2^{fl/fl} mouse to create the NG2CreERTM ; BMPR2^{fl/fl} (R2 floxed), respectively. Only homozygous floxed mice were used for experiments. In some cases, the ROSA^{EYFP/EYFP} [16] or ROSA^{mTmG} [17] floxed stop reporters were in the background, for example NG2CreERTM ; ROSA^{EYFP/EYFP} ; BMPR2^{fl/fl}. All mice were backcrossed to a C57Bl/6 background for at least 6 generations. Mice were housed in a facility with a 12-h light/dark cycle and allowed access to food and water ad libitum. Experiments were conducted according to protocols approved by the Northwestern University Institutional Animal Care and Use Committee and Northwestern Center for Comparative Medicine.

Neonatal HI Lesions

Postnatal day (P) 7 mice were subjected to right common carotid artery ligation and transection followed by hypoxia as previously described [12]. Floxed mice were treated with tamoxifen 60 mg/kg or vehicle (sterile corn oil: ethanol [9: 1]) by intraperitoneal injection once daily starting after HI on P7, P8, P9, and P10. Mice were sacrificed 24, 48, and 72 h or 7, 14, and 42 days post-lesion (dpl) for the different experiments.

Quantitative Real-Time Polymerase Chain Reaction

Wild-type (WT) pups were euthanized with carbon dioxide inhalation followed by decapitation; total RNA was obtained, cDNA generated, and quantitative real-time polymerase chain reaction (qRT-PCR) performed as previously described [12]. The following primers were used: BMPR1a: forward 5'-GTT CAT CAT TTC TCA TGT TCA AAC TA-3', reverse 5'-AAT CAG AGA CTT CAT ACT TCA TAC ACC-3' [18] ; BMPR1b: forward 5'-TCG ATG GAG CAG TGA TGA GTG TCT-3'; reverse 5'-GCT GAC GGC CCT GAG AGT TAA G-3'; BMPR2: forward 5'-ATG AAA GCT CTG CAG CTA GGT C-3', reverse 5'-CAT CAT TTA AAC, ATG, CAG, AAG ATG T-3'; and GAPDH: forward 5'-GTC GTG GAT CTG ACG TGC C-3', reverse 5'-TGC CTG CTT CAC CAC CTT C-3'.

Protein Extraction and Western Blot Analysis

Tissue was collected, and Western Blot analysis was performed as previously described [12]. The following antibodies were used: CNPase 1: 1,000 (Sternberger SMI91), GAPDH 1: 1,000 (Millipore MAB374), MBP 1: 1,000 (Sternberger SMI99), phosphorylated SMAD1/5/8 (pSMAD1/5/8) 1: 1,000 (Cell Signaling #9511), BMPR1a 1: 100 (Abgent AP2004a), BMPR1b 1: 1,000 (Abgent AP2005c), and BMPR2 1: 40 (Abgent AP2006a).

Magnetic Resonance Imaging

Mice were sacrificed 42 dpl for ex vivo imaging and transcatheterially perfused as for immunofluorescence studies. Brains were post-fixed for 7 days in 4% paraformaldehyde and

then transferred to 5 mM GdDTPA in PBS to rehydrate and enhance contrast. Brains were then immersed in Fomblin perfluoropolyether (Solvay Plastics, Cranbury, NJ, USA) for imaging. MRI was performed on the 9.4-T Bruker Biospec MRI System (Bruker Biospin, Billerica, MA, USA) using a 25-mm quadrature volume coil. Images were acquired using a 3D spin echo-based sequence with TR/TE = 250/17.9 ms, matrix $150 \times 100 \times 100$, $0.1 \times 0.1 \times 0.1$ mm³ isotropic resolution, and 3 averages. MRI was performed by the Center for Advanced Molecular Imaging at Northwestern University.

Digital Gait Analysis

Digital video images were captured at 14 dpl and analyzed as previously described [12].

RotaRod

Two-month-old mice were placed on rotating drums of the RotaRod system V4.1.4 (TSE Systems, Midland, MI, USA), and time was measured from the start to the time they fell off. Mice were trained for 3 consecutive days with 4 trials at the fixed speed of 12 rpm. On the 4th and 5th days, they were tested with an accelerating speed to a maximum speed of 27 rpm [19].

Immunofluorescence

Tissue was collected and fixed, and sections were prepared as previously described [12]. The following antibodies were used: CC1 1: 250 (Millipore OP80), CNPase 1: 200 (Sternberger SMI91) (Covance, Princeton, NJ, USA), GFAP 1: 200 (Dako Z0334), Iba1 1: 150 (Wako 019-19741), MBP 1: 500 (Sternberger SMI99), Olig1 1: 600 (Chemicon AB5540) (EMD Millipore, Billerica, MA, USA), Olig2 1: 400 (Chemicon AB9610), O4 1: 250 (EMD Millipore MAB345), PDGFRA 1: 200 (Fitzgerald CD140a) (Fitzgerald, North Acton, MA, USA), pSMAD1/5/8 1: 50 (Cell Signaling 9511) (Cell Signaling Technology, Danvers, MA, USA), Alexa Fluor 488 goat anti-rabbit 1: 500, Alexa Fluor 594 goat anti-mouse IgG1 1: 500, Alexa Fluor 594 goat anti-mouse IgG2b 1: 500, Alexa Fluor 594 goat anti-mouse IgM 1: 500, and Alexa Fluor 647 goat anti-mouse IgM 1: 500 (Invitrogen) (Life Technologies, Grand Island, NY, USA). Confocal images were obtained using the Zeiss LSM 880 microscope at the Stanley Manne Children's Research Institute.

Histochemistry

Frozen sections were hydrated sequentially from 70% ethanol for 30 min to 95% ethanol for 30 min, then they were placed in 1% Luxol fast blue solution (1 g; S3382; Sigma-Aldrich, St. Louis, MO, USA), 95% ethanol (100 mL), and 10% acetic acid (5 mL) at 60 ° C for 16 h. Slides were rinsed with 95% ethanol, then ddH₂O, then differentiated in 0.05% lithium carbonate solution (0.5 g; L4283; Sigma-Aldrich; 1,000 mL ddH₂O) for 5 s followed by 70% ethanol for 5s twice, then rinsed with ddH₂O. Differentiation steps were repeated until gray matter had cleared. Slides were counterstained with 0.25% cresyl violet solution (0.1 g cresyl violet acetate [C1791, Sigma-Aldrich], 100 mL ddH₂O, and 10 drops glacial acetic acid) for 40 s, then rinsed with ddH₂O and differentiated in 95% ethanol for 5 min, and then rinsed in 100% ethanol for 5 min twice. Slides were dehydrated from 95% ethanol through

100% ethanol and cleared with xylene; coverslips were mounted with Permount (SP15-500, Fisher Scientific, Pittsburgh, PA, USA).

Transmission Electron Microscopy

Samples were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.3) and postfixed with unbuffered 2% osmium tetroxide, rinsed with distilled water, en bloc stained with 3% uranyl acetate, again rinsed with distilled water, dehydrated in ascending grades of ethanol, transitioned with propylene oxide, embedded in resin mixture using the EMbed 812 kit, and cured in a 60°C oven. Samples were sectioned on a Leica Ultracut UC6 ultramicrotome; 1- μ m-thick sections were collected and stained with toluidine blue O, and 70-nm sections were collected on formvar carbon-coated film on slotted grids; thin sections were stained with uranyl acetate and Reynolds lead citrate. Thin sections were examined on a FEI Tecnai Spirit G2 TEM, and digital images were captured with an FEI Eagle camera (FEI, Burlington, MA, USA). Samples were processed by the Center of Advanced Microscopy at the Northwestern University Feinberg School of Medicine.

Statistics and Analysis

For all experiments, 3–11 mice were used per treatment group. MRI scans were analyzed using Amira software for segmentation using a semi-automated region-growing method, followed by volume measurements of the regions of interest. Western blots were quantified using ImageJ software (NIH, Bethesda, MD, USA). For immunofluorescence, mid-striatal coronal sections were collected between +0.98 mm anterior to bregma and +0.38 posterior to bregma. For each animal, four $\times 40$ fields were captured from corpus callosum dorsomedial to the right lateral ventricle using Axiovision 4.6 software (Carl Zeiss Vision, Thornwood, NY, USA). Relative fluorescence was measured using Metamorph software (Molecular Devices, Sunnyvale, CA, USA). For immunohistochemistry studies, sections were collected similarly and four $\times 20$ fields were captured using Spot software (Spot Imaging Solutions, Sterling Heights, MI, USA). Image files were encoded, areas were measured, and cells were counted using Axiovision 4.6 (Carl Zeiss) by investigators blinded to the treatment group. MRI measurements, cell counts, and relative expression were analyzed by ANOVA, using the Fisher PLSD test for post hoc comparisons with the significance level set at $p < 0.05$; for 2-group comparisons, unpaired t tests were used with the significance level set at $p < 0.05$ (Statview; SAS, Chicago, IL, USA).

Results

BMP Signaling Increases following Postnatal HI

Previously, we observed that BMP4 protein levels increase in the brain following postnatal HI [12]. To more specifically define the effect of HI on the activation of BMP signaling, we quantified pSMAD1/5/8, a component of the canonical BMP signaling pathway, using Western blot analysis. Protein blots of total cerebral lysates showed that pSMAD1/5/8 increased 1.5-fold 24 h after injury (Les 24 h) compared to 24 h after sham surgery followed by normoxia (sham 24 h) ($p = 0.05$) as well as compared to sham 72 h ($p = 0.04$) (Fig. 1a), sham 7 days ($p = 0.05$), and Les 7 days ($p = 0.05$). To examine BMP signaling specifically

within OLs, we performed double immunofluorescence studies and found increased Olig1+/pSMAD1/5/8+ cells within the corpus callosum of lesioned mice 48 h after injury compared to 24 h after sham injury ($p = 0.01$, Les 48 h: 55 ± 12 [mean $\pm$ SEM] Olig1+/pSMAD1/5/8+ cells/mm², sham 24 h: 2.7 ± 1.5 Olig1+/pSMAD1/5/8+ cells/mm²) and compared to 24 h after injury ($p = 0.01$, Les 48 h: 55 ± 12 Olig1+/pSMAD1/5/8+ cells/mm², Les 24 h: 10.3 ± 8.4 Olig1+/pSMAD1/5/8+ cells/mm²) (Fig. 1b–j). These observations indicate that BMP signaling is stimulated by HI in the brain, including Olig1+ cells of the corpus callosum.

To determine if BMPR subtypes were altered after HI, we assayed changes in mRNA and protein levels. Using qRT-PCR, we found that levels of BMPR1a and BMPR1b transcripts both remained relatively stable 24, 48, and 72 h, and 7 days after HI compared to sham controls at the same time points (Fig. 2a, b), but BMPR2 mRNA was significantly decreased 72 h after injury compared to sham controls at the same time point ($p = 0.02$, Les C_T: 0.01 ± 0.01 , sham C_T: 0.05 ± 0.01) (Fig. 2c). Using Western blot analysis, we found that BMPR1a (Fig. 2d) and BMPR1b (Fig. 2e) protein levels remained stable 24, 48, and 72 h, and 7 days after HI compared to sham controls at the same time points. Despite finding decreased BMPR2 transcript levels after 72 h, we did not observe significant changes in BMPR2 protein levels after injury compared to sham controls at the same time points (Fig. 2f). Thus, BMPR components do not appear to change up to 7 days after HI, although BMPR2 transcripts declined at one time point assayed. Coupled with increased pSMAD1/5/8 levels, pSMAD1/5/8 staining in Olig1+ cells of the corpus callosum and increased BMP4 [12], our findings supported a role for increased BMP signaling within a primary site of NPC differentiation in the postnatal mouse brain after HI injury.

Loss of BMPR2 in NPCs following Postnatal HI Ameliorates HI-Induced Ventriculomegaly

Since we had shown that overexpression of the BMP inhibitor noggin by neurons increased oligodendroglia and improved motor function [12], we wondered whether rescue was the result of a paracrine effect on oligodendroglia and whether we could elicit a more robust response by specifically targeting BMP signaling in NPCs. BMPRs are expressed in OPCs and mature OLs thus making it feasible to disrupt this pathway in mouse brains [7, 20]. BMPRs consist of 2 type I receptor subunits (BMPR1a or BMPR1b) and 2 type II receptor subunits (BMPR2). These BMPRs bind to either BMP ligands or to growth differentiation factors, members of the TGF β superfamily of ligands [21]. Therefore, to alter BMP signaling in NPCs after HI, we conditionally deleted BMPR2 in cells activating the NG2 promoter starting at P7, a critical time in white matter production. Cre expression was driven using the NG2CreERTM BAC transgene developed by the Nishiyama Laboratory. Cre activity is dependent on tamoxifen administration and was demonstrated to be present in cells derived from OLs and white matter cells in the postnatal mouse brain [13]. Pups were injected daily with tamoxifen for 4 days total starting on P7, with the first dose administered immediately after hypoxia. This was done to approximate a possible clinical intervention in babies with suspected hypoxic-ischemic brain injury. On this schedule, tamoxifen induced recombination in the corpus callosum as evidenced by the expression of enhanced green fluorescent protein (EGFP) from the ROSA^{mTmG} reporter in the corpus callosum and striatum detected by membrane-targeted GFP (Fig. 3a–e). In this way, we conditionally and temporally controlled BMP signaling after HI.

To assess whether conditional deletion of BMPR2 in oligodendroglia would be globally protective within the context of HI, we performed ex vivo MRI and compared brains of injured mice that had been treated with tamoxifen after surgery (LesTam) to injured mice that received only vehicle (LesVeh). We assessed mice 42 dpl and measured volumes of the lateral ventricles and the 3rd ventricle. We found that the lateral ventricles ipsilateral to injury in LesTam mice were significantly smaller than in LesVeh mice ($p = 0.01$, LesTam-ipsilateral $0.64 \text{ mm}^3 \pm 0.26 \text{ mm}^3$, LesVeh-ipsilateral $5.43 \text{ mm}^3 \pm 2.71 \text{ mm}^3$) (Fig. 4a–c). Although we observed a trend towards decreased 3rd ventricle size in LesTam mice compared to LesVeh, this did not meet significance. Thus, conditional deletion of BMPR2 after HI globally protects the brain as ventriculomegaly reflects loss of brain tissue.

Loss of BMPR2 in NPCs following Postnatal HI Improves Ambulation

To assess whether conditional deletion of BMPR2 in OLs would also be protective of motor function, we used digital gait analysis to assess ambulation. Paw area changes on the transparent treadmill reflect important changes to the posture and kinematics of the mouse. In an injured animal, the area of contact of the paw is expected to be uncoordinated and the area increased, reflecting a flatfooted gait required for stability. Here, we observed significant increases in paw area at peak stance for the left forelimb, likely reflecting abnormal weight bearing on the weakened side when comparing LesVeh groups to ShamVeh mice (Fig. 5c). This is the limb expected to be most affected by right common carotid artery ligation. These differences were rescued in the LesTam group (Fig. 5a–c) ($p = 0.04$, LesTam $0.21 \pm 0.01 \text{ cm}^2$, LesVeh $0.28 \pm 0.03 \text{ cm}^2$). We observed smaller but still significant decreases in paw area at peak stance for the right forelimb and the right hindlimb (Fig. 5c) (right forelimb: $p = 0.01$, LesTam $0.18 \pm 0.01 \text{ cm}^2$, LesVeh $0.25 \pm 0.02 \text{ cm}^2$; right hindlimb: $p = 0.02$; LesTam $0.23 \pm 0.02 \text{ cm}^2$, LesVeh $0.34 \pm 0.03 \text{ cm}^2$). Paw area variability at peak stance is also expected to be higher in an animal that is weak and uncoordinated. We observed increased left forelimb paw area variability in HI-injured relative to sham-operated mice, and this was rescued in the LesTam group (Fig. 5d) ($p = 0.03$, LesTam $0.03 \pm 0.00 \text{ cm}^2$, LesVeh $0.08 \pm 0.02 \text{ cm}^2$). We also found decreased (maximum) change in paw area per change in time (max. dA/dT) (Fig. 5e) ($p = 0.03$, LesTam $10.71 \pm 0.35 \text{ cm}^2/\text{s}$, LesVeh $15.11 \pm 1.44 \text{ cm}^2/\text{s}$) and an improved ataxia coefficient, which indicates step-to-step variability similar to stumbling (Fig. 5f) ($p = 0.02$, LesTam 1.22 ± 0.17 , LesVeh 1.86 ± 0.15). Together, our analysis indicated that HI injury affects the gait of the injured mouse and that conditional deletion of BMPR2 in NPCs after injury significantly improved specific gait parameters. To test whether deletion of BMPR2 alone has an effect on motor function, mice were injected daily (with Veh or Tam) from P7 to P10, then RotaRod testing was performed at 2 months of age. No significant differences in performance were observed between Veh and Tam mice (Fig. 5g) ($p = 0.9$, acceleration day 1: Veh $101.5 \pm 16 \text{ s}$ and Tam $76.3 \pm 17 \text{ s}$; $p = 1.0$, acceleration day 2: Veh $91.3 \pm 15 \text{ s}$ and Tam $96.4 \pm 16 \text{ s}$). This finding supports the idea that conditional loss of BMPR2 in NG2 cells and their descendants does not otherwise affect motor function in older mice.

Loss of BMPR2 following Postnatal HI Preserves Expression of Myelin Proteins

To determine if conditional loss of BMPR2 in the oligodendroglial lineage improved motor function by affecting expression of myelin proteins, we sacrificed mice 14 dpl (P21). First,

we examined brain sections by immunofluorescence, focusing on the corpus callosum. We probed for CNPase, a marker that is expressed by immature OLs [22], and MBP, a marker that is expressed by mature myelinating OLs [22, 23]. We compared protein accumulation by relative fluorescence and found no change in CNPase expression within the corpus callosum when comparing the LesTam group to the LesVeh group ($p = 0.4$, LesVeh: 102 ± 29 mean fluorescence intensity [MFI], LesTam: 141 ± 48 MFI) (Fig. 6a). We found a significant 1.75-fold increase in MBP fluorescence intensity in the corpus callosum of the LesTam group compared to the LesVeh group ($p = 0.02$, LesVeh 79 ± 15 MFI and LesTam 137 ± 4 MFI) (Fig. 6b–d). Histologic staining for myelin using Luxol fast blue also showed qualitatively improved myelin staining in the LesTam group compared to the LesVeh group (Fig. 6e, f).

Because we saw improvements in motor function in BMPR2 conditional knockout mice, we investigated CNPase and MBP levels in the internal capsule, which connects the motor cortex to the cerebral peduncles and the spinal cord. Western blot analysis of total protein from the ipsilateral internal capsule revealed no loss of CNPase when comparing LesTam to LesVeh ($p = 0.4$, LesVeh: $54,182 \pm 36,978$ arbitrary units [a.u.] and LesTam: $94,141 \pm 37,586$ a.u.) (Fig. 6g). Similarly, there was no loss of MBP in the internal capsule when comparing LesTam to LesVeh ($p = 0.2$, LesVeh: $92,670 \pm 70,626$ a.u., LesTam: $268,225 \pm 114,452$ a.u.) (Fig. 6h). Differences in protein levels between the corpus callosum and the internal capsule are likely related to the posterior-anterior progression of differentiation, e.g., HI insult at P7 would affect oligodendroglia at earlier stages of differentiation in the corpus callosum than in the more posterior internal capsule. The whole brain would contain a preponderance of white matter tracts posterior to the mid-striatal corpus callosum (coronal sections between +0.98 mm anterior to bregma and +0.38 posterior to bregma). Together, these observations suggest that loss of BMPR2 in NPCs protects against loss of CNPase and MBP after HI in the corpus callosum and internal capsule.

Conditional Loss of BMPR2 in NPCs following Postnatal HI Prevents Loss of Oligodendroglia but Does Not Change NPC Lineage Commitment

To determine if conditional loss of BMPR2 in the oligodendroglial lineage alters differentiation of NPCs following HI, we performed immunofluorescence on the corpus callosum at 7 dpl. We assayed changes in total oligodendroglia probing for Olig1 and Olig2, transcription factors expressed by cells along the continuum of the oligodendroglial lineage, from progenitors to mature OLs [24]. We compared 3 groups: ShamVeh (Sham), LesVeh, and LesTam. Here, we found no change in Olig1+ cells when comparing LesTam to LesVeh groups ($p = 0.6$, LesVeh: 285 ± 52 Olig1+ cells/mm² and LesTam: 315 ± 47 Olig1+ cells/mm²) (Fig. 7a, a', a'', b). We also observed no change in Olig2+ cells in the corpus callosum of LesTam mice compared to LesVeh mice (Fig. 7c, c', c'', d) ($p = 0.2$, LesVeh: 200 ± 46 Olig2+ cells/mm², LesTam: 353 ± 121 Olig2+ cells/mm²). Thus, conditional loss of BMPR2 after HI injury does not affect the number of total oligodendroglia.

To test if loss of BMPR2 in the context of HI causes a change in OPCs, we probed for PDGFRA, a growth factor receptor that marks OPCs [25]. Here, we observed no significant differences when comparing sham, LesVeh, and LesTam groups ($p = 0.6$, sham: 205 ± 48

PDGFRA+ cells/mm², LesVeh: 169 ± 47 PDGFRA+ cells/mm², and LesTam: 144 ± 33 PDGFRA+ cells/mm²) (Fig. 7e, e', e'', f). We tested if loss of BMPR2 following HI increased premyelinating OLs by probing for O4, a sulfatide expressed in cells committed to the oligodendroglial lineage [26]. Here, we found no differences in O4+ cells between sham, LesTam, and LesVeh mice ($p = 0.5$, sham: 49 ± 20 O4+ cells/mm², LesVeh: 74 ± 16 O4+ cells/mm², and LesTam: 82 ± 36 O4+ cells/mm²) (Fig. 7g).

We used the monoclonal antibody CC1 to examine the number of mature OLs. CC1 binds to the protein Quaking-7, an RNA-binding protein expressed in the cell bodies of myelinating OLs [27]. We observed that HI mice had significantly lower numbers of CC1+ cells in the corpus callosum 7 dpl in comparison to sham ($p = 0.04$, sham: 234 ± 60 CC1+ cells/mm² and LesVeh: 94.8 ± 19 CC1+ cells/mm²) However, conditional loss of BMPR2 did not protect against this loss ($p = 0.9$, LesVeh: 94.8 ± 19 CC1+ cells/mm² and LesTam: 81.2 ± 8 CC1+ cells/mm²) (Fig. 7h, h', h'', i).

To determine if conditional loss of BMPR2 following HI altered astrocyte differentiation within the corpus callosum, we probed for the astrocyte marker GFAP. We found no significant differences in GFAP+ cells in the corpus callosum when comparing ShamVeh, LesVeh, and LesTam groups ($p = 0.6$, ShamVeh: 2,096 ± 621 GFP+ cells/mm², LesVeh: 2,760 ± 353 GFP+ cells/mm², LesTam: 2,297 ± 285 GFP+ cells/mm²) (Fig. 7j, j', j'', k). Because BMPR2 deletion was conditional in our experiments, we expected that we might see a change in astrocytes but not microglia. We probed for Iba1 to test for noncell autonomous changes in microglia. We found no significant differences in Iba1+ cells in the corpus callosum when comparing sham, LesVeh, and LesTam groups ($p = 0.07$, sham: 79 ± 30 Iba1+ cells/mm², LesVeh: 205 ± 15 Iba1+ cells/mm², and LesTam: 172 ± 49 Iba1+ cells/mm²) (Fig. 7l, l', l'', m). Our findings suggest that loss of BMPR2 does not significantly alter oligodendrocyte lineage specification and maturation in the context of HI.

To explore whether tamoxifen alone had a confounding effect on OPC number, perhaps through toxicity, we compared lesioned NG2CreERTM; ROSA^{EYFP/EYFP}; BMPR2^{+/+} WT reporter mice treated with tamoxifen to lesioned BMPR2 floxed reporter mice treated with tamoxifen. We observed no difference in PDGFRA+/GFP+ cell counts in R2 floxed mice compared to reporter mice (Fig. 7n). Thus, outside of the high BMP milieu of HI, BMPR2 deletion in NG2-expressing cells may cause an increase in OPCs in the corpus callosum. However, in the context of HI, BMPR2 deletion is insufficient to significantly increase OPCs. Alternatively, it is known that PDGFRA+ OPCs increase in subventricular zone and striatum following HI, changes not observed within the corpus callosum of WT mice, possibly due to emigration from the corpus callosum to these adjacent regions [28].

Conditional Loss of BMPR2 following Postnatal HI Increases Noncompacted Myelin

Since our data supported a role for BMPR2 in protecting loss of CNPase and MBP by a mechanism that was independent of changes to the oligodendroglial lineage, we hypothesized that BMP signaling affects maturation of committed oligodendroglia. To test this, we used scanning electron microscopy to examine the myelin ultrastructure in mature OLs. We compared myelin between LesTam and LesVeh mice 14 dpl (Fig. 8a, b). Surprisingly, we observed no difference in myelin thickness, G-ratio (ratio of the cross-

sectional diameter of an axon to the diameter of that axon plus its surrounding myelin), axon counts, and axon diameters in LesTam compared to LesVeh mice (Fig. 8c–f), indicating that conditional loss of BMPR2 did not affect these parameters. Notably, with conditional loss of BMPR2, we found a significant increase in noncompacted myelin ($p = 0.01$, LesVeh: $35 \pm 8\%$ and LesTam: $73 \pm 8\%$) (Fig. 7g). This finding could be consistent with improved although not normal myelination.

Discussion

BMP signaling is known to be increased in several models of white matter injury. BMP7 transcripts were increased in rat spinal cord following compression injury [29]. BMP4, BMP6, and BMP7 were all increased in a mouse model of multiple sclerosis [30]. Similar increases in BMPs were observed in other models of spinal cord injury, demyelinating lesions, and HI in the brain. Previously, we reported increased BMP expression in HI-injured mouse brains [12]. Here, we found that an output of canonical BMP signaling (pSMAD1/5/8) was increased in the corpus callosum, indicating not only that BMP ligands are increased after HI but also that they signal within OLs of the corpus callosum. In addition, we obtained evidence that conditional ablation of BMPR2 specifically in NG2-expressing OPCs is sufficient to partially rescue myelination, limit ventriculomegaly, and improve ambulation in mice that have been subjected to perinatal HI. This suggests that while other cell types may be producing BMP ligands and/or responding to signals, OPCs specifically respond to this signal in this injury model.

If Increased Expression of BMPs and Increased Signaling by BMPs Is Common to Various Forms of CNS Injury, What Pathways Are Modified by BMPs?

In general, BMP signaling is thought to inhibit oligodendroglial lineage commitment during development, and this is supported by studies in which cultured NPCs are stimulated to differentiate into astrocytes after treatment with BMPs [7, 31, 32]. Furthermore, expression of noggin in areas of BMP expression overcomes the inhibitory nature of BMPs and allows OL formation [33]. Similarly, BMP4 overexpression in neurons increased astroglial lineage commitment in mouse brains [34]. Increased BMP signaling could thus be expected to inhibit or alter the maturation of oligodendroglial lineage cells following white matter injury.

While we were not entirely surprised to find that conditional ablation of BMPR2 partially protected against HI injury, the cellular effects we observed were unexpected. After finding that conditional loss of BMPR2 maintained MBP in the corpus callosum after HI, we expected that this effect would be reflected by a change in the lineage of NPCs to mature OLs. However, we found that total numbers of OPCs were not increased. Tamoxifen administration did not increase the numbers of OPCs as marked by Olig2 or PDGFRA when comparing LesTam to LesVeh mice. We were particularly surprised to find no increase in PDGFRA+GFP+ cells when comparing cell counts from NG2CreERTM; ROSA^{EYFP/EYFP}; BMPR2^{+/+} mice to NG2CreERTM; ROSA^{EYFP/EYFP}; BMPR2^{fl/fl} mice (Fig. 7n). It is possible that the timing of administration of tamoxifen played a key role here. In our model, recombination could not occur until after HI injury. This recombination is extensive, as we observed with the ROSA^{mTmG} reporter, but the NG2-expressing cells would not lose

BMPR2 function until some time after Cre was activated. Thus, the effects of loss of BMPR2 on the lineage may be lessened because tamoxifen was not injected until after the injury itself. On the other hand, there are examples where ablation of oligodendroglial-expressed genes did not significantly change OL numbers [35–38]. This may be also true for BMPR2.

Still another possibility is that the majority of NG2 cells in which recombination occurred were already committed to the oligodendroglial lineage and that the primary effect was on the rate of differentiation. Thus, BMP inhibition could change the rate of differentiation of OPCs into mature OLs during the critical period of postnatal myelination, similar to its increasing effects on the differentiation rate of adult hippocampal stem cells into neurons. Supporting this is the observation that Olig1 localization in the corpus callosum at P14 was almost entirely cytoplasmic (Fig. 7a, a' a''). Olig1 is shuttled between the cytoplasm and the nucleus, and nuclear export of Olig1 is important for promoting the differentiation of OLs [39–41]. Our observations were made in the context of constant numbers of OPCs, suggesting that increased BMP signaling in injured brain inhibits differentiation into mature OLs. This was supported by a significant decrease in the number of cells expressing CC1 in LesVeh mice as compared with sham mice. However, conditional loss of BMPR2 after HI did not ameliorate this loss suggesting that BMP signaling needs to be inhibited soon after HI to have its greatest effect.

Since the lineage was not altered by loss of BMPR2, our data suggest that another role of BMP signaling in HI is the suppression of production of myelinating proteins. It has been reported that BMP signaling in committed OLs modifies expression of genes encoding myelinating proteins. Cultured immature OLs were treated with BMP4 and, while they did express GalC, they did not express PLP, MBP, MAG, and CNPase. Similarly, BMP4-soaked beads suppressed myelinating proteins in cultured embryonic cervical spinal cords [9]. Importantly, we observed no loss of the myelinating proteins that we examined in either the corpus callosum or internal capsule after downregulation of BMPR2 in the injured animal. While MBP was significantly increased in the corpus callosum by fluorescence intensity, CNPase was not significantly changed in injured BMPR2 conditional knockout mice. This may reflect that CNPase is one of the first myelin proteins to be expressed and is synthesized by cells prior to full differentiation [42]. Neither MBP nor CNPase was significantly changed in the internal capsule by Western blot despite a loss of CC1+ cells in the lesioned condition. Together these observations suggest that BMP signaling after HI could play a role in the production of myelinating proteins such as CNPase and MBP.

Conditional Loss of BMPR2 in NPCs Partially Protects Brains from White Matter Loss and Motor Deficits

Ultimately, our goal is to develop clinical interventions that protect babies from white matter injury and cerebral palsy. Our findings support a role for BMP signaling in the pathogenesis of white matter injury, and that loss of BMPR2 partially protects white matter. This was also accompanied by improved ambulation and protection from ventriculomegaly. Therefore, attenuating BMP signaling soon after neonatal HI is likely to provide long-term benefits to

babies at risk for cerebral palsy. Moreover, we showed that motor function is not adversely affected in the long term by BMPR2 deletion.

It is interesting that the greatest changes caused by ablating BMPR2 were in improving ambulation and reducing ventriculomegaly. We considered several reasons for this. First, as for humans, the effects of neonatal HI in mice are somewhat variable. In mice, the severity of the lesion and survival after hypoxia can vary between background strains. This is thought to involve differences in collateral circulation in the brain [43]. In fact, in this study we needed to carefully titrate the length of time of hypoxia to which mice were subjected. Longer times resulted in frequent death and shorter times resulted in limited injury. Another important consideration, as we discussed above, is the degree to which tamoxifen is effective at stimulating recombination at a particular dose. A third point is when tamoxifen was administered. To reproduce the timing of possible clinical interventions for HI in babies, we induced recombination immediately after injury. Since recombination is likely to have begun to occur within hours of the first injection, and since cells were expressing BMPR2 during the time of the injury and for a short time after, it is likely that inhibition of the BMP signal caused by HI does not occur immediately – only after existing BMP receptors were turned over. Given the limitations of our approach, it is even more impressive that loss of BMPR2 had important protective effects on gait and ventriculomegaly. This suggests that even limited inhibition of BMP signaling in babies with HI will have important long-term benefits. This could be tested further by introducing pharmacologic inhibitors of BMP signaling immediately after HI.

Conclusions

We conclude that inhibition of BMP signaling specifically in NG2-expressing NPCs protects the brain and ameliorates detrimental effects of HI on motor function through an effect on OL differentiation.

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Fig. 1.

Postnatal hypoxia-ischemia (HI) increases SMAD signaling in the cerebrum and oligodendroglia. **a** Quantification of relative phosphorylated SMAD1/5/8 (pSMAD1/5/8) protein levels from total cerebral lysate following HI. Band intensity is relative to 24 h after sham surgery (* $p = 0.05$ and ** $p = 0.04$). Representative blots are shown below the graph. **b** Quantification of Olig1+ pSMAD1/5/8+ cells within the ipsilateral (injured/right side) corpus callosum (* $p = 0.01$ and ** $p = 0.01$). **a, b** $n = 3$ for each time point and treatment group. Error bars represent SEM. **c-j** Representative micrographs of Olig1+ pSMAD1/5/8+ cells within the ipsilateral (HI-injured/right side) corpus callosum 48 h after injury. White arrowheads indicate examples of cells with pSMAD1/5/8+ nuclei in images of the sham (noninjured/right side; c-f) and ipsilateral (HI-injured/right side; g-j) corpus callosum. c, g DAPI-stained nuclei (blue). d, h Olig1-stained cells (green). e, i pSMAD1/5/8-stained cells (red). f, j Color images demonstrate overlapping fluorescence in images c-e and g-i, respectively.

Fig. 2.

Bone morphogenetic protein receptor (BMPR) levels remain relatively stable after hypoxia-ischemia (HI) injury. Bar graphs representing quantification of BMPR1a (a), BMPR1b (b), and BMPR2 transcripts (c) following sham or HI surgery as determined by quantitative real-time polymerase chain reaction. y-axis represents change in threshold cycle (ΔC_T), x-axis indicates time after surgery. * $p = 0.02$. d–f Bar graphs represent relative levels of BMPR1a (d), BMPR1b (e), and BMPR2 (f) protein in total cerebral lysates from sham or HI surgery brains. Quantification is band intensity relative to sham at 24 h. Representative blots are shown below each graph. $n = 3$ for each time point and treatment group. Error bars represent SEM.

Fig. 3. Recombination in NG2CreERTM-expressing cells after postnatal tamoxifen induction. Representative confocal micrographs of NG2CreERTM ; ROSA^{mTmG} noninjured mouse brains demonstrating extensive recombination within the oligodendroglial lineage after daily injections of tamoxifen from postnatal days 7–10. **a** Low magnification, tiled image in which recombined cells are green (membrane-enhanced green fluorescent protein [mEGFP]), nuclei are blue (DAPI), and nonrecombined cells are red (mTdTomo). White boxes indicate areas shown at higher magnification in **b–e**. **b, c, e** Higher magnifications of the corpus callosum at 3 different positions. **d** Higher magnification of the striatum with recombination in Wilson's pencil fibers. **e** Arrowhead points to a blood vessel with recombined vascular cells.

Fig. 4.

Bone morphogenic protein receptor 2 (BMPR2) knockdown in NG2 cells following injury decreases ventriculomegaly. Magnetic resonance images 42 dpl showing ipsilateral lateral ventricle (red), contralateral lateral ventricle (yellow), and third ventricle (blue). **a**, **a'**, **a''** Three different brains from lesioned, vehicle-treated mice (LesVeh). **b**, **b'**, **b''**, **b'''** Four different brains from lesioned, tamoxifen-treated mice (LesTam). **c** Quantification of lateral ventricle volumes. Error bars represent SEM. contra, contralateral lateral ventricle; ipsi, ipsilateral lateral ventricle. Four LesTam-ipsilateral, 4 LesTam-contralateral, 3 LesVeh-ipsilateral, and 3 LesVeh-contralateral ventricles. * $p = 0.01$, ** $p = 0.01$, *** $p = 0.01$. **d** Quantification of 3rd ventricle volumes. Three LesVeh and 4 LesTam. Error bars represent SEM.

Fig. 5.

Bone morphogenic protein receptor 2 (BMPR2) knockdown in NG2 cells following injury improves motor function. Representative left forelimb gait tracings for a nonlesioned, vehicle-treated mouse (ShamVeh) (a), a lesioned, vehicle-treated mouse (LesVeh) (b), and a lesioned, tamoxifen-treated mouse (LesTam) (c) at 14 dpl are shown. The height of each peak is the area of the footprint at peak stance. The number of peaks is the number of steps the animal takes within the time shown. **d** Quantification of mean paw area at peak stance. Error bars represent SEM. L, left; R, right. * $p = 0.04$, ** $p = 0.02$, *** $p = 0.02$. **e** Quantification of paw area variability at peak stance for the left forelimb. * $p = 0.03$. **f** Quantification of maximum change in paw area per change in time (Max. dA/dT) for the left forelimb. * $p = 0.03$. **g** Ataxia coefficient for the left forelimb. Sample sizes were as follows: 3 sham, 8 LesVeh, and 6 LesTam. Error bars represent SEM. * $p = 0.02$, ** $p = 0.01$. **h** RotaRod testing of 2-month-old uninjured vehicle-treated mice ($n = 7$) compared to tamoxifen-treated mice ($n = 5$). T1–T3, training days 1–3; A1/A2, acceleration days 1/2.

Fig. 6.

Bone morphogenic protein receptor 2 (BMPR2) knockdown in NG2 cells following injury partially protects against myelin loss. Veh, vehicle; Tam, tamoxifen. $n = 4$, respectively. **a** Quantification of CNPase expression by mean relative immunofluorescence. Error bars represent SEM. **b** Quantification of MBP expression by mean relative immunofluorescence. $*p = 0.02$. **c, d** Representative micrographs of MBP expression within the corpus callosum 14 dpl. $\times 40$. Scale bar, 25 μm . **e, f** Representative micrographs of Luxol fast blue (LFB) staining within the corpus callosum 14 dpl. Pink is cresyl violet. $\times 20$. Scale bar, 50 μm . **g, h** Quantification of CNPase (**g**) and MBP expression (**h**) by Western blot of the ipsilateral internal capsule and representative blots. Four nonlesioned, vehicle-treated mice (LesVeh) and 5 lesioned, tamoxifen-treated mice (LesTam). Error bars represent SEM. a.u., arbitrary units.

Fig. 7.

Bone morphogenic protein receptor 2 (BMPR2) knockdown in NG2 cells following injury does not affect oligodendroglial lineage. Representative confocal micrographs of cells within the corpus callosum 7 dpl. Markers used are indicated on the left of each set of panels. ShamVeh, sham surgery, vehicle-treated; LesVeh, hypoxia-ischemia (HI) surgery, vehicle treated; LesTam, HI surgery, tamoxifen treated. Antibodies tested were Olig1 (a, a', a''), Olig2 (c, c', c''), PDGFRα (e, e', e''), CC1 (h, h', h''), GFAP (j, j', j''), and Iba1 (l, l', l''). b, d, f, g, i, k, m, n Quantification of cells in the corpus callosum. Error bars represent SEM. b Olig1 ($n = 7$ sham, 10 LesVeh, and 11 LesTam). d Olig2 ($n = 5$ sham, 11 LesVeh, and 10 LesTam), f PDGFRα ($n = 4$ sham, 6 LesVeh, and 9 LesTam). g O4 ($n = 9$ sham, 7 LesVeh, and 6 LesTam). i CC1 ($n = 5$ sham and 5 LesTam). * $p = 0.04$, ** $p = 0.03$. k GFAP ($n = 5$ sham, 5 LesVeh, and 7 LesTam). m Iba1 ($n = 5$ sham, 5 LesVeh, and 5 LesTam). n PDGFRα/GFP (green fluorescent protein) co-staining ($n = 3$ WT mice and 5 BMPR2 conditional knockout mice).

Fig. 8. Knockdown of bone morphogenic protein receptor 2 (BMPR2) following postnatal hypoxia-ischemia (HI) increases noncompacted myelin. Representative electron micrographs of the internal capsule 14 dpl in lesioned, vehicle-treated mice (LesVeh) (a) and lesioned, tamoxifen-treated mice (LesTam) (b). Quantification of myelin thickness (c), the G-ratio (ratio of the cross-sectional diameter of an axon to the diameter of that axon plus its surrounding myelin) (d), axon count (e), axon diameter (f), and noncompacted myelin (g). c–g $n = 4$ LesVeh and 4 LesTam. Error bars represent SEM. * $p = 0.01$.