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Inhibiting bone morphogenetic protein 4 type I receptor signaling promotes remyelination by potentiating oligodendrocyte differentiation

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Title page

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Abstract

Blocking inhibitory factors within central nervous system (CNS) demyelinating lesions is regarded as a promising strategy to promote remyelination. Bone morphogenetic protein 4 (BMP4) is an inhibitory factor present in demyelinating lesions. Noggin, an endogenous antagonist to BMP, has previously been shown to increase the number of oligodendrocytes and promote remyelination *in vivo.* However, it remains unclear how BMP4 signaling inhibits remyelination. Here we investigated the downstream signaling pathway that mediates the inhibitory effect that BMP4 exerts upon remyelination through pharmacological and transgenic approaches. Using the cuprizone mouse model of central demyelination, we demonstrate that selectively blocking BMP4 signaling via the pharmacological inhibitor LDN-193189 significantly promotes oligodendroglial differentiation and the extent of remyelination *in vivo*. This was accompanied by the downregulation of transcriptional targets that suppress oligodendrocyte differentiation. Further, selective deletion of BMPRIA receptors within primary mouse OPCs significantly enhanced their differentiation and subsequent myelination *in vitro*. Together, results of this study identify that BMP4 signals via BMPRIA within OPCs to inhibit oligodendroglial differentiation and their capacity to myelinate axons, and suggest that blocking BMP4/BMPRIA pathway in OPCs is a promising strategy to promote CNS remyelination.

Significance Statement

Blocking inhibitory factors within central demyelinating lesions is a promising strategy to promote remyelination. Previous studies have established that exogenous BMPs inhibit oligodendrocyte differentiation during CNS development and after injury. Here, we demonstrate that blocking endogenous BMP4 signaling via a selective pharmacological approach promotes oligodendroglial differentiation and the rate of remyelination after a central demyelinating insult *in vivo.* Using *in vitro* analysis, we identify that OPC-expressed BMPRIA receptors mediate this effect. Together, our data propose that blocking the BMP4 signaling pathway and/or BMPRIA receptors in OPCs is a promising strategy to promote CNS remyelination.

Introduction

In central demyelinating diseases such as multiple sclerosis (MS), oligodendrocytes (OLs) are targeted through inflammatory activity and the myelin sheath surrounding axons is degraded (Noseworthy, 1999; Weiner, 2009). The degree of remyelination within demyelinating lesions is variable; although MS lesions remyelinate relatively efficiently early on in disease, at later stages many lesions remain chronically demyelinated (Trapp & Nave, 2008). These chronically demyelinated lesions typically contain oligodendrocyte progenitor cells (OPCs) and premyelinating oligodendrocytes that have "stalled" in their differentiation, implicating blocked oligodendrocyte differentiation as a major contributing factor to remyelination failure (Chang, Tourtellotte, Rudick, & Trapp, 2002; Kuhlmann et al., 2008). Although the full complement of factors that inhibit oligodendrocyte differentiation and remyelination in the context of MS are yet to be completely elucidated, they most likely include a variety of inhibitory signals present within the lesion environment as well as an absence of positive signals (Fancy, Chan, Baranzini, Franklin, & Rowitch, 2011; Franklin, ffrench-Constant, Edgar, & Smith, 2012; Kotter, Stadelmann, & Hartung, 2011). Thus, blocking the action of inhibitory factors is regarded as a leading strategy to promote endogenous CNS remyelination (Franklin & Gallo, 2014).

The bone morphogenetic proteins (BMPs) are a group of secreted proteins that are part of the larger TGF-β superfamily (Chen, Zhao, & Mundy, 2004), and play critical roles in neural development and gliogenesis (Bond, Bhalala, & Kessler, 2012; Cole, Murray, & Xiao, 2016). Of the 20 BMPs, BMP4 has a prominent role in promoting astroglial, and inhibiting oligodendroglial specification (Gomes, Mehler, & Kessler, 2003; Grinspan, 2015). *In vitro*, BMP4 exerts stage-specific inhibitory effects on OPCs (Grinspan et al., 2000), in particular inhibiting the production of myelin proteins by immature oligodendrocytes (See et al., 2004). *In vivo,* transgenic overexpression of BMP4 led to an increase in the number of astrocytes and a decrease in the number of oligodendrocytes in the murine CNS (Gomes et al., 2003). In the context of demyelinating disease, BMP4 mRNA is detected in human demyelinated MS lesions (Deininger, Meyermann, & Schluesener, 1995), and is expressed by astrocytes, microglia and infiltrating immune cells (Harnisch et al., 2019). Astrocytes also express a high level of BMP4 in chronic lesions that have failed to remyelinate

(Harnisch et al., 2019). Through using the cuprizone induced murine model of CNS demyelination, we have previously found that BMP4 mRNA is upregulated in the mouse corpus callosum following a demyelinating insult *in vivo* (Cate et al., 2010). Furthermore, we demonstrated that inhibiting BMP4 signaling following cuprizone-induced CNS demyelination via infusion of its extracellular antagonist noggin resulted in more mature oligodendrocytes and more remyelinated axons (Cate et al., 2010; Sabo et al., 2011). However, in addition to BMP4, noggin also inhibits other BMPs such as BMP2, 7, 13 and 14 (Krause, Guzman, & Knaus, 2011). Due to the promiscuous inhibitory effect of noggin, and the potential effects it exerted upon oligodendroglia, astrocytes and microglia, the precise influence that inhibition of BMP4 exerts upon remyelination, and the cell type mediating the effect remains unclear.

BMP4 signals through membrane-bound receptor complexes comprised of two Type I receptors and two Type II receptors. Whilst several Type I receptors exist, BMP4 has greatest affinity for the Type I receptors BMPRIA (also known as ALK3) and BMPRIB (also known as ALK6) (Knaus & Sebald, 2001; Liu, Ventura, Doody, & Massague, 1995). In the presence of BMP4, BMPRIA and BMPRIB initiate signalling via phosphorylation of SMADs 1, 5 and 8 (SMAD1/5/8) (Cuny et al., 2008) and the pharmacological inhibitor LDN-193189 selectively blocks phosphorylation SMAD1/5/8 (Cuny et al., 2008). In order to specifically interrogate the influence that BMP4 signalling exerted upon remyelination, we infused LDN-193189 into the brain following cuprizone-induced demyelination and found it significantly enhanced oligodendroglial differentiation and their subsequent remyelination following the demyelinating insult *in vivo*. This finding is also supported *in vitro* in which LDN-193189 significantly enhanced OPC differentiation and myelination. Further, by utilizing a tamoxifen-dependent inducible conditional knockout mouse strategy (*Pdgfra-CreER*^{T2}::*Bmpr1a*$^{fl/fl}$) to specifically ablate BMPRIA expression within OPCs, we identified that selectively deleting of BMPRIA in OPCs significantly potentiated their differentiation into mature oligodendrocytes and increased myelin formation *in vitro*. Together, our findings indicate that BMP4 acts on OPC-expressed BMPRIA receptors to inhibit oligodendroglial differentiation and myelination, and that blocking BMPRIA signalling OPCs is a promising strategy to promote CNS remyelination.

Methods:

Animals and reagents

All animal procedures were performed in accordance with the [Facility acknowledged in Title Page 'Acknowledgements' section] animal care committee's regulations.. Female mice aged 7-8 weeks old were used for *in vivo* cuprizone experiments and postnatal day 5 (P5)-P7 mice of either sex were used for *in vitro* experiments. C57BL/6 mice were purchased from the Animal Resource Centre (Canning Vale, WA, Australia). *Pdgfra-CreER*^{T2}::*Bmpr1a*$^{fl/fl}$ mice were generated by crossing *Pdgfra-CreER*^{T2} mouse line (Rivers et al., 2008) (kindly provided by Dr Kaylene Young of the University of Tasmania, Australia) with *Bmpr1a*$^{fl/fl}$ mouse colony (also known as *Alk3*$^{fl/fl}$; kindly provided by Professor Yuji Mishina of the University of Michigan, USA) (Mishina, Hanks, Miura, Tallquist, & Behringer, 2002). *Pdgfra-CreER*^{T2}::*Bmpr1a*$^{fl/fl}$ mice have a tamoxifen-inducible deletion of the *Bmpr1a* allele from the start of the sequence to the end of exon 2, rendering it untranscribable (Mishina, Suzuki, Ueno, & Behringer, 1995). All animals used for this study were bred at the Animal Facilities of the [Author University]. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

Cuprizone protocol

Cuprizone mediated demyelination was induced by feeding 8–10 week old female mice (C57/B6) powdered feed (Barastoc, Pakenham, Victoria, Australia) containing 0.2% cuprizone (w/w: bis-cyclohexanoneoxaldihydrazone) for five weeks, as previously described (Cate et al., 2010; Sabo et al., 2011). Mice were then returned to a normal diet for either 0 or 1 week(s), according to the experimental paradigm. During the 5-week demyelination phase, feed was refreshed every three days, with approximately 20g provided per mouse for this period. Mice were weighed daily to monitor extreme fluctuations in weight and ensure no mouse lost more than 15% of its initial weight during the protocol. Unchallenged control mice were fed identical feed without added cuprizone.

Intracerebroventricular infusion

Following cuprizone feeding, animals received either LDN-193189 (Stemgent, 400ng/day) or artificial

cerebrospinal fluid (aCSF) via intracerebroventricular osmotic pumps (Alzet, Cat#: 1007D, CA, USA). The concentration of LDN-193189 was based on our previous study using noggin (Sabo et al., 2011). Mice were deeply anaesthetised using 2.5% isofluorane and attached to a stereotactic frame. The scalp was cut sagittal to the cervical spine. The pumps were used in conjunction with Alzet Brain Infusion Kit III to implant a cannula into an entry point drilled 0.5mm anterior to Bregma, 0.7mm laterally from the longitudinal midline and at a depth of ~1-2mm. Canullae were fused to the skull using araldite and the incision was sutured with Vicryl veterinary sutures and disinfected using Betadine iodine solution. Mice were allowed to recover for >30mins at 30^{o}C before returning to cage. Mice were monitored daily to observe any symptoms of distress or infection. After 7 days of continuous infusion, animals were sacrificed and the brain removed for immunohistochemical and histological analysis.

Post-cuprizone tissue collection

Following cuprizone withdrawal, mice were transcardially perfused using 0.1M mouse-tonicity PBS (MT-PBS) as a buffer and 4% PFA (in MT-PBS, 15mL per mouse) as a fixative. Brains were dissected and post-fixed overnight with 4% PFA in MT-PBS and rinsed the following day with MT-PBS before being cut coronally into 1mm sections. For electron microscopy, sections containing the most caudal region of the CC (~Bregma: -2.12mm) were trimmed to expose the splenium of the caudal CC and placed in Karnovsky's buffer (4% PFA, 2.5% glutaraldehyde in 0.1M sodium cacodylate) overnight before being rinsed three times in 0.1M sodium cacodylate. For immunohistochemical analyses, sections containing the caudal corpus callosum (~Bregma: -1.12mm) were placed in 30% sucrose (in MT-PBS with 0.1% sodium azide) overnight. Sucrose-treated sections were frozen in Tissue-Tek Optimum Cutting Temperature (O.C.T., Sakura) solution using chilled iso-pentane and stored at -80^{o}C.

Immunohistochemistry

Coronal brain sections were cut at 10μm or 12μm thin and blocked for 1h in antibody diluent (10% normal goat serum, 0.3% Triton-X100 in MT-PBS) at room temperature (RT) before exposure to primary antibodies diluted in antibody diluent overnight at 4^{o}C. The following primary antibodies were used at a dilution of

1:200: rat anti-myelin basic protein (MBP, Abcam, Cat.#:MAB386), rabbit anti-OLIG2 (Millipore, Cat.#:ab9610), rat anti-CC1/APC (Calbiochem, Cat.#:D35078), mouse anti-platelet-derived growth factor receptor alpha (PDGFRα, R&D Systems, Cat.#:AF1062), mouse anti-glial fibrillary acidic protein (GFAP, Millipore, Cat.#:MAB360), and goat anti-IBA-1 (Abcam, Cat.#:ab5076). Cryosections were then rinsed with MT-PBS three times for ~5mins followed by the appropriate fluorophore-conjugated secondary antibodies (all 1:500 in antibody diluent, Thermofisher Scientific) for 60mins at RT. Sections were rinsed twice in MT-PBS before adding Hoechst (1:10000 in MT-PBS, Cat#:33342, Invitrogen) for 10 minutes. Cryosections were rinsed twice in MT-PBS and a coverslip was mounted with Cytomation™ fluorescence mounting medium (Dako). Six sections per animal from a minimum of three animals per group were analyzed, and images captured by Carl Zeiss LSM 780 confocal fluorescent microscopy. All images were acquired using the same settings and analyzed by an operator blinded to conditions using FIJI (ImageJ 1.51K, National Institutes of Health) software (Schindelin et al., 2012). For OLIG2+/CC1+/PDGFRα+ cell counts, cells were counted from the entire visible corpus callosum per image field with the same size of area. For MBP immunostaining, a central area of 200 μm^2 was measured for integrated density (the product of the mean grey value of each pixel, ranging from 0 to 255, and the total area) using the 'Measure' function in FIJI. For GFAP and IBA1 immunostaining, the entire corpus callosum was measured using the 'Trace' function.

Spectral confocal reflection (SCoRe) microscopy

SCoRe imaging was performed on brain sections to assess the extent of myelin damage in cuprizone mice using published methods (Gonsalvez, De Silva, et al., 2017; Gonsalvez, Tran, et al., 2017; Schain, Hill, & Grutzendler, 2014). Briefly, mice were perfused with 4% PFA, and their brains were dissected, frozen and cryosectioned at 12μm. Coronal sections of caudal brains were imaged via a Zeiss 780 LSM confocal microscope with a water immersion objective (Zeiss W Plan-Apochromat 20×/1.0 NA DIC M27 70mm) using 458, 561 and 633-nm laser wavelength through the Tunable Lazer In Tune 488-640 filter/splitter wheel and a 20/80 partially reflective mirror. The reflected light was collected using three photodetectors set to collect light through narrow bands defined by prism and mirror-sliders, centered around the laser wavelengths 488nm, 561nm and 633nm. Sections were immersed in MT-PBS and a 20X dipping objective

was equipped prior to imaging. The midline corpus callosum was located and a 3x2 tile scan image was taken of each section. The channels from each photodetector were then additively combined as a one color composite. Myelinated area was calculated using ImageJ by firstly applying a Z-stack transformation and then setting a threshold of 50 pixels. Measurements of the resulting area were obtained with the 'Measure' function and divided by the total area of the region of interest (ROI). The percentage area of positive signal was computed for each image. For quantification, a minimum 3 separate ROIs per image and 3 images per tile (using a 20x/1.0NA objective at a z-depth 4µm from the tissue surface) per treatment group were used and statistically analyzed.

Transmission Electron Microscopy (TEM)

Mouse caudal CC samples were embedded in resin for five days before trimming and sectioning using an ultramicrotome. Semi-thin sections (0.5um) were taken and imaged using toluene blue staining to identify ROI. Ultra-thin sections (70nm) were then taken and imaged using a TEM. Images were taken at 5000X and 10,000X magnification per animal using JEOL 1011 transmission electron microscope. Three 10,000X images were taken per hexagonal bounding grid (corresponding to a size of $250um^2$, with six distinct fields of view were imaged at 10000x magnification per animal. Images were used to count myelinated axons, measure axon diameters, and g-ratios in FIJI. For g-ratios analysis, a minimum of 90 axons per animal from minimum 3 mice per group were measured.

Primary mouse OPC culture

Oligodendrocyte progenitor cells were isolated from P5-6 wildtype or transgenic mouse pups using a previously published protocol (Emery & Dugas, 2013). Cultures were grown on poly-D-lysine (pDL)-coated vessels in defined serum-free media and supplied daily with PDGF (10ng/mL, Peprotech), Neurotrophin 3 (NT-3, 1ng/mL, Peprotech) and Ciliary Neurotrophic Factor (CNTF, 10ng/mL, Peprotech). For the differentiation assay, PDGF is withdrawn from OPCs culture and cells were cultured in Sato media containing oligodendrocyte differentiation factor thyroid hormone T3 (3,3',5-Triiodo-L-thyronine sodium**,** 4ng/mL in Sato media; Sigma-Aldrich), CNTF (10ng/mL), forskolin (5µM) and NT-3 (1ng/mL). For small

molecule inhibitor experiments, OPCs were either cultured in the differentiating condition (see above) with LDN-193189 (0.2µM, Stemgent) or vehicle (DMSO) being added 30 minutes prior to BMP4 addition (R&D Systems, Cat#:314-BP, 1ng/mL). In some cultured, OPCs were isolated from *Pdgfra-CreER*^{T2}::*Bmpr1a*$^{fl/fl}$ (Cre[+]) and *Bmpr1a*$^{fl/fl}$ control (Cre[-]) mice. These OPCs were treated with 4-hydroxy-tamoxifen (referred to as '4OHT', 500nM in EtOH, Sigma) to induce knockout of BMPRIA or an equal volume of vehicle (ethanol). For the differentiation assay, OPCs were either treated with BMP4 (1ng/mL) or vehicle (0.1% BSA in D-PBS) with the addition of differentiation Sato media containing T3. For some experiments, 4OHT or vehicle (ethanol) were added 24 hours prior to BMP4 addition (R&D Systems, Cat#:314-BP, 1ng/mL). After a set time point as indicated, cells were fixed in 4% PFA for 20 minutes followed by immunocytochemical staining (see below). For differentiation assays, three technical replicates and a minimum of 3 mice per condition or genotype were used.

Dorsal root ganglion (DRG)/OPC co-culture

DRG/OPC co-cultures were established based on published techniques (J. Xiao et al., 2010). Briefly, OPCs were isolated as detailed above and seeded onto coverslips containing purified DRGs at a density of $2x10^5$ OPCs per 22-mm poly-ornithine (Sigma)/pDL-coated coverslip and incubated overnight to facilitate attachment. DRG-OPC co-cultures were maintained for 14 days in a defined co-culture media containing a 1:1 ratio of Sato medium/Neurobasal medium (Gibco) with 2% NeuroCult™ SM1 supplement (Stem Cell Technologies). Media was changed every 2-3 days. For small molecule inhibitor experiments, cells were either cultured with LDN-193189 (0.2µM, Stemgent) or vehicle (DMSO) for 30 minutes prior to BMP4 addition (R&D Systems, Cat#:314-BP, 1ng/mL) at each feed. For transgenic experiments, OPCs that isolated from *Pdgfra-CreER*^{T2}::*Bmpr1a*$^{fl/fl}$ and *Bmpr1a*$^{fl/fl}$ control (Cre[-]) mice were treated with 4OHT or vehicle control (ethanol) for the first 48 hours following co-culturing with neurons. After 14 days, co-cultures were immunostained, and protein extracted for western blotting as described below.

Immunocytochemistry

After fixation with 4% PFA for 18 mins, cells were rinsed three times in MT-PBS. Cells were blocked with

10% normal goat serum with 0.3% Triton-X 100 in MT-PBS for 60mins at RT, followed an incubation with primary antibodies against GFAP (mouse, Millipore, Cat.#:MAB360, 1:200; rabbit, DAKO, Cat.#:Z03374, 1:200), MBP (mouse, Millipore, Cat.#:MAB381, 1:50; rat, Millipore, Cat.#:ab980, 1:100) or rabbit anti-Neurofilament (Millipore, Cat.#:AB1987, 1:200). Cells were then rinsed with MT-PBS followed by the appropriate fluorophore-conjugated secondary antibodies (all 1:500 in antibody diluent, Thermo Fisher Scientific) for 60mins at RT. Cells were rinsed twice in MT-PBS before adding Hoechst (1:10,000 in MT-PBS, Cat#:33342, Invitrogen) for 10 mins. Cells were rinsed twice in MT-PBS and mounted with Cytomation™ fluorescence mounting medium (Dako) on SuperFrost Plus™ glass slides (ThermoFisher). Six fields per culture, and three technical replicate from a minimum of three animals per condition or genotype were analyzed, and images captured by a Carl Zeiss™ Axioplan 2 epi-fluorescence upright microscope.

Immunocytochemical quantification

For OPC culture images, all Hoechst-positive nuclei were counted using Adobe Photoshop (Adobe Inc., Version CS5), and the morphology of each Hoechst-positive cell was designated as either an astrocyte (GFAP+), immature oligodendrocyte (MBP+) or mature oligodendrocyte (MBP+), or unclear (MBP/GFAP-negative). These populations (excluding the 'unclear' cells) were then graphed as a proportion of all Hoechst+ cells. For the DRG/OPC co-culture analysis, an average length for a clearly defined segment was subjectively defined at the start of counting using the ImageJ measure tool, and then the same length is used to count further segments. This was consistently applied throughout all treatments by one counter over one session.

Western blotting analysis

Total protein of OPC/DRG co-cultures was extracted using TNE buffer supplemented with proteinase inhibitor (Roche), separated by SDS-PAGE (200V, approximately 30-40 minutes) and transferred to PVDF membrane using an iBlot® quick transfer dry blot system (Life Technologies). Protein blots were blocked with 5% non-fat milk powder in Tris-buffered saline/Tween-20 (TBST, 50mM Tris, 150mM NaCl, 0.05% Tween-20, all Sigma) for 5-10 minutes, followed by three rinses with TBST. Blots were subsequently

probed with antibodies against myelin proteins MBP (1:50, Cat#:AB980, Chemicon), MOG (1:50, Cat#:MAB5680, Millipore) or BMPRIA (1:200, Cat#:38560, Abcam) overnight at 4°C. An antibody against β-actin (1:5000 in TBST+2%BSA; Cat#:A5441, Sigma) was also added as an internal loading control. Following three rinses with TBST, blots were incubated with HRP-conjugated secondary antibodies (1:5000; Cell Signaling Technologies).

RNA isolation and q-RT-PCR analysis

Following differentiation assay, OPCs were rinsed once with cold D-PBS and lysed using a cell scraper with addition of 600µL RLT-plus buffer (Qiagen) supplemented with 1% 2-mercaptoethanol (Sigma) as an RNase inhibitor. Pure OPC RNA was acquired by following RNeasy Plus Mini protocol (Qiagen). RNA was reverse-transcribed using Applied Biosystems reagents and following manufacturer's protocol. Following synthesis of cDNA, samples were loaded undiluted into 96-well plates and SYBR™Green Master Mix (Applied Biosystems) was added along with primers. The plate was sealed with optical film (Applied Biosystems) and centrifuged for 1min at 1000rpm. It was then loaded into an Applied Biosystems ViiA™7 quantitative real-time PCR system. Average expression of housekeeping gene 18S was used to normalise gene expression using the ΔΔCt method. Primer sequences used were shown in Table 1 (all primers are specific for *Mus musculus*).

Analyzing multiple transcriptional changes using RT^2 Profiler PCR Array

Purified mRNA reverse-transcribed using the RT^2 First Strand kit (Qiagen, Cat#:330401) according to the manufacturer's instructions. A mouse TGF-β/BMP Signaling Pathway RT^2 Profiler PCR Array (SABioScience, Cat#: PAMM-035C) was used to assess the expression of 84 gene specific to TGF-β/BMP signaling activity. Reverse-transcribed cDNA was added to SYBR™Green ROX Master mix (Qiagen, Cat#:330520) as per manufacturer's instructions and loaded into the 96-well plate PCR array. Samples were run on an Applied Biosystems ViiA™7 quantitative real-time PCR system (experimental setup settings were provided by SABioSciences and are listed in the Appendix). Average transcription of housekeeping genes provided in the

PCR Array was used to normalise gene expression using the ΔΔCt method. Data were analysed using an online software program provided by the manufacturer. Data are reported as changes in fold regulation, defined as equal to the fold change when the fold change value is positive, and the negative inverse of the fold change when the fold change value is negative. A full list of genes analysed using this method can be found at https://www.qiagen.com/us/shop/pcr/primer-sets/rt2-profiler-pcr-arrays/?catno=PAMM-035Z#geneglobe.

Statistical analysis

All statistical tests were performed using GraphPad Prism 7 (GraphPad Software). Assessors were blinded to conditions, groups or genotypes during analysis. All data are presented as mean±S.E.M.

Test identifier	***Type of test***	***Sample size***	***Confidence intervals***
a0	*Student's unpaired two-tailed t-test*	*Three animals per treatment; six technical replicates per animal.*	*-11.1 to 37.1*
a	*Student's unpaired two-tailed t-test*	*Four control and cuprizone-fed animals, six for vehicle- and LDN-treated animals; three technical replicates per animal.*	*14.81 to 55.60*
b	*Student's unpaired two-tailed t-test*	*Four control and cuprizone-fed animals, six for vehicle- and LDN-treated animals; three technical replicates per animal.*	*-20.02 to -2.95*
b1	*Student's unpaired two-tailed t-test*	*Four vehicle- and five LDN-treated animals; six technical replicates per animal, approximately 100 axons counted per*	*-45.96 to 11.67*

		animal.	
b2	*Student's unpaired two-tailed t-test*	*Three vehicle- and three LDN-treated animals; six technical replicates per animal, approximately 100 axons counted per animal.*	*0.017 to 0.091*
b3	*Two-way ordinary ANOVA with Tukey's multiple corrections test*	*Three vehicle- and three LDN-treated animals; six technical replicates per animal, approximately 100 axons counted per animal.*	*-23.39 to -0.6092*
b4	*Student's unpaired two-tailed t-test*	*Four control and cuprizone-fed animals, six for vehicle- and LDN-treated animals; three technical replicates per animal.*	*0.76 to 123.91*
b5	*Student's unpaired two-tailed t-test*	*Four control and cuprizone-fed animals, six for vehicle- and LDN-treated animals; three technical replicates per animal.*	*-81.21 to 26.21*
c	*Student's unpaired two-tailed t-test*	*Four control and cuprizone-fed animals, six for vehicle- and LDN-treated animals; three technical replicates per animal.*	*-94.40 to -60.46*
d	*Student's unpaired two-tailed t-test*	*Four control and cuprizone-fed animals, six for vehicle- and LDN-treated animals; three technical replicates per*	*38.05 to 91.35*

		animal.	
e	*Student's unpaired two-tailed t-test*	*Four control and cuprizone-fed animals, six for vehicle- and LDN-treated animals; three technical replicates per animal.*	*4.59 to 17.66*
f	*Student's unpaired two-tailed t-test*	*Four control and cuprizone-fed animals, six for vehicle- and LDN-treated animals; three technical replicates per animal.*	*-9.19 to -1.36*
g	*Student's unpaired two-tailed t-test*	*Four control and cuprizone-fed animals, six for vehicle- and LDN-treated animals; three technical replicates per animal.*	*-4980.00 to 2499.00*
h	*Student's unpaired two-tailed t-test*	*Four control and cuprizone-fed animals, six for vehicle- and LDN-treated animals; three technical replicates per animal.*	*-33305.00 to -21828.00*
I	*Student's unpaired two-tailed t-test*	*Four control and cuprizone-fed animals, six for vehicle- and LDN-treated animals; three technical replicates per animal.*	*-7695.00 to 2665.00*
j	*Two-way ordinary ANOVA with Tukey's multiple corrections test*	*Four independent cultures; three technical replicates per treatment; approximately 500-600 cells counted per*	*-81.20 to -66.60*

		treatment.	
k	*Two-way ordinary ANOVA with Tukey's multiple corrections test*	*Four independent cultures; three technical replicates per treatment; approximately 500-600 cells counted per treatment.*	*-68.00 to -53.30*
l	*Two-way ordinary ANOVA with Tukey's multiple corrections test*	*Four independent cultures; three technical replicates per treatment; approximately 500-600 cells counted per treatment.*	*-23.10 to -8.46*
m	*One-way ordinary ANOVA with Tukey's multiple corrections test*	*Three independent cultures; eight 10x image fields counted per treatment group.*	*19.43 to 51.24*
n	*One-way ordinary ANOVA with Tukey's multiple corrections test*	*Three independent cultures; eight 10x image fields counted per treatment group.*	*-51.15 to -19.35*
o	*One-way ordinary ANOVA with Tukey's multiple corrections test*	*Three independent cultures; eight 10x image fields counted per treatment group.*	*-33.99 to -2.18*
p	*One-way ordinary ANOVA with Tukey's multiple corrections test*	*Three independent cultures; three technical replicates per treatment.*	*-5.49 to -2.99*
q	*One-way ordinary ANOVA with Tukey's multiple corrections test*	*Three independent cultures; three technical replicates per treatment.*	*-7.02 to -3.91*
r	*One-way ordinary ANOVA with Tukey's multiple corrections test*	*Three independent cultures; three technical replicates per treatment.*	*1.11 to 3.61*
s	*One-way ordinary ANOVA with Tukey's multiple*	*Three independent cultures; three technical*	*2.84 to 5.95*

	corrections test	*replicates per treatment.*	
t	*One-way ordinary ANOVA with Tukey's multiple corrections test*	*Three independent cultures; three technical replicates per treatment.*	*-2.77 to -0.35*
u	*One-way ordinary ANOVA with Tukey's multiple corrections test*	*Three independent cultures; three technical replicates per treatment.*	*-0.17 to 2.25*
v	*One-way ordinary ANOVA with Tukey's multiple corrections test*	*Three independent cultures; three technical replicates per treatment.*	*-1.36 to -0.16*
w	*One-way ordinary ANOVA with Tukey's multiple corrections test*	*Three independent cultures; three technical replicates per treatment.*	*-2.47 to -0.51*
x	*Student's unpaired two-tailed t-test*	*Three independent cultures; three technical replicates per treatment.*	*Not available*
y	*Two-way ordinary ANOVA with Tukey's multiple corrections test*	*Three independent cultures; three technical replicates per treatment; approximately 500-600 cells counted per treatment.*	*-89.42 to -43.00*
z	*Two-way ordinary ANOVA with Tukey's multiple corrections test*	*Three independent cultures; three technical replicates per treatment; approximately 500-600 cells counted per treatment.*	*-32.21 to 14.20*
aa	*Two-way ordinary ANOVA with Tukey's multiple corrections test*	*Four independent cultures; three technical replicates per treatment; approximately 500-600 cells counted per treatment.*	*-23.20 to 23.30*
ab	*Two-way ordinary ANOVA with Tukey's multiple*	*Four independent cultures; three technical*	*-19.65 to -2.29*

	corrections test	*replicates per treatment; approximately 500-600 cells counted per treatment.*	
ac	*Two-way ordinary ANOVA with Tukey's multiple corrections test*	*Four independent cultures; three technical replicates per treatment; approximately 500-600 cells counted per treatment.*	*13.07 to 30.42*
ad	*Student's unpaired two-tailed t-test*	*Three independent cultures for both* Pdgfra-CreER^{T2}::Bmpr1a$^{fl/fl}$ *and* Bmpr1a$^{fl/fl}$ *co-cultures; eight 10x image fields counted per treatment group.*	*14.65 to 70.88*
ae	*Student's unpaired two-tailed t-test*	*Three independent cultures for both* PdgfraCreER^{T2}::Bmpr1a$^{fl/fl}$ *and* Bmpr1a$^{fl/fl}$ *co-cultures; eight 10x image fields counted per treatment group.*	*-39.73 to 36.81*
af	*Linear regression*	*Four vehicle- and five LDN-treated animals; six technical replicates per animal, 262 individual values for vehicle- and 259 individual values for LDN-treated mice.*	*Vehicle:* *slope value: 0.15±0.011, 95% confidence interval: 0.13 to 0.17.* *LDN:* *slope value: 0.15±0.0095, 95% confidence interval: 0.13 to 0.17.*

304

Results

LDN-193189 infusion promotes remyelination following cuprizone-induced demyelination in vivo

To investigate the influence that BMP4/BMP4 Type I receptor (BMPRI) signalling exerts upon remyelination, we subjected C57/BL6 mice to cuprizone-induced demyelination as published previously (Sabo et al., 2011). Mice were fed cuprizone for 5 weeks to induce demyelination in several white matter tracts of the brain including the corpus callosum (CC). Following cuprizone withdrawal, mice were infused with either LDN-193189 (400ng/day), a previously characterised inhibitor of BMPRIA and BMPRIB receptor signaling (Boergermann, Kopf, Yu, & Knaus, 2010), or vehicle (0.1% DMSO in aCSF) for seven days and allowed to recover. A parallel cohort of control mice were fed cuprizone and sacrificed at the end of 5 week period (with no recovery) to assess the extent of demyelination.

The extent of demyelination in the medial caudal corpus callosum of 5-week cuprizone-fed mice (no recovery), and the extent of remyelination in cuprizone-fed mice following seven days infusion with vehicle or LDN-193189 after cuprizone withdrawal was assessed three ways. We first performed immunohistochemical analysis of the myelin protein marker MBP, as an indicator of myelination. Unchallenged age-matched mice were used as healthy controls to assess the basal level of myelination. Whilst there were clear qualitative effects on MBP staining following Cuprizone exposure (Figure 1A, top panels) and LDN infusion (Figure 1A, bottom panels), assessment of the intensity of MBP staining revealed no significant difference between the groups (Figure 1B). This could be due to the presence of myelin debris (positive for MBP) after cuprizone-induced demyelination. We did observe a trend difference between vehicle- and LDN-infused mice following one week of recovery from cuprizone, but this was not significant (Figure 1B, right histogram, $p=0.21^{a0}$). We next used spectral confocal reflection (SCoRe) imaging to assess the extent of remyelination. SCoRe imaging is a label-free (antibody-free) technique allowing for high-resolution quantitative *in vivo* imaging of substantial areas of myelinated white matter tracts such as the CC (Schain et al., 2014). Using the SCoRe imaging, at the end of five weeks of cuprizone feeding, we observed a significant reduction (>10 fold) in the percentage of myelinated area in the corpus callosum of cuprizone-fed mice compared to healthy control mice (Figure 1C top panels, quantified in D,

p=0.0047[a]). When assessing the one-week recovery groups, we found that mice infused with LDN-193189 for 7 days showed a significant increase (~2 fold) in the myelinated area compared to vehicle infused control mice (Figure 1C bottom panels, quantified in D, p=0.014[b]), indicating a greater extent of remyelination.

To ascertain the effect of LDN-193189 on the extent of remyelination and ultrastructure of myelinated axons, sagittal sections of caudal corpus callosum were assessed using transmission electron microscopy (TEM). Comparing the raw counts of total myelinated axons per image field in the corpus callosum of mice treated with either LDN-193189 or vehicle revealed a trend increase in the percentage of myelinated axons compared to the control group (Figure 1E, quantified in G, p=0.20[b1]). However, when we compared g-ratios (as an indicator of myelin thickness), both the average g-ratio (Figure 1H) and distribution of g-ratios relative to axonal diameter (Figure 1F), were greater in mice infused with LDN-193189 compared to vehicle-infused controls, indicative of thinner myelin (Figure 1F,H, p=0.016[b2]). Thinner myelin sheaths are likely to have been recently myelinated after a demyelinating insult, as they have not completed the full number of wraps around the axon compared to myelin sheaths formed during development (Franklin, Zhao, Lubetzki, & others, 2013). Importantly, analysis of the number of myelinated axons grouped by the range of g-ratios demonstrated that the LDN-infused group had significantly more myelinated axons with g-ratios greater than 0.81 (Figure 1I, p=0.035[b3]) compared to the control group, indicative of more axons with thin myelin sheathes. Therefore, our EM results together with the SCoRe imaging data collectively suggest that LDN infusion significantly enhances the extent of remyelination, resulting in more remyelinating axons than the control group.

LDN-193189 infusion promotes oligodendrocyte differentiation following demyelination in vivo

Having shown that infusion of LDN-193189 significantly enhanced the extent of myelin repair *in vivo*, we next sought to determine the effect that infusion exerted upon oligodendroglial populations. To address this, we assessed the number of OLIG2+ oligodendroglia as well as the proportion of OLIG2+/CC1+ mature oligodendrocytes and OLIG2+/PDGFRα+ OPCs in the medial caudal corpus callosum (Figure 2). As

359 expected, there was significantly fewer OLIG2+ oligodendroglial cells in the corpus callosum of mice treated
360 with cuprizone for five weeks versus control mice (Figure 2A, quantified in B, p=0.030[b4]). Interestingly,
361 there was no significant difference in the number of OLIG2+ cells between the vehicle- and LDN-treated
362 mice (Figure 2A, quantified in C, p=0.26[b5]), suggesting that LDN infusion does alter the overall number of
363 oligodendroglial lineage cells during remyelination. Consistent with previous studies (Chari & Blakemore,
364 2002; Keirstead, Levine, & Blakemore, 1998), there was a significant reduction in the proportion of
365 OLIG2+/CC1+ mature oligodendrocytes at the peak of demyelination (five weeks of cuprizone) compared to
366 non-cuprizone challenged healthy control mice (Figure 2A, quantified in D, p=0.0002[c]), which is
367 accompanied by a significantly higher proportion of OLIG2+/PDGFRα+ OPCs (Figure 2A, quantified in F
368 p=0.0025[d]). Interestingly, after one week of recovery following cuprizone withdrawal, LDN-193189-infused
369 mice had a significantly higher proportion of OLIG2+/CC1+ mature oligodendrocytes compared to the
370 vehicle infused mice (Figure 2A, quantified in E, p=0.0059[e]). This is accompanied by fewer OPCs in these
371 animals compared to the vehicle control group (Figure 2A, quantitated in G, p=0.016[f]). Thus, our results
372 show that blocking BMP4/BMPRI signaling enhances the differentiation of OPCs into mature
373 oligodendrocytes during remyelination *in vivo*. Coupled with the SCoRe and TEM analysis, it suggests that
374 inhibiting BMPRIA/B signalling with LDN-193189 leads to a greater number of OPCs contacting axons,
375 differentiating, and forming new myelin; this subsequently leads to a greater number of axons with high g-
376 ratios, indicative of remyelination.
377
378 It has been previously identified that exogenous BMP4 promotes astrogliogenic effect *in vitro* and *in vivo,*
379 whereas blocking its signaling inhibits this effect (Grinspan et al., 2000; Sabo et al., 2011; See et al., 2004).
380 Thus we next investigated whether LDN-193189 infusion also affected astrocytes (Figure 3A,C).
381 Immunostaining of caudal corpus callosum sections of normal control mice for GFAP showed a low level of
382 positive immunostaining. As astrocytes can both proliferate and ramify in response to injury (Williams,
383 Piaton, & Lubetzki, 2007), we assessed the integrated density of GFAP fluorescence of the section, and
384 observed a substantial increase in GFAP immunofluorescence signal at the end of 5-weeks cuprizone
385 feeding compared to healthy control (Figure 3A, quantified in C). This is expected, as astrogliosis is

observed from three to four weeks after cuprizone (Hibbits, Yoshino, Le, & Armstrong, 2012). Interestingly, the administration of LDN-193189 resulted in no significant effect upon GFAP immunofluorescence intensity compared to the vehicle control (Figure 3A, quantified in C[g], p=0.85), suggesting that blocking BMP4/BMPRI signaling via LDN-193189 infusion exerted little influence upon astrogliosis during remyelination *in vivo.*

As microglia represent a considerable proportion of cells in the corpus callosum during cuprizone-induced demyelination (Gudi, Gingele, Skripuletz, & Stangel, 2014), we then assessed whether LDN-193189 infusion affected microglia by quantifying the degree of IBA+ immunofluorescence in the corpus callosum (Figure 3B, D). As expected, there is a significant increase in the integrated density of IBA-1 immunofluorescence in the caudal corpus callosum of mice at the peak of demyelination (following 5 weeks of cuprizone) compared to healthy controls (Figure 3B, quantified in D, $p<0.0001$[h]), indicating a dramatic increase in the inflammatory response to demyelination. However, there was no significant difference in the integrated density of IBA-1 immunofluorescent between mice infused with LDN-193189 and vehicle following one week of recovery (Figure 3B, quantified in D, $p=0.61$[i]), suggesting that LDN-193189 exerted no significant influence upon microglia during remyelination *in vivo.* Taken together, these data suggest that blocking BMP4/BMPRI signaling in the murine cuprizone model of demyelination exerts little effect upon either astrocytes or microglia, but rather selectively enhances OPC differentiation to promote myelin repair *in vivo*.

Inhibiting BMP4/BMPRI signaling promotes oligodendroglial differentiation and myelination in vitro

The *in vivo* data suggest that LDN-193189 is exerting its effects selectively upon OPC differentiation to promote remyelination. To further establish whether LDN-193189 mediates its pro-myelinating effect directly upon oligodendroglia, we used an *in vitro* OPC monocultures and myelinating co-cultures to examine the effect of BMP4 and LDN-193189 on OPC differentiation and myelination, respectively. To assess differentiation, isolated primary mouse OPCs were exposed to T3 to initiate differentiation, in the presence of either LDN-193189, BMP4, both (LDN+BMP4, with BMP4 being added after 30 minutes after

LDN-193189) or vehicle for 72 hours (Figure 4). The majority (~70%) of vehicle-treated OPCs differentiated into MBP+ mature oligodendrocytes (Figure 4A, quantified in B, E), characterised by a flat morphology as the cells extended their developing myelin sheath across the 2D surface of the coverslip. This contrasted with the immature phenotype, where the processes of differentiating oligodendrocytes have extended, but have not begun spreading out and fusing. Concordant with previous studies (Grinspan et al., 2000; Mabie et al., 1997), OPCs treated with BMP4 primarily (~70%) differentiated into GFAP+ astrocytes compared to vehicle control OPC cultures (Figure 4A, quantified in B, C, $p<0.0001^{j}$). While LDN-193189 treatment alone did not significantly influence OPC differentiation at the basal level, it significantly blocked the astrogliogenic effect that BMP4 exerted upon the OPC cultures, as evidenced by significantly more oligodendrocytes (both immature and mature phenotypes) in LDN plus BMP4 treated cultures than BMP4 alone cultures (Figure 4A, quantified in B, D, E, $p<0.0001^{k,l}$). These data demonstrate that blocking BMPRI signaling in OPCs reduces the astrogliogenic effect of BMP4 and promotes the differentiation of OPCs into mature oligodendrocytes, suggesting that BMP4 signals via BMPRI receptors in OPCs to exert an inhibitory effect upon oligodendrocyte differentiation.

We next assessed whether the effect that LDN-193189 exerts on potentiating oligodendrocyte differentiation also enhances myelination utilizing the well-established DRG neuron / OPC myelinating co-culture assay (Xiao et al., 2010) (Figure 5). Consistent with a previous report (See et al., 2004), there is significantly fewer MBP+ myelinated axonal segments (~ 3 fold reduction) in exogenous BMP4-treated co-cultures compared to vehicle treated control co-cultures (Figure 5A-B, $p<0.0001^{m}$), suggesting that BMP4 inhibits myelination *in vitro*. Importantly, this BMP4-induced inhibitory effect upon myelination is blocked by pre-treatment with LDN-193189 prior to BMP4 exposure (Figure 5A-B, $p<0.0001^{n}$), suggesting that BMP4 signals via BMPRI to exert this inhibitory effect. Interestingly, LDN-193189 treatment alone also resulted in a significant increase in number of myelinated segments compared to baseline vehicle controls (Figure 5A-B, $p=0.019^{o}$), suggesting there is some endogenous BMP4 present in the co-cultures. Together, our results suggest that BMP4 signals via BMPRI in OPCs to inhibit their differentiation into mature oligodendrocyte and subsequent myelination.

Inhibiting BMP4/BMPRI signaling in OPCs alters the expression of the transcriptional repressor Id4

Previous research strongly suggests that BMP4 inhibits the differentiation of oligodendrocyte-lineage cells by upregulating Id4, a transcription factor that inhibits oligodendrocyte differentiation (Samanta & Kessler, 2004). To understand whether the effect observed on OPC differentiation and myelination was mediated, at least partially, by Id4, we used quantitative real-time polymerase chain reaction (q-RT-PCR) to examine changes in transcription levels of *Id4* as well as *Gfap*, *Mbp* and myelin regulatory factor (*Myrf)* in OPCs treated with BMP4 and/or LDN-193189. To do this, we repeated the differentiation assay in OPC monocultures in the presence or absence of LDN-193189 and BMP4 over various time points (Figure 6). We found there was a significant increase in the level of *Id4* transcription in BMP4-treated OPCs compared to control untreated cultures at 2 hours (~5-fold, Figure 6A, $p<0.0001^{p}$) which peaked at 24 hours (~ 6 fold, Figure 6A, $p<0.0001^{q}$). Interestingly, this BMP4-induced increase in *Id4* transcription is abolished by pre-treatment with LDN-193189 at both the 2-hour and 24-hour timepoint (Figure 6A, $p=0.0014^{r}$ (2-hr), $p<0.0001^{s}$ (24-hr)). BMP4 treatment also led to a significant increase in *Gfap* transcription at 24 hours (Figure 6B, $p=0.014^{t}$), which was attenuated by the pre-treatment with LDN-193189, but not significantly (Figure 6B, $p=0.094^{u}$). Further, BMP4 treatment significantly reduced the expression of *Mbp* and *Myrf* transcripts at the 24 hour mark compared to vehicle treated cultures (Figure 6C-D, $p=0.016^{v}$ (*Mbp)*, $p=0.0053^{w}$ (*Myrf)*). Collectively, these data suggest that BMP4 signals to BMPRI in OPCs to upregulate *Id4*, coinciding with an increase in *Gfap* transcription and downregulation of myelin genes *Mbp* and *Myrf*.

We further explored the downstream transcriptional effects that BMP4 and LDN-193189 exerted upon OPCs utilising the RT2 PCR Profiler Array Kit measuring the transcription of 84 genes related to the TGF-β/BMP signaling family. To address this, OPC monocultures were treated with either LDN-193189, BMP4, both (LDN+BMP4) or vehicle and allowed to differentiate for 24 hours before RNA analysis. We compared changes in transcription within three comparisons: i) control OPCs versus BMP4-treated OPCs; ii) control

OPCs versus LDN-193189-treated OPCs; iii) BMP4-treated OPCs versus LDN-193189+BMP4-treated OPCs. A summary of genes with a significant fold regulation of greater than two is presented in Table 2[x]. We found that BMP4-treated OPCs significantly increased transcription of several TGF-β target genes, as well as *Id1* and *Id2*. Interestingly, genes of several BMP signaling regulatory proteins such as noggin, BAMBI and BMP binding endothelial regulator (BMPER) were also upregulated, suggesting the possibility that exogenous BMP4 treatment of OPCs also activates intrinsic self-feedback mechanisms to modify levels of BMP4 signaling. The transcription of *Bmp4* itself was downregulated by exogenous BMP4 treatment in OPCs. Interestingly, BMP4 treatment significantly upregulates *Smad1* but not *Smad5*; this is reversed in OPC cultures pre-treated with LDN-193189 prior to BMP4 exposure. *Smad2,* which is not typically used by BMP4 (Miyazono, Kamiya, & Morikawa, 2009), was also downregulated, suggesting that *Smad5* may also be similarly unused by BMP4 in OPCs.

Furthermore, we found that OPCs treated with LDN-193189 significantly downregulated *Id1* and *Id2*, as well as levels of the BMP antagonist noggin. Pre-treatment of OPCs with LDN-193189 prior to BMP4 exposure reversed the transcriptional levels of several genes differentially regulated by BMP4 treatment, including *Bmper, Bambi,* and *Emp1.* Levels of *Id1* and *Id2* were not significantly downregulated as a result of LDN-193189 pre-treatment, in contrast with decreased *Id4* transcription in OPC cultures pre-treated with LDN-193189 prior to BMP4 exposure (identified by an individual *Id4* q-RT-PCR). Taken together, the data suggest that BMP4 inhibits OPC differentiation and their subsequent capacity to myelinate axons via signaling through BMPR1 and regulating an array of downstream signalling molecules and transcription factors in OPCs.

Deleting OPC-expressed BMPRIA receptors promotes differentiation and myelination in vitro

LDN-193189 is known to disrupt BMP4 signaling by inhibiting both BMPRIA and BMPRIB, and while mouse OPCs express both BMPRIA and BMPRIB; BMPRIA is expressed at a substantial higher level than BMPRIB (Zhang et al., 2014). Thus, it remains unclear whether the aforementioned effect of LDN-193189 upon OPC differentiation and myelination are mediated via BMPRIA or BMPRIB or both. Further, it also remained

possible that BMP4 signaling in neurons may influence myelination in the co-culture setting. To unequivocally determine whether BMP4 selectively signals to BMPRIA in OPCs to regulate their differentiation and myelination, we adopted a genetic approach, specifically deleting BMPRIA from OPCs. The BMPRIA KO mice are embryonic lethal: thus, we generated *Pdgfra-CreER*^{T2}::*Bmpr1a*$^{fl/fl}$ conditional KO mice allowing 4OHT-dependent Bmpr1a deletion in *Pdgfra*-expressing OPCs. We firstly confirmed 4OHT-mediated knockout of *Bmpr1a* in primary OPCs using PCR. OPCs were isolated from *Pdgfra-CreER*^{T2}::*Bmpr1a*$^{fl/fl}$ (Cre[+]) and *Bmpr1a*$^{fl/fl}$ control (Cre[-]) mice, treated with 4OHT followed by RNA extraction. PCR analysis confirmed expression of Cre-recombinase in the 4OHT-treated cells, as well as deletion of exon 2 of the Bmpr1a sequence (Figure 7F, ΔBMPRIa panel), while Bmpr1b transcription was unaffected. Analysis of 18S confirmed similar levels of RNA were analysed (Figure 7F, 18s panel).

To investigate the effect that BMPRIA signaling exerts on OPC differentiation, cells were isolated from Cre[+] and Cre[-] control mice and exposed to 4OHT or vehicle for 24 hours, followed by a 72 hour differentiation assay in the presence or absence of BMP4. Cultures were assessed for the proportion of postmitotic oligodendrocytes and astrocytes via immunostaining for MBP and GFAP, respectively (Figure 7A). Consistent with previous results (Figure 4), in the control condition, the majority (>60%) of OPCs differentiated into mature myelinating oligodendrocytes after 72 hours at the basal level (Figure 7A, quantified in B, D, E). As expected, exogenous BMP4 significantly inhibited OPC differentiation compared to the vehicle control, with the vast majority (~80%) of cells being GFAP+ astrocytes in BMP4 alone treated cultures after 72 hours (Figure 7A, quantified in B, C, $p<0.0001^{y}$). Treatment with 4OHT exerted no effect upon the proportion of oligodendrocytes (Figure 7A, quantified in B, D, E, $p=0.711^{z}$) or astrocytes (Figure 7A, quantified in B, C, $p>0.999^{aa}$), but importantly it resulted in significantly more oligodendrocyte differentiation and less astrogliogenesis following BMP4 treatment (Figure 7E, $p=0.011^{ab}$), potentiating astrogliosis (Figure 7B, D, E, $p<0.0001^{ac}$). These results collectively suggest that BMP4 signals via BMPRIA within OPCs to inhibit their differentiation.

To investigate whether BMPRIA also mediates the subsequent capacity to myelinate axons, we repeated

the myelinating co-cultures containing OPCs isolated from Cre[+] and Cre[-] control mice. Co-cultures were exposed to 4OHT or vehicle for 24 hours and maintained for 14 days followed by immunocytochemical and biochemical analyses of myelination *in vitro.* We found that, in co-cultures containing BMPRIA-null OPCs (isolated from Cre[+] mice), 4OHT treatment resulted in significantly more MBP+ myelinated axonal segments compared to vehicle treated control cultures (Figure 8A-B, $p=0.0098^{ad}$). Concordant with this, western blot analysis of myelin proteins MBP and MOG show there was qualitatively more myelin protein expression in 4OHT-treated co-cultures compared to vehicle controls (Figure 8C). In contrast, 4OHT exerted no effect upon myelin formation in co-cultures containing OPCs from *Bmpr1a*$^{fl/fl}$ control (Cre[-]) mice (Figure 8D-E, $p=0.92^{ae}$). Together, our data suggest that selectively blocking BMP4 signaling in OPCs through ablating BMPRIA promotes oligodendroglial differentiation and reduces astrogliogenesis, and leads to a greater extent of myelination *in vitro*, indicating that BMP4 selectively signals via BMPRIA in OPCs to block oligodendroglial differentiation and myelination.

Discussion

Identifying the mechanisms that inhibit oligodendrocyte differentiation and remyelination is crucial for developing future strategies that directly target myelin repair in MS. Here we have identified that inhibiting BMP4/BMPRI signaling following cuprizone-induced central demyelination significantly enhances oligodendroglial differentiation and promotes myelin repair in the brain *in vivo*. We have further determined that BMP4 signals to BMPRIA receptors in OPCs to inhibit oligodendrocyte differentiation and myelination *in vitro*. Together, results of this study identify that inhibiting BMP4/BMPRIA signaling within OPCs promotes CNS remyelination via potentiating oligodendrocyte differentiation, and that blocking this pathway within OPCs is a potential strategy to enhance remyelination.

Disrupting BMP4/BMPR1 signaling promotes remyelination via potentiating oligodendrocyte differentiation in vivo

Results of this study strongly support a role for blocking BMP4/BMPR1 receptor signalling in promoting CNS remyelination. BMP4/BMPRI signaling is upregulated during the remyelinating phase after myelin injury

548 (Cate et al., 2010) and blocking BMP signalling via noggin significantly enhances remyelination following
549 demyelination *in vivo* (Sabo et al., 2011). Whilst these studies firmly identify BMP signalling as refractory to
550 remyelination, the fact that noggin promiscuously inhibits multiple BMPs, and thus signalling through
551 several receptor classes, ultimately means the molecular mechanisms mediating this effect remain to be
552 elucidated. In this study, we took advantage of pharmacological developments in small molecule inhibitors
553 of the TGF-β signaling pathway and adopted an approach more specific to BMP4/BMPRI signalling (Cuny et
554 al., 2008). LDN-193189 primarily inhibits BMPRIA and BMPRIB, with some inhibition of ACVRL1 (ALK1) and
555 ACVR1 (ALK2) demonstrated in C2C12 osteoblast and chondroblast cell lines(Boergermann et al., 2010).
556 The mechanism of inhibition involves competitive binding of the compound to the kinase domain of the
557 Type I subunits, preventing phosphorylation of downstream SMAD molecules and restricting the signalling
558 cascade (Boergermann et al., 2010). Concordant with previous studies (Karni, Amir Levi, Urshansky, &
559 Bernadet-Fainberg, 2013; Sabo et al., 2011), here we have shown that inhibiting BMP4/BMPRI signalling
560 with LDN-193189 significantly increased remyelination after central demyelination. This beneficial effect is
561 achieved via selectively promoting oligodendrocyte differentiation, as evidenced by significantly more
562 mature oligodendrocytes after LDN-193189 administration whereas the number of other glial cells such as
563 astrocytes and microglia remained unchanged. This is also supported by the analysis of cultured primary
564 OPCs, in which LDN-193189 significantly potentiated oligodendrocyte differentiation and their subsequent
565 myelination, and importantly, blocked the astrogliogenic effect of BMP4 on OPCs.
566
567 It is interesting that LDN-193189 did not exert any significant effect upon astrocytes during remyelination *in*
568 *vivo,* whereas in our previous studies noggin infusion significantly inhibited the proliferation of GFAP+
569 astrocytes (Sabo et al., 2011; Wu et al., 2012). One consideration regarding the different astroglial effect is
570 likely the timing of infusion. In this study, LDN-193189 was administered following a five-week cuprizone
571 challenge (first week after cuprizone withdrawn) to assess its effect upon early myelin repair. However, in
572 our previous studies, noggin was infused into the murine corpus callosum during the final third of a six-
573 week cuprizone challenge, when there is ongoing demyelination (Sabo et al., 2011; Wu et al., 2012). Thus,
574 the role of BMP4 in relation to astrocytes may be proliferative in the context of acute CNS injury and be

575 more apparent earlier in disease course. Potentially, astrocyte proliferation and gliosis may be modified by
576 inhibiting BMP4 signaling activity at specific stages during demyelination and remyelination. Collectively,
577 the results of this study, together with our previously published work, indicate that the major role of BMP4
578 is promoting astrogliogenesis/astrocyte proliferation when there is active demyelination, but having
579 relatively little effect upon astrogliosis during remyelination following CNS injury. Additionally, the
580 differential effects of noggin and LDN-193189 on OPCs may be due to the broader inhibitory effect of
581 noggin. During remyelination, we found that LDN-193189 inhibition of BMP4/BMPRI/SMAD signalling
582 selectively promotes OPC differentiation but has no effect upon the generation of astrocytes. In contrast,
583 studies using noggin to inhibit the generation of astrocytes may be achieving this through by inhibiting
584 other BMP signalling pathways. This also supported by *in vitro* evidence, in which noggin inhibits astroglial
585 production *in vitro* (Grinspan et al., 2000), whereas in this study, we found LDN-193189 or deleting BMPRIA
586 receptor exerted little effect upon astrocytes in OPC cultures where exogenous BMP4 is absent (although
587 this may also be due to subtleties in culturing conditions). Together, our results together with previous
588 data suggest that the influence that BMP4 signalling effects upon OPCs is context dependent, promoting
589 astrogliogenesis when there is active demyelination while inhibiting OPCs differentiation during
590 remyelination following CNS injury.
591
592 ***BMP4 signals via BMPRIA in OPCs to inhibit oligodendrocyte differentiation and myelination***
593 Consistent with previous studies (Grinspan et al., 2000; See et al., 2004), we found that exogenous BMP4
594 promoted the majority of OPCs to differentiate into GFAP+ expressing astrocytes, while inhibiting
595 BMP4/BMPRI signalling using LDN-193189 prior to BMP4 exposure is sufficient to block this effect and
596 enhance myelination *in vitro*. Transcriptional analysis of OPCs revealed that LDN-193189 significantly
597 downregulated the expression of *Id* family genes including *Id4*, which strongly inhibits oligodendrocyte
598 differentiation *in vitro* (Samanta & Kessler, 2004). The resulting culture environment was such that
599 astrogliogenesis was mostly inhibited, but residual BMP4 signaling activity prevented full differentiation of
600 OPCs into mature oligodendrocytes. It is speculated this may be due to two separate mechanisms: an Id4-
601 mediated sequestering of oligodendrocyte transcription factor OLIG2, and synergy of BMP4-activated

SMADs with astrogliogenic pathway JaK-STAT. The action of LDN-193189 likely affects both pathways, as BMP4-induced phosphorylation of SMADs occurs upstream of both mechanisms. Different minimum thresholds of SMAD activation for each mechanism may mean that LDN-193189 has varying efficacy for inhibiting the separate effects of BMP4 signaling in OPCs. Here, we note again that *in vivo,* we did not observe decreased GFAP+ immunostaining in mice infused with LDN-193189 following cuprizone challenge in the corpus callosum compared to vehicle-infused mice. Thus, the environmental context in which OPCs are interacting with BMP4 likely influences the specific mechanism of action directing differentiation of these cells**.** Notably, BMP4 treatment *in vitro* exerted a marked inhibitory effect upon the expression of MBP proteins while a relatively less robust effect was observed on MBP transcription. The precise reason behind this relatively different transcriptional and translational regulation of MBP is unclear, but suggests that BMP4 signaling exerts greater influences that target translational regulation of MBP expression. Gene function is ultimately determined by the level of protein expression. In our study, the strong effect that BMP4 exerts upon suppressing MBP protein expression is consistent with its marked influence on inhibiting the differentiation of OPCs into mature oligodendrocytes.

Data obtained from the myelinating co-cultures was in accordance with that obtained from the OPC mono-cultures, with BMP4 decreasing and LDN-193189 increasing myelination respectively. Importantly, LDN-193189 blocked the inhibitory effect of BMP4 upon myelination *in vitro* (See et al., 2004). One interesting observation was the significantly higher number of MBP+ myelinated axonal segments in co-cultures treated with LDN-193189 compared to control. This is likely due to the increased levels of endogenous BMP4 expressed by neurons and OPCs in the co-culture setting. Indeed, OPCs themselves express a high level of BMP4 as they begin to differentiate (Zhang et al., 2014).

Historically, related but individual roles for BMPRIA and BMPRIB have been well-identified in the regulation of various aspects of chondrogenesis and osteogenesis (Lin, Svoboda, Feng, & Jiang, 2016). Precisely understanding the differential influences that BMPRIA and BMPRIB receptor signalling exerts in the context of oligodendrocyte differentiation is a key step towards identifying the most suitable therapeutic targets

for promoting myelination and remyelination. However, the effects of BMP4 signalling on oligodendrocyte differentiation have been inconsistent in the field, largely due to the complexity in the nature of BMP4 signalling, the genetic tools being used to target mixed cell lineages, and a diverse range of age and regions of animals being analysed. Given that global genetic knockout of BMP4 and its receptors is embryonic-lethal (Mishina et al., 1995; Winnier, Blessing, Labosky, & Hogan, 1995), conditional genetic ablation driven by expression of lineage markers offers a more nuanced approach to understanding BMP4 signalling in oligodendrocyte development. Previous to this study, See *et al.* used Cre-*loxP*-mediated transgenic excision of the *Bmpr1a* gene from cells expressing BRN4, a broad neural transcription factor activated in early embryogenesis (See et al., 2007). This was crossed with a conventional *Bmpr1b* KO mouse to generate mice with a *Bmpr1a-Bmpr1b* double KO in the neural tube by E10.5. This leads to loss of BMPRIA/BMPRIB function in all subsequent spinal cord and hindbrain cells, causing several developmental defects and lethality at P0. Cultures of *Bmpr1a-Bmpr1b* double KO OPCs did not display phospho-SMAD1/5/8 immunoreactivity when treated with 50ng/mL of BMP4, suggesting complete loss of the SMAD-dependent BMP4 signalling pathway in these mice. While the number of astrocytes in the spinal cord decreased at P0 compared to controls, disrupted BMP4 signalling through BMPRIA/B does not appear to affect the total number of spinal cord OPCs. Intriguingly, while the number of immature O4+ oligodendrocytes was unchanged, the number of mature oligodendrocytes expressing common myelin proteins including myelin basic protein (MBP) was reduced at P0. Counter-intuitively, this suggests that some level of BMP4 signalling through BMPRIA/B is required for oligodendrocyte maturation in the spinal cord and hindbrain (See et al., 2007), either through a direct effect or in combination with other synergistic pathways regulating oligodendrocyte development. Importantly, the lack of BMP4 signalling did not appear to affect the number of OPCs specified, conflicting with previous research indicating an inhibitory effect on OPC specification from neural stem cells *in vitro* (Gross et al., 1996) and in overexpression studies *in vivo* (Gomes et al., 2003).

A further study by Samanta *et al.* deleted BMPRIA only from neural precursor cells expressing OLIG1 in the neural tube from E13.5, which can differentiate into neurons, astrocytes or oligodendrocytes. This did not

affect the subsequent number of OPCs at birth or P20 (Samanta et al., 2007). However, at P20, there was an increase in mature oligodendrocytes in the BMPRIA KO group; this was at odds with the previous study, where mature oligodendrocytes were reduced at the much earlier timepoint. This study did not discount the possibility of increased compensatory signalling through BMPRIB, as phospho-SMADs 1, 5 and 8 were still detected. A third study by Araya *et al.* deleted *Bmpr1a* in *Emx-1-Cre* expressing NSCs of the murine telencephalon. These cells develop into neurons, astrocytes and oligodendrocytes in the telencephalon, with *Cre* recombination occurring at the peak of neurogenesis but preceding gliogenesis in the mouse. It was found that subsequent astrocytes derived from these NSCs aberrantly expressed vascular endothelial growth factor (VEGF) at P10, leading to the disruption of cerebrovascular angiogenesis as well as impaired blood-brain barrier formation (Araya et al., 2008). Interestingly, while previous studies using *Olig1-Cre*-driven *Bmpr1a* deletion showed increases in mature O4+ oligodendrocytes at P20, no differences in O4+ cells was observed at P20 in this study. In addition, compared to the earlier study deleting both *Bmpr1a* and *Bmpr1b* from BRN4-expressing cells in which GFAP+ astrocytes are reduced, no such decreases were observed here.

In summary, embryonic overexpression of BMP4 before or during gliogenesis clearly decreases subsequent oligodendrogliogenesis; inhibition of BMP4 signalling embryonically using noggin has the opposite effect and increases the number of oligodendrocytes. However, See *et al.* demonstrated that inhibiting BMP4-SMAD signalling by deleting BMPRIA/B prior to OPC specification reduces the number of mature oligodendrocytes at P0. Importantly, this was not due to reduction in the number of OPCs specified, as no changes in the number of OPCs were detected. Additionally, the study by Samanta *et al.* found that reduction, but not complete suppression, of BMP4 signalling through BMPRIA deletion in E13.5 neural precursor cells has no effect on the number of OPCs at P0. However, deleting BMPRIA at E13.5 increases mature oligodendrocyte number by P20. The reasons for this remain unclear. However, observations from all three studies suggest that BMP signalling through BMPRIA/BMPRIB does not play a role in specification of OPCs from NSCs, but has a strong negative effect on subsequent OPC differentiation (See & Grinspan, 2009). Only one study specifically targeted oligodendrocyte lineage cells using an *Olig1-Cre* driver;

683 however, this targets all oligodendrocytes as well as some neuronal populations. Prior to our study
684 presented here, the effect of inhibiting BMPRIA in postnatal, lineage-committed OPCs had not been
685 examined.
686
687 Using a conditional and inducible transgenic approach to specifically ablate BMPRIA expression in OPCs, we
688 have identified that BMPRIA has a critical role in mediating the inhibitory BMP4 signal in OPC lineage
689 progression within the postnatal CNS. We used the *Pdgfra-CreER*^{T2} driver of Cre expression to specifically
690 ablate expression of *Bmpr1a* in postnatally derived OPCs, rather than in neural progenitor cells, or in all
691 oligodendrocyte lineage cells as seen with the more commonly used *Olig2-Cre* driver. As PDGFRα is
692 downregulated in OPCs prior to differentiation (Ellison & de Vellis, 1994; Zhang et al., 2014), BMPRIA
693 expression is ablated prior to the differentiation process occurring. This allowed us to examine the
694 influence of BMPRIA specifically on this process, in the absence of any confounding effects of coincident
695 deletion in mature oligodendrocytes. We found that OPCs with a BMPRIA deletion significantly attenuated
696 the inhibitory effect of BMP4 on OPC differentiation into mature oligodendrocytes, as seen in OPC cultures
697 treated with LDN-193189. Moreover, we found that 4OHT-treated myelinating co-cultures containing
698 BMPRIA KO OPCs showed an increased capacity to myelinate, suggesting that inhibiting BMP4/BMPRI
699 signaling in OPCs promotes the basal level of myelination. Noticeably, the magnitude of the effect of
700 disrupting BMPRIA expression in OPCs was lower than that seen in experiments where the signalling of
701 BMPRI receptors is inhibited pharmacologically using LDN-193189, both at the transcriptional and protein
702 level. For instance, the astrogliogenic effect of BMP4 treatment (as measured by differentiation of OPCs
703 into GFAP-expressing astrocytes) was approximately 25% less in BMPRIA KO OPCs compared to control
704 cultures, in contrast to a near-total reduction in the LDN-193189-treated OPCs. Similarly, a greater number
705 of OPCs differentiated into either immature or mature oligodendrocytes in the LDN-193189-treated OPCs
706 compared to the BMPRIA null OPCs. This differential effect may be due to the latency of the turnover and
707 replacement of functional BMPRIA receptors. The rate of BMP receptor turnover is governed by either
708 clathrin-dependent or caveolin-dependent endocytosis depending on whether the BMP ligand initially
709 binds to the Type I subunit, or to a pre-formed complex of Type I/Type II subunits (Sieber, Kopf, Hiepen, &

Knaus, 2009). Secondly, it is possible that the *Pdgfra-CreER*^{T2} Cre driver used did not generate a full knockout of *Bmpr1a*. We observed residual BMPRIA protein expression in 4OHT-treated DRG/OPC co-cultures using western blotting (although this may have been contributed by DRG neurons). The original study characterizing the *Pdgfra-CreER*^{T2} Cre driver found approximately 45-50% successfully recombination of floxed DNA regions (Rivers et al., 2008). Thus, there is likely to be remaining BMPRIA expression on the OPC cell surface that may have attenuated the observed effect of inhibiting BMPRIA signaling on OPC differentiation. Further, LDN-193189 inhibits BMPRI receptors including BMPRIA and BMPRIB, whereas BMPRIB remains active in BMPRIA KO OPCs. This finding suggests that BMPRIB may also play a role in mediating BMP4-induced inhibitory effect upon oligodendrocyte differentiation and myelination in the postnatal CNS, which warrants future investigation. Both the use of LDN-193189 and OPC-targeted transgenic ablation of BMP receptor subunits may enhance the current state of knowledge regarding the role of BMP4 signalling on embryonic oligodendrocyte development, as detailed above.

In summary, our results show that inhibiting BMP4/BMPRI signaling in OPCs promotes remyelination following myelin injury *in vivo*. This beneficial effect is likely mediated by potentiating OPCs differentiation into mature myelinating oligodendrocytes. Further, we have identified that BMPRIA in OPCs plays a critical role in mediating the inhibitory effect of BMP4 upon OPCs differentiation and myelination. Together, our work presented here indicate that targeting BMP4/BMPRIA signaling in OPCs is a potential strategy for enhancing remyelination following a demyelinating insult.

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Figures and figure legend

Figure 1: Inhibiting BMP4/BMPRI signaling following demyelination promotes remyelination *in vivo*.

(A): Representative MBP IHC images showing myelin protein in the caudal corpus callosi of healthy control (control) and 5-weeks cuprizone-challenged mice (Cuprizone 5w, top panels); and 5 weeks cuprizone-challenged mice followed by one-week recovery with vehicle (Vehicle recovery) or LDN-193189 (LDN recovery) infusion (bottom panels).

(B) Quantification of integrated density of MBP immunostaining. No significant differences were observed between control and cuprizone-fed mice, or between vehicle- and LDN-infused mice.

(C): Representative SCoRe images to identify myelin in the caudal corpus callosi of healthy control (control) and 5 weeks cuprizone-challenged mice (Cuprizone (5w), top panels); and 5 weeks cuprizone-challenged mice followed by one-week recovery with vehicle (Vehicle recovery) or LDN-193189 (LDN recovery) infusion (bottom panels).

(D) Quantification of myelinated area (SCoRe signal that is pixelated) as a percentage of total area measured. The SCoRe signal is significantly reduced in 5 weeks cuprizone-challenged mice (Cuprizone (5w) compared to healthy control (Ctrl) mice, confirming demyelination (**B**). LDN-193189 infused mice display a significantly greater SCoRe signal than the vehicle infused control group (**C**), indicating greater remyelination.

(E): TEM cross-sectional images of caudal corpus callosum axons of 5 weeks cuprizone-challenged mice followed by one-week recovery with vehicle (Vehicle recovery) or LDN-193189 (LDN recovery) infusion.

(F): A scatterplot comparison of g-ratio distribution relative to axonal diameter. LDN-infused mice had a significantly higher average g-ratio than vehicle-infused controls (p=0.016).

(G): Proportion of total myelinated axons in the caudal corpus callosum of vehicle- and LDN-infused mice following five weeks of cuprizone. A trend but non-significant intrend increase was observed in LDN-infused mice compared to vehicle controls.

(H): The average g-ratio of axons in the caudal corpus callosum of vehicle- and LDN-infused mice following five weeks of cuprizone. Mice treated with LDN-193189 after five weeks of cuprizone had more thinly myelinated axons (high g-ratio) in the corpus callosum compared to vehicle-infused mice.

(I): Number of axons in corresponding g-ratio range for vehicle- versus LDN-infused mice following five weeks of cuprizone. EM analysis indicated a higher number of axons with thinner myelin in the LDN-treated group, indicating greater remyelination.

(N=4-6 animals per group for SCoRe, N=3 animals per group for EM, *p<0.05, ****p<0.0001, scale bar for SCoRe images: 50µm, scale bar for TEM images: 2µm).

Figure 2: Inhibiting BMP4/BMPRI signaling following demyelination promotes oligodendrocyte differentiation *in vivo*.

(**A**): Representative micrographs of immunostaining in the caudal corpus callosi of healthy control mice (control), mice subjected to 5 weeks cuprizone (Cuprizone 5w), and mice subjected to 5 weeks cuprizone with either vehicle (Vehicle recovery) or LDN-193189 (LDN recovery) infusion for one week, and immunostained with OLIG2 and either PDGFRα or CC1.

(**B,C**):Analysis of OLIG2+ cell number in healthy control mice (control), mice subjected to five weeks of cuprizone (Cuprizone 5w), mice infused with either vehicle (Vehicle recovery) for one week, or with LDN-193189 (LDN recovery) for one week. As expected, the total number of OLIG2+ cells is significant decreased after five weeks of cuprizone compared to controls.

(**D,E**): Quantification of the proportion of OLIG2+/CC1+ mature oligodendrocytes showing a significant reduction at the end of cuprizone feeding (**D**). LDN-193189-infused mice have a significantly higher prorpotion of mature oligodendrocytes compared to the vehicle control group following one week recovery (**E**).

(**F,G**): Quantification of the proportion of OLIG2+/PDGFRα+ OPCs showing a significant increase at the end of cuprizone feeding (**F**). LDN-193189-infused mice have a significantly small fraction of OPCs (**G**) compared to the vehicle control group following recovery.
(N=4-6 animals per group, *p<0.05, ** p<0.01, ***p<0.001, scale bar for all images: 50μm).

Figure 3: Inhibiting BMP4/BMPRI signaling exerts no influence on astrocytes or microglia *in vivo*
(A-B): Representative micrographs of immunostaining in the caudal corpus callosi of healthy control mice (control), mice subjected to 5 weeks cuprizone (Cuprizone 5w), and mice subjected to 5 weeks cuprizone with either vehicle (Vehicle recovery) or LDN-193189 (LDN recovery) infusion for one week, and immunostained with GFAP (**A**) or IBA1 (**B**).
(C): Quantification of the integrated density of GFAP immunofluorescence. There is no significant change in GFAP immunofluorescence at peak demyelination (Cuprizone 5w) (left panel) or following infusion of LDN-193189 (LDN recovery) for one week compared to control groups (Control, Vehicle) (right panel).
(D): Quantification of the integrated density of IBA-1 immunofluorescence. There is a significant increase in IBA-1 immunofluorescence in the corpus callosum at peak demyelination (Cuprizone 5w) (left panel), however there is no significant difference increase in IBA-1 immunofluorescence between vehicle (Vehicle recovery) or LDN-193189 (LDN recovery) infused during one-week recovery after cuprizone (right panel).
(N=4-6 animals per group, ****p<0.0001, scale bar= 50μm for all images).

Figure 4: BMP4 signals via BMPR1 in OPCs to enhance oligodendrocyte differentiation and reduce astrogliogenesis *in vitro*
(A): Representative micrographs of immunostaining of differentiated OPC cultures for MBP and GFAP under untreated (Control) conditions, or following treatment with BMP4, LDN-193189 (LDN) or both BMP4 and LDN-193189 (LDN+BMP4).
(B): Quantification of cell phenotypic distribution for each condition based on GFAP expression and MBP+ morphology. MBP+ cells were classified as either mature (flattening of branched extracellular membrane) or immature (branched morphology but not fused layers).
(C): Quantification of the proportion of GFAP+ cells in the cultures. BMP4 significantly increased the proportion of GFAP+ cells compared to untreated (Control) cultures. While LDN-193189 (LDN) alone exerted no significant effect, pre-treatment with LDN prior to BMP4 (LDN+BMP4) significantly abrogated BMP4's effect on astrocytes.
(**D**): Quantification of the proportion of immature oligodendrocytes in the cultures. Treatment with BMP4 or LDN-193189 (LDN) exerted no significant effect, whereas pre-treatment with LDN-193189 prior to BMP4 (LDN+BMP4) significantly increased the proportion of immature oligodendrocytes.
(**E**): Quantification of the proportion of mature oligodendrocytes in the cultures. Treatment with BMP4 significantly blocked OPC differentiation, whereas LDN-193189 (LDN) alone exerted no significant effect. Pre-treatment with LDN prior to BMP4 (LDN+BMP4) significantly abrogated BMP4's effect on oligodendrocyte differentiation.
(N=4 animals per group, ****p<0.0001, scale bar for all images: 20μm).

Figure 5: BMP4 signals via BMPR1 in OPCs to promote myelin formation *in vitro*.
(A): Representative micrographs of myelinating DRG/OPC co-cultures treated with vehicle (control), BMP4, LDN-193189 (LDN) or BMP4+LDN-193189 for 14 days and immunostained for MBP and Neurofilament. Arrows indicate MBP+ myelin segments co-labelled with NFL+ axons.
(B): Quantification of the number of MBP+ myelinated axonal segments per field from these co-cultures. BMP4 treatment significantly reduced the number of MBP+ myelin segments compared to co-cultures, which is blocked by the pre-treatment of LDN-193189 (LDN+BMP4). (N=4 independent co-cultures per

997 group, *p<0.05, ****p<0.0001, scale bar for all images: 30µm).
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Figure 6: Inhibiting BMP4/BMPRI signaling in OPCs alters the expression of transcription factor *Id4* and *Gfap*, but not *Mbp* or *Myrf*.

(A-B): Q-RT-PCR analysis of *Id4* and *Gfap* transcript levels from OPCs cultured in differentiation media and treated with LDN-193189, BMP4, both, or vehicle (control) over various time points. BMP4 significantly increased the level of *Id4* transcripts at 2 and 24 hours compared to the control, and this upregulation is blocked by pre-treatment with LDN-193189 prior to BMP4 exposure. *Gfap* expression was also significantly reduced by pre-treatment of OPCs with LDN-193189 prior to BMP4 exposure.

(C-D): Q-RT-PCR analysis of myelin protein gene *Mbp* and key myelination transcription factor *Myrf* from OPCs treated with LDN-193189, BMP4, both, or vehicle over 24 hours. BMP4 significantly reduced the expression level of both *Mbp* and *Myrf* genes, with this effect reduced by LDN-193189 pre-treatment. (N=3 independent cultures per group, *p<0.05, **p<0.01).

Figure 7: BMP4 signals via BMPR1A in OPCs to potentiate oligodendrocyte differentiation and reduce astrogliogenesis *in vitro*

(A): Representative micrographs of immunostaining of differentiated OPC cultures (isolated from *Pdgfra-CreER^{T2}::Bmpr1a$^{fl/fl}$* mice) for MBP and GFAP under untreated (Control) conditions, or following treatment with BMP4, 4-Hydroxytamoxifen (4OHT) or both BMP4 and 4OHT (+4OHT+BMP4).

(B): Quantification of cell phenotypic distribution for each condition based on GFAP expression and MBP+ morphology as described above (see Figure 4B).

(C): Quantification of the proportion of GFAP+ cells in the cultures. BMP4 significantly increased the proportion of GFAP+ cells compared to untreated (Control) cultures, whereas 4OHT alone exerted no significant effect. Pre-treatment with 4OHT prior to BMP4 (+4OHT+BMP4) significantly attenuated BMP4's effect.

(D): Quantification of the proportion of immature oligodendrocytes in the cultures. Treatment with BMP4, 4OHT, or both BMP4 and 4OHT (+4OHT+BMP4) exerted no significant effect.

(E): Quantification of the proportion of mature oligodendrocytes in the cultures. Treatment with BMP4 significantly decreased OPC differentiation whereas 4OHT alone exerted no significant effect. Pre-treatment with 4OHT prior to BMP4 (+4OHT+BMP4) significantly attenuated BMP4's inhibitory effect on OPC differentiation.

(F): PCR analysis of 4OHT-treated OPCs to assess *Bmpr1a* knockout. *Pdgfra-CreER^{T2}::Bmpr1a$^{fl/fl}$* and Cre[-] OPCs were isolated and treated with 4OHT for 24 hours and analysed for transcription of a sequence corresponding to *Bmpr1a-ex2*, rendering the resulting protein untranscribable. (N=4 animals per group, *p<0.05, ****p<0.0001, scale bar for all images: 20μm).

Figure 8: BMP4 signals via BMPR1A in OPCs to promote myelination *In vitro*

(A): Representative micrographs of immunostaining for MBP and Neurofilament (NFL) in myelinating co-cultures containing OPCs isolated from *Pdgfra-CreER^{T2}::Bmpr1a$^{fl/fl}$* mice. The co-cultures were treated with or without 4OHT for 24 hours prior to 14 days of myelination. Arrows indicate MBP+ myelinated axons segments co-labelled with NFL+ axons.

(B): Quantification of MBP+ myelinated axonal segments from these co-cultures. 4OHT-induced BMPRIA ablation in OPCs causes significantly more MBP+ myelinated axonal segments compared to controls.

(C): Western blot analysis of BMPRIA and myelin proteins (MOG and MBP) in sister co-cultures from (A-B), treated with either 4OHT or vehicle. Treatment with 4OHT substantially reduced BMPRIA expression and leads to qualitatively more myelin proteins (MBP and MOG) expression compared to controls.

(D): Representative micrographs of immunostaining for MBP and NFL in myelinating co-cultures containing

OPCs isolated from *Bmpr1a*$^{fl/fl}$ (Cre negative control) mice. The co-cultures were treated with or without 4OHT for 24 hours prior to 14 days of myelination. Arrows indicate MBP+ myelin segments co-labelled with NFL+ axons. **(E):** Quantification of MBP+ myelinated axonal segments from co-cultures. Treatment with 4OHT did not exert a significant effect upon myelination in the Cre- co-cultures.
(N=3 independent cultures per treatment group, **p<0.01, scale bar for all images: 30µm).

Tables

Table 1 – Primer sequences used for q-RT-PCR

Gene name	Forward primer	Reverse primer
18S	5'CGAACGTCTGCCCTATCAACTT3'	5'ACCCGTGGTCACCATGGTA3'
Myelin basic protein (*Mbp*)	5'CCCGTGGAGCCGTGATC3'	5'TCTTCAAACGAAAAGGGACGAA 3'
Glial fibrillary acidic protein (*Gfap*)	5'CGTTTCTCCTTGTCTCGAATGA3'	5'CCCGGCCAGGGAGAAGT3'
Inhibitor of DNA binding (*Id4*)	5'TTTGCACGTTCACGAGCATT3'	5'GCGGTCATAAAAGAAGAAACGAA3'
Myelin regulatory factor (*Myrf*)	5'AAGGAGCTGCCTATGCTCACCT3'	5'GCCTCTAGCTTCACACCATGCA3'
BMPRIA (*Bmpr1a*)	5'TCATGTTCAAGGGCAGAATCTAGA3'	5'GGCAAGGTATCCTCTGGTGCTA3'
BMPRIB (*Bmpr1b*)	5'GCGCACCCCGATGTTG3'	5'CATGTCCCCTAAGAAGCTTTCTG3'
BMPRIA-ex2	5'GTTCATCATTTCTCATGTTCAAACTA3'	5'AATCAGAGCCTTCATACTTCATACACC3'

Table 2 - ***Summary of differentially regulated BMP/TGF-β signaling pathway genes in OPCs cultured in LDN-193189, BMP4, both, or vehicle for 24 hours in differentiating conditions***. (⋆ p<0.05, ⋆⋆ p<0.01, ⋆⋆⋆ p<0.001, ⋆⋆⋆⋆ p<0.0001)

BMP4 versus control			
Gene Name	**Up/down**	**Fold regulation**	**p-value**
Epithelial membrane protein 1 (*Emp1)*	↑	9.19	⋆
Noggin (*Nog)*	↑	6.20	⋆⋆⋆
Growth arrest and DNA-damage-inducible 45 beta *(Gadd45b)*	↑	5.17	⋆
Cyclin-dependent kinase inhibitor 1A *(Cdkn1a)*	↑	5.11	⋆⋆
Transforming growth factor, beta 3 *(Tgfb3)*	↑	4.37	⋆
Jun-B oncogene *(Junb)*	↑	4.27	⋆
Latent transforming growth factor beta binding protein 1 *(Ltbp1)*	↑	3.94	⋆⋆
BMP and activin membrane-bound inhibitor (*Bambi)*	↑	3.84	⋆⋆
BMP-binding endothelial regulator *(Bmper)*	↑	3.40	⋆⋆
Inhibitor of DNA binding 2 (*Id2)*	↑	2.25	⋆
Distal-less homeobox 2 *(Dlx2)*	↑	2.10	⋆⋆

TGFβ-1-induced transcript (*Tgfb1i1)*	↑	2.06	⋆
Inhibitor of DNA binding 1 (*Id1)*	↑	1.94	⋆
FBJ osteosarcoma oncogene *(Fos)*	↑	1.94	⋆⋆
SRY-box containing gene 4 *(Sox4)*	↑	1.87	⋆
Small MAD homolog 1 *(Smad1)*	↑	1.50	⋆
BMP receptor 1A (*Bmpr1a)*	↑	1.54	⋆
Small MAD homolog 5 *(Smad5)*	↓	-1.27	⋆
Signal transducer and activator of transcription *(Stat1)*	↓	-1.35	⋆
TGF-β receptor I *(Tgfbr1)*	↓	-1.60	⋆
Small MAD homolog 2 *(Smad2)*	↓	-1.66	⋆⋆
Small MAD homolog 7 *(Smad7)*	↓	-1.74	⋆
SMAD specific E3 ubiquitin protein ligase 1 *(Smurf1)*	↓	-2.13	⋆⋆⋆⋆
Plasminogen activator, urokinase *(Plau)*	↓	-5.16	⋆⋆
Bone morphogenetic protein 4 *(Bmp4)*	↓	-5.53	⋆

Table 2 (cont'd). ⋆ p<0.05, ⋆⋆ p<0.01, ⋆⋆⋆ p<0.001, ⋆⋆⋆⋆ p<0.0001.

LDN-193189 versus control			
Gene Name	**Up/down** (compared to control)	**Fold regulation** (compared to control)	**p-value**
Epithelial membrane protein 1 (*Emp1)*	↓	-2.07	⋆
Inhibitor of DNA binding 2 (*Id2)*	↓	-3.15	⋆⋆
BMP-binding endothelial regulator *(Bmper)*	↓	-3.63	⋆⋆
Inhibitor of DNA binding 1 *(Id1)*	↓	-3.65	⋆
Noggin (*Nog)*	↓	-6.35	⋆
MDS1 and EVI1 complex locus *(Mecom)*	↓	-22.15	⋆⋆

LDN-193189+BMP4 versus BMP4			
Gene Name	**Up/down** (compared to BMP4)	**Fold regulation** (compared to BMP4)	**p-value**
Bone morphogenetic protein 4 *(Bmp4)*	↑	3.32	⋆
TGF-β receptor I *(Tgfbr1)*	↑	1.69	⋆
Small MAD homolog 5 *(Smad5)*	↑	1.58	⋆
Signal transducer and activator of transcription *(Stat1)*	↑	1.41	⋆

Noggin (*Nog)*	↓	-1.36	⋆
Distal-less homeobox 2 *(Dlx2)*	↓	-1.40	⋆
FBJ osteosarcoma oncogene *(Fos)*	↓	-1.42	⋆
Col1a1	↓	-1.64	⋆
BMP-binding endothelial regulator *(Bmper)*	↓	-2.12	⋆
BMP and activin membrane-bound inhibitor (*Bambi)*	↓	-2.22	⋆
Transforming growth factor beta-1-induced transcript 1 (*Tgfb1i1)*	↓	-2.50	⋆
Cyclin-dependent kinase inhibitor 1A *(Cdkn1a)*	↓	-3.23	⋆⋆
Insulin-like growth factor 1 *(Igf1)*	↓	-3.50	⋆⋆
Jun-B oncogene *(Jun)*	↓	-4.19	⋆
Latent transforming growth factor beta binding protein 1 *(Ltbp1)*	↓	-5.46	⋆⋆⋆
Epithelial membrane protein 1 (*Emp1)*	↓	-9.63	⋆

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