Regulation of Myelination by Exosome Associated Retinoic Acid Release from NG2-Positive Cells

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In the CNS, oligodendrocytes are responsible for myelin formation and maintenance. Following spinal cord injury, oligodendrocyte loss and an inhibitory milieu compromise remyelination and recovery. Here, we explored the role of retinoic acid receptor-beta (RARβ) signaling in remyelination. Using a male Sprague Dawley rat model of PNS-CNS injury, we show that oral treatment with a novel drug like RARβ agonist, C286, induces neuronal expression of the proteoglycan decorin and promotes myelination and differentiation of oligodendrocyte precursor cells (NG2⁺ cells) in a decorin-mediated neuron–glia cross talk. Decorin promoted the activation of RARα in NG2⁺ cells by increasing the availability of the endogenous ligand RA. NG2⁺ cells synthesize RA, which is released in association with exosomes. We found that decorin prevents this secretion through regulation of the EGFR–Ca²⁺ pathway. Using functional and pharmacological studies, we further show that RARα signaling is both required and sufficient for oligodendrocyte differentiation. These findings illustrate that RARβ and RARα are important regulators of oligodendrocyte differentiation, providing new targets for myelination.

Key words: decorin; exosome; myelination; NG2⁺ cells; RARβ; RARα; retinoic acid

Significance Statement
This study identifies novel therapeutic targets for remyelination after PNS-CNS injury. Pharmacological and knock-down experiments show that the retinoic acid (RA) signaling promotes differentiation of oligodendrocyte precursor cells (OPCs) and remyelination in a cross talk between neuronal RA receptor-beta (RARβ) and RARα in NG2⁺ cells. We show that stimulation of RARα is required for the differentiation of OPCs and we describe for the first time how oral treatment with a RARβ agonist (C286, currently being tested in a Phase 1 trial, ISRCTN12424734) leads to the endogenous synthesis of RA through retinaldehyde dehydrogenase 2 (Raldh2) in NG2 cells and controls exosome-associated RA intracellular levels through a decorin–Ca²⁺ pathway. Although RARβ has been implicated in distinct aspects of CNS regeneration, this study identifies a novel function for both RARβ and RARα in remyelination.

Introduction
After spinal cord injury (SCI), there is widespread oligodendrocyte cell death and demyelination. Remyelination is important to restore reliable axonal transmission (Smith et al., 1979) and protect axons from degeneration (Irvine and Blakemore, 2008). When demyelinating lesions occur, newly generated oligodendrocytes (OLs), mostly derived from oligodendrocyte precursor cells (OPCs), can repair or replace damaged myelin (Salgado-Ceballos et al., 1998; Zawadzka et al., 2010). Nevertheless, due to external and intrinsic inhibitory cues, the amount of new myelin is unable to cover all exposed axons, generating a negative myelin balance that increases the number of naked axons and thereby results in degeneration and sensory and motor deficits in residual nerves. The microenvironment after SCI is not particularly favorable to remyelination: chondroitin sulfate proteoglycans (CSPGs) in the extracellular matrix can inhibit remyelination by blocking OPC migration (Siebert and Osterhout, 2011), the glial scar hinders OPC proliferation and migration, and downregulation of nutrients and growth factors also limit remyelination (Meletis et al., 2008; Gauthier et al., 2013; Lukovic et al., 2015). Here, we sought to identify mechanisms and their putative therapeutic targets that can re-
lieve inhibition to myelination and promote proliferation and differentiation of OPCs.

Several studies have shown that retinoic acid receptor-beta (RARβ) activation is beneficial in a variety of CNS injuries (Dmetrichuk et al., 2005; Wong et al., 2006; Yip et al., 2006; Agudo et al., 2010; Puttagunta et al., 2011; Koriyama et al., 2013; Hernández-Pedro et al., 2014; Goncalves et al., 2015, 2018). We have previously shown that stimulation of the RARβ signaling pathway in an animal model of dorsal root avulsion (DRA)-induced recovery of motor and sensory functions and this was accompanied by an increase in remyelination across the dorsal root entry zone (DREZ) (Goncalves et al., 2015).

Here, we show that the RA signaling pathway promotes remyelination in a twofold way: eliminating the inhibitory CSPGs from the extracellular milieu and driving the differentiation of NG2+ cells into myelinating OLs. We found that activation of neuronal RARβ results in the upregulation of decorin. This small leucine proteoglycan that can suppress the core protein levels of the CSPGs at sites of acute rat SCI resulting from suppression of CSPG synthesis and/or a potential increase in CSPG degradation (Davies et al., 2004, 2006). Furthermore, decorin has also been shown to inhibit the activity of transforming growth factor β (TGFβ) (Yamaguchi et al., 1990) and the epidermal growth factor receptor (EGFR) (Csorda’s et al., 2000), both of which regulate the synthesis of axon growth inhibitory CSPGs (Asher et al., 2000; Dobbertin et al., 2003). Additionally, we show that activation of the RARα in NG2+ cells, which is expressed in OPCs (Tripathi and McTigue, 2007; Barnabe-Heider et al., 2010; Hughes et al., 2013), drives their differentiation into myelin-producing oligodendrocytes in vivo. In vitro, deletion of RARα was sufficient to abrogate oligodendrocyte genesis from NG2+ cells. We further show that endogenous RA is essential to drive RARo signaling in vivo because the transduction of a lentivirus overexpressing Raldh2 silencing RNA (shRNA) in RARβ-agonist-treated, avulsed rats prevented myelination. Likewise, treatment with disulfiram, an aldehyde dehydrogenase 2 inhibitor (Lipsky et al., 2001), yielded similar results. RARβ signaling results in the increased expression of Raldh2 in NG2+ cells responsible for the synthesis of RA, which is secreted in association with exosomes during axonal regeneration (Goncalves et al., 2018). Here, we found that decorin reduces intracellular Ca2+ in NG2+ cells cultured in the presence of retinal and this prevents the release of exosome-associated RA. The same effect was seen with an intracellular Ca2+ chelator.

Collectively our results demonstrate the dual decorin-mediated effect of RA signaling in remyelination after SCI and sheds novel insights into the cross talk between RARβ and RARα endogenous pathways. Additionally, we herein identify two therapeutic targets, RARα and RARβ, of interest for the treatment of demyelination.

Materials and Methods

Surgery and drug treatments. All surgeries, behavioral testing, and analysis were performed using a randomized block design and in a blinded fashion. Allocation concealment was performed by having the treatments stocks coded by a person independent of the study. The blinded treatment code for each rat was drawn at random from a hat without replacement. Codes were only broken after the end of the study. All procedures were in accordance with the UK Home Office guidelines and Animals (Scientific Procedures) Act of 1986. All animal care and experimental procedures complied with the Animals (Scientific Procedures) Act of 1986 of the UK Parliament, Directive 2010/63/EU of the European Parliament, and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath and Lilley, 2015).

Animals were housed in groups of three to four in Plexiglas cages with tunnels and bedding, on a 12/12 h light/dark cycle and had access to food and water ad libitum.

For the DRA model, male Sprague Dawley rats weighing 220–250 g (n = 8 per treatment group for each set of experiments) were anesthetized with an intramuscular injection of 60 mg/kg ketamine and 0.4 mg/kg medetomidine and their backs were shaved and aseptically prepared.

After opening the skin and muscle layers, C5–C8 and T1 dorsal roots were cut flush with the spinal cord (SC) surface. The cut ends of the dorsal roots were subsequently introduced through slits in the pia mater and positioned superficially in the SC adjacent to where they had been cut (Goncalves et al., 2015). During the surgery, the rats were placed on a controlled heating pad to maintain temperature at 37 ± 1°C. After the surgery, the rats were hydrated with physiological saline (2 ml, s.c.). Anesthesia was reversed with an intramuscular injection of 0.05 ml (1 mg/kg) atipamezole hydrochloride (Antisedan; Pfizer Animal Health). Animals were kept in a heated recovery box until fully conscious and analgesia (buprenorphine, 0.01 mg/kg, subcutaneously) was given after suturing and recovery. All rats survived this surgery.

For Raldh2 loss-of-function studies, 5 μl of lentivirus (titer: 3.67 × 106 TU/ml) was injected manually at the DREZ of the severed sensory roots using a 20 μl Hamilton syringe at 0.5 μl min−1 and the needle was left in place for 5 min to limit diffusion through the needle tract. Lentiviruses were provided by GeneC opoeia. The shRNA to rat Raldh-2 sequence gatccggcagatagcaagattgcattctcaagaggaatgcaatcttgtctatgcctttttg was ligated into psi-LVRHIGH. The lentiviral particles were then generated by following a standardized protocol using highly purified plasmids and En doFec tin-Lenti and TiterBoost reagents. The LV transfer vector was cotransfected into 293Ta cells with the Lenti-Pac HIV packaging mixture.

Drug treatments for in vivo studies. Rats were treated with: vehicle or the novel selective RARβ agonist C286 (synthesized by S ignature Chemical Services) given by oral gavage 3 times a week for 4 weeks at 3 mg/kg (n = 8 per treatment group) or with the aldehyde dehydrogenase inhibitor disulfiram (10 mg/kg) (Mittal et al., 2014) by intraperitoneal injection 3 times a week for 4 weeks. C286 has a high potency at RARβ (similar potency to all-trans-RA) and behaves as a full agonist, showing a half-maximal effective concentration (EC50) of 1.94 nM at the mouse RARβ receptor, a selectivity for RARβ over RARα of 13.4, and a selectivity for RARβ over RARγ of 5.6-fold.

Animals were perfused after 4 weeks of treatment. Rats were perfused transcardially with heparinized 0.9% NaCl solution and 4% PFA. The cervical cords with attached DRGs were dissected, rapidly removed, and postfixed with 4% PFA for at least 2 d at room temperature. Tissue was then embedded in paraffin wax and 5 μm longitudinal sections cut through each block. Sets of consecutive sections comprising the lesioned area and taken from equivalent regions of the spinal cord were used for immunostaining.

Histological and quantitative analysis of proteins in the rat tissue. Quantification of immunofluorescence CS56, decorin, olig1, myelin-associated protein (MAG), NG2, Raldh2, proteolipid protein (PLP), RARα, and RARβ was done as described previously (Herrmann et al., 2010).

In brief, positively stained areas were quantified as the pixels of immunoreactivity above a threshold level per unit area. The threshold value was set to include fluorescent positive signal and to exclude background staining. Threshold values for a given section and stain remained the same throughout the study. The number of pixels was measured in a 300 μm2 area comprising the DREZ of the implanted severed dorsal roots and the contiguous spinal cord. At least 10 sections per rat were used for these quantifications.

Primary neuronal cell cultures. Mouse primary cortical neurons were prepared as described previously (Goncalves et al., 2013). Cells were plated onto 5 μg/ml poly-d-lysine-coated 100 mm dishes at a density of 6 × 104 cells/dish. Cells were cultured in Neurobasal medium (Invitrogen) containing 2% (v/v) B27 serum-free supplement, 2 mm l-glutamine, 1.5% glucose, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO2/95% air. Cultures
were used after 1 week and were >98% neurons judged by βIII-tubulin staining. Treatments were as follows: mouse primary cortical cultures (0 d in vitro) were treated with vehicle or 10^−7 M C286 in the presence or absence of decorin neutralizing antibody (NAb, 5 μg/ml) for 72 h. The conditioned medium was then collected and added to astrocyte cultures for another 72 h as indicated. Culture conditions were three wells per treatment performed three times independently.

Astrocyte cultures. Primary mixed glial cultures were prepared as described previously (Goncalves et al., 2013) using a modified protocol. Briefly, mixed glial cultures were obtained from the cortices of postnatal day 5 (P5) to P8 mice. Cultures were maintained at 37°C (5% CO2/95% O2) in DMEM/F12 medium containing 15% fetal bovine serum (FBS) (Invitrogen) and 1% penicillin–streptomycin (Sigma-Aldrich) for 10–14 d. Microglial and NG2+ cells were then harvested by forceful shaking for 1 min by hand. After harvesting of microglia and NG2+ cells, done every 3 d for 9 d, 0.25% trypsin EDTA was added to each flask for 5 min. Cells were then centrifuged at 1000 rpm for 5 min and plated on poly-D-lysine-coated 8-well slides in 5% FBS. To obtain reactive astrocytes, cells were treated with 100 ng/ml lipopolysaccharides (LPS) for 24 h (Tarassishin et al., 2014) before any other treatment was initiated. Treatments were as follows: vehicle, C286 (10^−7 M), decorin (20 μg/ml, Sigma D84428), neuronal conditioned media from neurons (that had been treated as indicated), and in the presence or absence of decorin NAb (5 μg/ml). Astrocytes were treated for 72 h, after which cells were fixed and immunostained.

**NG2+ cell cultures.** Primary mixed glial cultures were prepared as described previously (Goncalves et al., 2013) using a modified protocol. Briefly, mixed glial cultures were obtained from the cortices of P5 mice. Cultures were maintained at 37°C (5% CO2/95% O2) in DMEM/F12 medium supplemented with 15% fetal bovine serum (FBS) (Invitrogen, Life Technologies), 9 μM glucose (Sigma-Aldrich), 2 mM L-glutamine (Invitrogen), and 1% penicillin–streptomycin (Sigma-Aldrich) on poly-D-lysine (PDL, 5 μg/ml, Sigma-Aldrich) precoated 75 cm² flasks for 10 d. Microglial cells were then harvested by forcefully tapping at the side of the flasks six times. Medium was collected and centrifuged at 1000 rpm for 5 min. The cells were plated in a 75 cm² flask at 37°C for 30 min to allow microglial cells to attach. Medium was collected again and centrifuged at 1000 rpm for 5 min. Cells were resuspended in NG2 growth medium [DMEM/F12 supplemented with 1% N2, 2% B27, 2 mM L-glutamine, 1% penicillin–streptomycin, 10 ng/ml FGF2 (R&D Systems) and 10 ng/ml PDGF-AA (Cell Guidance Systems)] and plated in PDL-precoated culture vessels at the density of 30,000 cells/cm² for NG2–neuron coculture or 70,000 cells/cm² for siRNA transfection. The purity of the NG2 cultures was confirmed by immunostaining, with >95% of the cells being NG2+ . For the F9-RARE LacZ reporter assay, 99% confluent NG2+ cells were cultured in PDL-precoated 75 cm² Thermo Scientific/Nunc cell culture flasks with Transfection of NG2+ cells. Mouse NG2+ cells were transfected with mouse RARα siRNA with the duplex sequence rGrCrArArGrUrArCrAr CrUrArCrGrArArCrArGrCrGrCTC or scrambled mouse siRNA (Origene) using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, mouse NG2+ cells were plated in PDL-coated culture vessels with density of 70,000 cells/cm² in NG2 growth medium. The next day, culture medium was changed to NG2 medium without penicillin–streptomycin 2 h before transfection; 0.5 μl of Lipofectamine 2000 transfection reagent was used for every 6.25 pmol of siRNA. Six hours after transfection, cells were applied to different treatment conditions.

**qRT-PCR.** Total RNA was extracted from siRNA-transfected NG2+ cells treated with vehicle using the RNeasy mini kit (Qiagen) 24, 48, and 72 h after transfection. Five hundred nanogram total RNA was used for first-strand cDNA synthesis with high-capacity cDNA reverse transcription kit (Applied Biosystems). qRT-PCR was performed using LightCycler 480 SYBR Green I master mixture (Roche) and gene-specific primers on a LightCycler 480 II (Roche). Primers used were as follows: forward 5′-CTGATTACAAATGTTGGAAGT-3′, reverse primer 5′-GACTCCAGCAGACTCAGC-3′ for RARα, forward primer 5′-AGATAGTACCCGCCCTC-3′, reverse primer 5′-TTCTT CTGGATGCTGTCTGG-3′. Levels of RARα mRNA were normalized to the reference gene GAPDH and relative change in mRNA levels following treatment was calculated using the 2^ΔΔCT method.

**Quantification of immunofluorescence in astrocyte cultures and decorin in neuronal cultures.** Ten random fields per treatment condition were captured using a 63× oil-immersion Achromat objective (Carl Zeiss) and the total pixel values for each antibody immunofluorescence staining (CD44, CSPG, and decorin) were measured using Axiosiovision software. The experimental conditions were done in triplicate and repeated three times independently and the quantifications were done by an operator blinded to the treatments.

**Quantification of immunofluorescence in NG2+ cell cultures.** For quantification of oligodendrocyte-specific protein (OSP), NG2, EGFR, intracellular Ca2+ dye (Fluo-4AM), and RARα in NG2+ cell cultures, high-magnification images were obtained and mean intensity values of immunoreactivity were normalized for DAPI staining in each field. Five fields per culture condition, which were done in triplicate, were taken by a blinded operator and this was repeated independently three times.

**F9-RARE LacZ reporter assay.** Murine F9 embryonal carcinoma cells, which stably express an RARβ2-promoter construct, were used as described previously (Sonneveld et al., 1999). Cells were scored on a blue versus not blue basis and the number or percentage of F9-RARE LacZ-positive cells were determined from five random fields from three independent experiments (Sonneveld et al., 1999).

**Exosomes.** Exosomes were prepared from conditioned media from cell cultures using total exosome isolation (TEI) reagent (Invitrogen, Life Technologies) in accordance with the manufacturer’s instructions. Conditioned media were centrifuged at 2000 × g for 30 min at 4°C to remove cell debris and used to separate extracellular vesicles. Resulting supernatants were mixed with 0.5 volumes of TEI reagent and centrifuged at 10,000 × g for 1 h at 4°C following overnight 4°C incubation. Exosome pellets were twice washed by resuspension in ice-cold PBS followed by centrifugation at 100,000 × g for 1 h at 4°C. Unused intact exosomes were stored at −80°C as PBS suspensions. For F9-RARE-lacZ RA reporter cell experiments, a pool of exosomes isolated from a 75 cm² Thermo Scientific/Nunc cell culture treated flasks with filter caps of 99% confluent NG2+ cells were used.

**Western blotting.** Proteins were separated by SDS-PAGE on 10% (w/v) polyacrylamide gels and then transferred to a 0.45 μm pore size nitrocellulose membrane (BA85; Schleicher and Schuell) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad Laboratories) Nitrocellulose membranes were incubated in blocking solution consisting of 3% (w/v) skimmed milk powder in PBS/Tween-20 (0.1%, w/v) (PBS-T) for 1 h at room temperature, followed by incubation with the appropriate primary antibody diluted in blocking solution overnight at 4°C. Membranes were washed in PBS-T and then incubated with species-specific secondary antibodies in blocking solution for 1 h at room temperature in the dark, after which the membranes were washed as above. Proteins were detected by scanning at 700 and 800 nm using the Odyssey detection system (LI-COR Biosciences).

**Electron microscopy.** Aliquots of 2.5 μl of exosomes were placed on Formvar-coated grids and allowed to settle for 1–2 min without being
allowed to dry. Exosomes were fixed with 2% glutaraldehyde for 5 min, washed 3 times with distilled deionized water, and then contrasted with 1 part 3% uranyl acetate in 9 parts 2% methyl cellulose for 10 min. Visualization of exosomes was by a FEI Tecnai T12 BioTWIN transmission electron microscope fitted with an AMT camera.

**Immunocytochemistry/immunohistochemistry and antibodies.** Immunocytochemistry was performed as described previously (Goncalves et al., 2005). Paraaffin wax-embedded spinal cord tissue was first dewaxed in xylene and 100% industrial methylated spirit (IMS, Sigma-Aldrich), heated in citric acid (10 mM, pH 6) until boiling, and then washed under a running tap for 5 min. The sections were washed 3× for 5 min each in PBS before incubation with primary antibody in PBS/0.02% Tween at 4°C overnight. Primary antibody was removed by washing 3× for 5 min each in PBS. The sections were incubated in the secondary antibody for 1 h at room temperature in PBS/0.02% Tween and then washed in PBS 3× for 5 min. Antibodies used were as follows: mouse monoclonal anti-BII tubulin (Promega, 1:1000, catalog #G7121, RRID: AB_430874); chicken polyclonal anti-GFAP (Abcam, 1:300, catalog #ab4674, RRID: AB_304558); rabbit polyclonal anti-GFAP (DAKO, 1:2500); mouse monoclonal anti-GFAP (Sigma-Aldrich, 1:100, catalog #Z0334, RRID: AB_10013382); rabbit polyclonal anti-RARβ (Santa Cruz Biotechnology, 1:100, catalog #sc-552, RRID: AB_2175379); rabbit polyclonal anti-NG2 (Millipore, 1:100, catalog #05-710, RRID: AB_309925); goat polyclonal anti-aldyde dehydrogenase1A2 (Raldh2) (Santa Cruz Biotechnology, Inc, 1:100, catalog #sc-22591, RRID: AB_1224036); mouse monoclonal anti-CSF (CS-56) (Sigma-Aldrich, 1:100, catalog #C8035, RRID: AB_476879); goat polyclonal anti-decorin (R&D Systems, 1:20, for immunocytochemistry and 5 µg/ml as a neutralizing antibody; catalog #AF143, RRID: AB_354790); rabbit polyclonal anti-decorin (Abcam, 1:50, catalog #ab175404); rat monoclonal anti-CD44 (eBioscience, 1:150, catalog #14-0441-86, RRID: AB_869579); rabbit monoclonal anti-RA (Abnova, 1:300, catalog #PAB15482, RRID: AB_10759405); goat polyclonal anti-GFAP (DAKO, 1:2500, catalog #AB_467248), mouse monoclonal anti-MAG (Abcam, 1:2000, catalog #ab89780, RRID: AB_2042411); goat polyclonal anti-Olig1 (Abcam, 1:1000, catalog #ab68105, RRID: AB_1142042); rabbit polyclonal anti-myelin PLP (Abcam, 1:1000, catalog #ab105784, RRID: AB_10973392); goat polyclonal anti-RARα (Abcam, 1:100, catalog #ab28767, RRID: AB_777684); rabbit polyclonal anti-OSP (Abcam, 1:2000, catalog #ab53041, RRID: AB_2276205); rabbit polyclonal anti-RA (Abnova, 1:300, catalog #PAB15482, RRID: AB_10759405); rabbit monoclonal anti-EGFR (Abcam, 1:500, catalog #ab52894, RRID: AB_869579); rabbit polyclonal anti-AIP1/Alx1 (1:1000 for Western blotting, catalog #ABC40, RRID: AB_10806218). Secondary antibodies for immunohistochemistry were Alexa Fluor 594 and Alexa Fluor 488 (1:1000, Invitrogen, Life Technologies) and Alexa Fluor 647 (1:1000, Invitrogen, Life Technologies). DAPI was used to stain nuclei (1 µg/ml, Sigma-Aldrich).

**Confocal microscopy.** Multichannel fluorescence (DAPI–FITC–Texas Red– Cy3 channel) was captured using a Zeiss LSM 700 laser-scanning confocal microscope. For high-magnification images, a 63× oil-immersion Achromat objective (Zeiss) was used. Settings for gain, aperture, contrast, and brightness were optimized initially and held constant throughout each study so that all sections were digitized under the same conditions of illumination. Channels were imaged sequentially to eliminate bleed-through and multichannel image overlays were obtained using Adobe Photoshop version 7.0. Axiomio software was used to collect information on pixel immunoreactivity used for quantitative purposes.

**Experimental design and statistical analysis.** Data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis and Abernethy, 2015). Data analysis was performed in a blinded fashion. Group sizes were estimated based on previous experiments (Goncalves et al., 2015, 2018); eight rats per treatment condition were used for each experiment. All statistical analysis was performed using SigmaStat software (SPSS). Data are expressed as mean ± SEM. Histological data were analyzed using one-way or two-way repeated-measures ANOVA or Student’s t test. Post hoc comparisons were performed where appropriate and all statistical tests are stated in the text. Post hoc tests were run only if F achieved p < 0.05 and when no significant variance in homogeneity was observed. Significance was accepted for p < 0.05. Exact p-values are shown except for ***p ≤ 0.001.

**Results**

**RARB signaling downregulates CSPGs by endogenous upregulation of decorin.** We have used a model of DRA for our experimental in vivo studies. A DRA causes the disruption of the DR and contributing roolets of the DREZ and injury to the dorsal column and horn along the SC segment connected to the avulsed root. This results in neurodegeneration, glial scar formation, and demyelination across the PNS-CNS interface, which is a well delineated area, so this model offers a good pathological template to assess remyelination and the mechanisms involved.

The accumulation of glial-scar-derived CSPGs at the injury site after SCI is a major obstacle to remyelination. CSPGs start being secreted in excess by reactive astrocytes within 24 h of the lesion and can persist for months afterward (Pindzola et al., 1993; Jones et al., 2003). We had previously observed remyelination of regenerated axons across the DREZ in this animal model after stimulation of RARβ signaling for 4 weeks. A possible mechanism whereby this pathway could be promoting remyelination is by CSPG clearance. To assess the effect, if any, of RARβ signaling on CSPGs, avulsed rats were treated orally with vehicle or a novel RARβ agonist C286 (Fig. 1A). Treatment was 3 mg/kg administered 3 times a week from day 2 after injury for 4 weeks because we found that this was the optimal dosing regimen to restore locomotor and sensory functions (Goncalves et al., 2019) that arestringently correlated with remyelination (McDonald and Belegu, 2006). We first analyzed the total CSPG levels determined by immunohistochemistry with CS56 antibody (Avnur and Geiger, 1984; Silver and Miller, 2004) from a 300 µm² area of the DREZ (Fig. 1B, C). Vehicle-treated rats showed intense CS56 immunostaining [fluorescence intensity: 49 ± 3.93 arbitrary units (a.u.)] in the GFAP-enriched lesioned area, but the RARβ-treated rats had significantly lower levels of CS56 (fluorescence intensity: 15.75 ± 5.76 a.u.) (Fig. 1D). To assess the excess of CSPGs in astrocytes in the vehicle-treated rats compared with the rats treated with RARβ agonist, we calculated the ratios of fluorescence intensity of CSPGs/GFAP (Fig. 1E) and found that these were significantly higher in the vehicle-treated group (fluorescence intensity: 0.96 ± 0.35 a.u.) than in RARβ-treated rats (fluorescence intensity: 0.44 ± 0.13 a.u.). The modulation of CSPGs by an RARβ agonist had never been described before, so we next searched for molecular mechanisms that might explain this effect. Because decorin is modulated by RA (Pearson and Sasse, 1992) and is a potent endogenous CSPG scavenger (Esmaeili et al., 2014), we hypothesized that neuronal RARβ, which is upregulated by the RARβ agonist treatment in this animal model (Goncalves et al., 2018), could be inducing decorin. We assessed decorin expression in neurons immunohistologically (Fig. 1F). Quantification of total decorin in the same area used for CS56 analysis (Fig. 1B) was significantly higher in RARβ-treated rats (fluorescence intensity: vehicle, 15.25 ± 1.79 a.u.; RARβ agonist, 52.5 ± 4.17 a.u.). When the levels of BII-tubulin, which were higher in RARβ-agonist-treated rats due to increased regeneration (Goncalves et al., 2015) were taken into account, the ratio decorin/BII-tubulin was still significantly higher upon agonist treatment (fluorescence intensity: vehicle, 0.76 ± 0.31 a.u.; RARβ agonist, 2.2 ± 0.31 a.u.) (Fig. 1G,H), strongly suggesting that neuronal decorin is a major source of the RARβ-induced decorin upregulation.

To underpin a direct effect of neuronal RARβ signaling and decorin, we assayed for decorin expression in RARβ-agonist-
treated neuronal cultures. Treatment with the agonist for 3 d significantly increased the expression of decorin compared with vehicle (fluorescence intensity: vehicle, 8 ± 0.4 a.u.; RARβ agonist, 20.25 ± 1.18 a.u.) (Fig. 1I,J).

Human recombinant decorin has been shown to inhibit inflammation, glial scar formation, and CSPG expression after SCI (Davies et al., 2004). To determine whether the neuronal RARβ-agonist-induced decorin release was sufficient to elicit anti-

**Figure 1.** C286 reduces CSPGs expression and upregulates decorin expression after sensory root avulsion. A, Chemical structure of the novel RARβ agonist C286. B, Representative images of the expression of CSPG (CS-56) and GFAP at the PNS-CNS in injured vehicle- and C286-treated rats. C, Representation of the area of the lesioned DREZ where the molecules of interest were quantified (300 μm²). D, E, Quantification of immunofluorescence intensity of total CS-56 (D) and CS-56 in astrocytes (E) shown in arbitrary units (a.u.) shows that CSPGs levels are significantly lower in C286-treated rats compared with vehicle-treated rats (n = 5 per treatment group, results are mean of fluorescence pixels ± SEM taken from 10 sections per animal, ***p ≤ 0.001, Student’s t test). F–H, Expression of decorin and βIII tubulin (F) and quantification of immunofluorescence intensity of total decorin (G) and decorin in neurons (H) shown in arbitrary units (a.u.) show that decorin is significantly higher in C286-treated rats compared with vehicle treated. Results are mean of fluorescence pixels ± SEM taken from 10 sections per animal and from five rats per treatment group, ***p ≤ 0.001, Student’s t test. C286 induces upregulation of decorin in cultured neurons. I, Expression of decorin in neurons cultured for 72 h with vehicle or C286 (10^-7 M) is significantly higher in the latter (colocalization indicated by white arrows). Scale bar, 20 μm. J, Quantification of decorin expression taken from 10 fields per culture condition from three independent experiments. Data represent mean of fluorescence intensity for decorin in arbitrary units ± SEM, ***p ≤ 0.001, Student’s t test.
inflammatory effects and downregulation of CSPGs in reactive astrocytes, we cultured rat astrocytes and, after 24 h activation with LPS (Tarassishin et al., 2014), treated the cultures for 72 h with either decorin or RARβ agonist or conditioned media from neurons that had been treated with the agonist (RARβ ag-CM) or vehicle (V-CM) in the presence and absence of a decorin NAb (Li et al., 2008) (Fig. 2A). The cultures were then immunoassayed for total CSPGs (CS56) and for CD44, a transmembrane glycoprotein associated with reactive astrocytes (Girgrah et al., 1991). We found that there was a significant reduction of both CS56 and CD44 in the decorin (fluorescence intensity: CS56: 10.25 ± 3.03 a.u.; CD44: 10.12 ± 1.92 a.u) and RARβ ag-CM (fluorescence intensity: CS56: 11.25 ± 2.13 a.u.; CD44: 11.25 ± 0.92 a.u) treated cultures compared with vehicle (fluorescence intensity: CS56: 43.75 ± 2.78 a.u.; CD44: 44.57 ± 2.22 a.u), RARβ agonist (fluorescence intensity: CS56: 34.25 ± 1.65 a.u.; CD44: 34.75 ± 1.98 a.u), or V-CM (fluorescence intensity: CS56: 41.75 ± 3.9 a.u.; CD44: 40.87 ± 2.37 a.u) (Fig. 2B–D). To be certain that the reduction of CS56 and CD44 was a decorin-mediated effect, we repeated the experiment adding a decorin NAb to the cultures to block both exogenous and endogenous decorin because neurons and astrocytes can secrete this proteoglycan (Esmaeili et al., 2014). Remarkably, when the reactive astrocytes were treated with RARβ ag-CM in the presence of the decorin NAb, no significant decrease was seen on CS56 (fluorescence intensity: 40.25 ± 4.47 a.u) nor on CD44 (fluorescence intensity: 41.75 ± 2.37 a.u)

Figure 2. C286 suppresses CSPGs in reactive astrocytes via neuronal release of decorin. A, Illustration of the cell culture treatments: LPS-activated astrocytes were treated with vehicle, decorin (20 μg/ml), or C286 or with conditioned medium from neurons previously treated with vehicle (V-CM) or C286 (C286-CM) in the presence or absence of decorin NAb, which was added to C286-treated neurons and astrocyte cultures that were treated with C286 + decorin NAb-CM. B, Expression of CD44 and CSPGs in the astrocyte cultures. Scale bar, 20 μm. C, D, Decorin and C286-CM treatment significantly suppresses CSPGs and CD44, an effect that is abolished by the addition of decorin NAb to the cultures. Data represent mean of fluorescence intensity for CSPGs in arbitrary units (a.u.) ± SEM taken from 10 fields per culture condition from three independent experiments. ***p ≤ 0.001, one-way ANOVA followed by Fisher’s post hoc test. E, F, LPS-activated astrocytes treated with vehicle or C286 and immunoassayed for GFAP (E) and decorin (F) showed no significant difference in decorin expression between the two treatments. Data represent mean of fluorescence intensity for decorin in arbitrary units (a.u.) ± SEM taken from 10 fields per culture condition from three independent experiments. n.s., Not significant, Student’s t test.
RARα-mediated myelination in RARβ-agonist-treated avulsed rats. A, Expression of both PLP and MAG are upregulated in the RARβ-treated rats and both types of myelin show an unbroken pattern (inset). B, C, Quantification of PLP and MAG shows a significant increase in response to RARβ treatment. Data show mean ± SEM, n = 8 per treatment group, **p ≤ 0.01, Student’s t test. D, RARα, but not RARβ, is upregulated in NG2 

Two years after the publication of the above, we have expanded our understanding of the role of RARα in regenerative myelination. We have shown that RARα signaling is induced in NG2+ cells in the dorsal horn of RARβ-agonist-treated rats, and that this induction of RARα is followed by an increase in oligodendrocyte progenitor cell differentiation into oligodendrocytes. Furthermore, we have shown that the expression of RARα in the NG2+ cells is significantly higher in RARβ-agonist-treated rats compared with vehicle-treated rats, as is the total number of oligodendrocytes per field (J). Data are shown as mean ± SEM, n = 8 ***p ≤ 0.001, Student’s t test. Scale bars, 100 μm.

RARβ signaling activates RARα in regenerative myelination after DRA

To directly assess the effect of RARβ signaling on remyelination in vivo, we compared the levels of myelination in the avulsed rats that had received vehicle or RARβ agonist treatment. We immunoassayed the injured SCs and dorsal roots for MAG, which is present in both the PNS and CNS (Quarles, 2002), and for PLP, which is the major myelin protein in the CNS (Kamholz et al., 1992). We had previously observed myelin sheaths around regenerated axons across the DREZ in response to RARβ application using the same animal model (Goncalves et al., 2015). Unsurprisingly, we found that both PLP and MAG were significantly higher in RARβ-agonist-treated compared with vehicle-treated rats (fluorescence intensity: PLP: vehicle, 12 ± 0.91 a.u.; RARβ agonist, 18.75 ± 1.10 a.u.; MAG: vehicle, 18.22 ± 1.12 a.u.; RARβ agonist, 39 ± 8.13 a.u.) (Fig. 3A–C).

Because remyelination is preceded by the invasion and differentiation of oligodendrocyte progenitors at the lesion site, we investigated whether an increase in oligodendrocyte genesis could also contribute to the RARβ-agonist-induced remyelination observed. Previously, we had shown that RARα activation induces neural progenitor cell differentiation into oligodendrocytes (Goncalves et al., 2005) and RARα has been shown to be expressed in OPCs (Laeng et al., 1994), so we first looked at the expression of RARα and RARβ in the NG2+ cells in the injury area of myelinating rats. In agreement with previous in vitro observations (Laeng et al., 1994; Goncalves et al., 2005), we found...
that RARα was highly associated with NG2-immunoreactive cells, whereas little or no RARβ was detected in the NG2⁺ cells (Fig. 3D). We then compared the expression of RARα in NG2⁺ cells between vehicle-treated and RARβ-agonist-treated groups (Fig. 3E) and found that RARα immunostaining was significantly higher in NG2⁺-expressing cells in the NG2⁺-agonist-treated group (fluorescence intensity: 0.66 ± 0.04 a.u.) compared with vehicle (fluorescence intensity: 0.38 ± 0.03 a.u.) (Fig. 3F). This was also true for total RARα expression (fluorescence intensity: vehicle, 17.63 ± 5.53 a.u.; C286, 30.99 ± 5.40 a.u.) (Fig. 3G). This suggests that RARβ drives RARα in this injury model and supports a function for RARα in promoting differentiation of the precursors into myelinating oligodendrocytes. Next, to determine whether the presence of RARα was associated with the differentiation process in vivo, we double stained tissue from myelinating and non-myelinating rats with RARα and Olig1, which is a transcription factor expressed from the early stages of oligodendrocyte lineage development (Woodruff et al., 2001) and essential to their differentiation into remyelinating oligodendrocytes (Arnett et al., 2004). Both RARα and Olig1 expression were significantly higher in the SCs of myelinating rats compared with non-myelinating and ~50% of Olig1-expressing cells also expressed RARα (vehicle, 3.88 ± 0.73%; RARβ agonist 47.8 ± 2.65%), with the total number of Olig1⁺ cells at the DREZ in the-agonist-treated rats also being significantly higher (vehicle, 1.94 ± 1.10 and RARβ agonist, 23.88 ± 3.97 mean number of cells per field) (Fig. 3H–J), suggesting that this signaling pathway may be required throughout oligodendrocyte differentiation. Together, these data suggest that treatment with an RARβ agonist leads to an increase in myelinating oligodendrocytes driven by the removal of CSPGs and by the repopulation of oligodendrocytes at the lesioned site. However, the latter is not driven directly by RARβ, but rather by RARα, in the NG2⁺ cells.

Intracellular calcium levels modulate the secretion of exosome-associated RA in NG2⁺ cells and determine their differentiation into oligodendrocytes via RARα signaling. A, Expression of RARα, OSP, and RA in NG2 cultures treated for 3 d with: vehicle, BAPTA-AM, GW4869, AM 580, retinal + BAPTA-AM, or retinal + GW4869. Scale bar, 50 μm and for the bottom 300 μm. B, C, Quantification of OSP (B) and RARα (C) expression showed that both were significantly higher in the presence of AM580 or when increased intracellular concentrations of RA occurred (retinal + BAPTA-AM and retinal + GW4869). Data are shown as mean ± SEM taken from five random fields from three independent experiments. **p ≤ 0.01, One-way ANOVA followed by Tukey’s test. D, Electron microscopy images of exosomes (arrows) isolated from vehicle-treated NG2⁺ cells. Scale bar, 50 nm. E, β-galactoside staining of RARE LacZ-transfected F9 cells showing RA in exosomes isolated from NG2⁺ cell cultures treated with: vehicle, retinal, retinal + BAPTA-AM, GW4869, or retinal + GW4869. F, Quantification of RA plotted as percentage of LacZ⁺ cells shows that preventing exosome release from the NG2⁺ cells results in a significant decrease in secreted RA. Data are shown as mean ± SEM taken from five random fields from three independent experiments, ***p ≤ 0.001, one-way ANOVA, followed by Tukey’s test.

The above results pose the question: how are these two signaling pathways connected? We had previously shown that treatment of avulsed rats with the RARβ agonist leads to an increase of Raldh2 activity in the NG2⁺ cells that populate the injury site and an increase in the release of RA associated with exosomes that serve as guidance cues for the regenerating axons (Goncalves et al., 2018). Because we did not administer an RARα agonist in addition to the RARβ agonist, we reasoned that RA as the endogenous

**Figure 4.** Intracellular calcium levels modulate the secretion of exosome-associated RA in NG2⁺ cells and determine their differentiation into oligodendrocytes via RARα signaling. A, Expression of RARα, OSP, and RA in NG2 cultures treated for 3 d with: vehicle, BAPTA-AM, GW4869, AM 580, retinal + BAPTA-AM, or retinal + GW4869. Scale bar, 50 μm and for the bottom 300 μm. B, C, Quantification of OSP (B) and RARα (C) expression showed that both were significantly higher in the presence of AM580 or when increased intracellular concentrations of RA occurred (retinal + BAPTA-AM and retinal + GW4869). Data are shown as mean ± SEM taken from five random fields from three independent experiments. **p ≤ 0.01, One-way ANOVA followed by Tukey’s test. D, Electron microscopy images of exosomes (arrows) isolated from vehicle-treated NG2⁺ cell cultures. Scale bar, 50 nm. E, β-galactoside staining of RARE LacZ-transfected F9 cells showing RA in exosomes isolated from NG2⁺ cell cultures treated with: vehicle, retinal, retinal + BAPTA-AM, GW4869, or retinal + GW4869. F, Quantification of RA plotted as percentage of LacZ⁺ cells shows that preventing exosome release from the NG2⁺ cells results in a significant decrease in secreted RA. Data are shown as mean ± SEM taken from five random fields from three independent experiments, ***p ≤ 0.001, one-way ANOVA, followed by Tukey’s test.
ligand had to be driving RARα signaling in the NG2⁺ cells. A shift in RA traffic by curtailing exosome release could be the cause of RARα activation. Calcium is known to govern exosome release, with increased intracellular concentrations inducing its secretion (Savina et al., 2003). To determine whether a change in intracellular calcium and exosome release was involved in a RARα-mediated NG2⁺ cell differentiation, we treated NG2⁺ cells with vehicle, a calcium chelator (BAPTA-AM), or GW4869, which is the most widely used pharmacological agent for blocking exosome biogenesis/release (Essandoh et al., 2015), a specific RARα agonist (AM 580), or BAPTA-AM plus retinal to provide the substrate for RA synthesis, or retinal plus GW4869. After 3 d in culture, we looked at the oligodendrocyte marker, OSP (Sallis et al., 2006) and RARα expression. Only AM 580 and BAPTA-AM plus retinal or retinal plus the exosome inhibitor resulted in the upregulation of OSP and RARα (fluorescence intensity: OSP—vehicle, 0.78 ± 0.06 a.u.; BAPTA-AM, 0.81 ± 0.07 a.u.; GW4869, 0.75 ± 0.09 a.u.; AM 580, 1.37 ± 0.13 a.u.; retinal + BAPTA-AM, 1.46 ± 0.19; retinal + GW4869, 0.59 ± 0.05 a.u.; RARα - vehicle, 0.41 ± 0.29 a.u.; BAPTA-AM, 0.45 ± 0.26 a.u.; GW4869, 0.56 ± 0.23 a.u.; AM 580, 1.80 ± 0.47 a.u.; retinal + BAPTA-AM, 1.53 ± 0.38; retinal + GW4869, 0.77 ± 0.27 a.u.) (Fig. 4A–C). In the treatments that lead to an increase in OSP, we also observed an intracellular increase in RA (Fig. 4A, middle), possibly due to cytoplasmic accumulation because it has not been secreted. To confirm that the NG2⁺ cells retain intracellular RA that would otherwise be secreted in association with exosomes as a response to intracellular calcium changes, we isolated exosomes as de-
Decorin regulates the release of exosome-associated RA in NG2 \(^+\) cells via the EGFR–calcium pathway

Decorin has been shown to downregulate EGFR within 3 h (Zhu et al., 2005). EGFR activation can induce Ca\(^{2+}\) release from endoplasmic reticulum stores (Wang et al., 2005), thus increasing intracellular Ca\(^{2+}\) concentrations. To determine whether decorin could regulate exosome release from the RA synthesizing NG2 \(^+\) cells by inducing changes in intracellular Ca\(^{2+}\), we cultured NG2 \(^+\) cells on a CSPG matrix to mimic the extracellular milieu after a SCI using retinal as the Raldh2 substrate and treated the cells with vehicle or decorin. After 2 h, we added a Ca\(^{2+}\) dye (Fluo-4AM) to the medium and allowed the cells to incubate for another hour before assessing the levels of intracellular Ca\(^{2+}\) by immunocytochemistry. We found that decorin significantly reduced intracellular Ca\(^{2+}\) (fluorescence intensity: retinal + vehicle, 12.2 ± 0.91 a.u.; retinal + decorin, 4 ± 0.83 a.u.) and EGFR (fluorescence intensity: retinal + vehicle, 8.6 ± 1.2 a.u.; retinal + decorin, 4 ± 0.54 a.u.) (Fig. 5A–C). To evaluate the effect of decorin on oligodendrocyte differentiation, we used cultures treated the same way and looked at the expression of NG2 and OSP after 3 d in culture. Decorin significantly increased the differentiation of NG2 \(^+\) cells (fluorescence intensity: retinal + vehicle, 13.48 ± 1.53 a.u.; retinal + decorin, 6.85 ± 1.06 a.u.) into OSP \(^+\) cells (fluorescence intensity: retinal + vehicle, 3.9 ± 10.97 a.u.; retinal + decorin, 22.52 ± 5.43 a.u.) (Fig. 5D, E). Finally, to directly assess the effect of decorin in the exosome-associated RA

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**Figure 6.** RAR\(\alpha\) is required for the differentiation of NG2 \(^+\) cells into oligodendrocytes. A, Immunostaining of NG2 \(^+\) cell cultures transfected with scrambled or RAR\(\alpha\) siRNA showing that transfection with RAR\(\alpha\) siRNA resulted in the loss of RAR\(\alpha\). B, Quantification of RAR\(\alpha\) expression normalized to GAPDH by qRT-PCR confirm the suppression of RAR\(\alpha\) over 72 h. C, Quantitative analysis of RNA levels normalized to GAPDH by qRT-PCR confirm the suppression of RAR\(\alpha\) expression normalized to number of cells in the cultures taken from five fields per culture condition from three independent experiments, Data are shown as mean ± SEM, **p \(\leq\) 0.01, Student’s t test. D, Expression of RAR\(\alpha\) and OSP in scrambled and RAR\(\alpha\) siRNA-transfected NG2 \(^+\) cells in the presence of vehicle, BAPTA-AM, or BAPTA + retinal. Only scrambled siRNA-transfected NG2 \(^+\) cells treated with BAPTA-AM + retinal differentiate into oligodendrocytes. E, Quantification of OSP \(^+\) normalized for number of cells in the cultures taken from five fields per culture condition from three independent experiments, Data are shown as mean ± SEM, ***p \(\leq\) 0.001. n.s., Not significant, Student’s t test. Scale bars, 50 \(\mu\)m.
release, we collected the conditioned media from these cultures and isolated exosomes as described previously (Goncalves et al., 2018). Western blotting with the exosome marker AIP1/Alix (Willms et al., 2016) was used to confirm exosome isolation (Fig. 5F). These were then added to F9 RARE-lacZ RA reporter cells as described above for quantification of RA. As anticipated, we found that exosomes from decorin-treated NG2/H11001 cells had significantly lower levels of RA than retinal plus vehicle ones, as shown by the percentage of LacZ/H11001 cells: retinal + vehicle, 419.81 ± 2.8; retinal + decorin, 6.43 ± 0.72) (Fig. 5G,H).

RARα is required for the differentiation of NG2+ cells into oligodendrocytes

Next, to confirm the requirement of RARα signaling to drive the differentiation process, we transfected NG2+ cells with an RARα siRNA or a scrambled siRNA and cultured them with vehicle, BAPTA-AM, or BAPTA-AM plus retinol for 4 d. First, we con-
firmed that RARα was knocked down by immunocytochemical RARα expression (fluorescence intensity: scrambled siRNA, 0.43 ± 0.02 a.u.; RARα siRNA, 0.26 ± 0.04 a.u.) (Fig. 6A,B), and by qPCR of RARα over 72 h (relative expression of RARα RNA/GAPDH: 24 h, scrambled siRNA, 1.0 ± 0.20 and RARα siRNA 0.3 ± 0.11; 48 h, scrambled siRNA, 0.99 ± 0.04 and RARα siRNA 0.4 ± 0.04, 72 h, scrambled siRNA, 0.76 ± 0.23 and RARα siRNA, 0.27 ± 0.03) (Fig. 6C). We next looked at the differentiation into oligodendrocytes in the same culture conditions. In RARα siRNA-transfected NG2+ cells, treatment with BAPTA-AM and retinal yielded a significantly lower number of OSP+ cells than in the scrambled or nontransfected cultures (fluorescence intensity: scrambled siRNA: vehicle, 0.79 ± 0.07 a.u.; BAPTA-AM, 0.81 ± 0.06 a.u.; BAPTA-AM+ retinal, 1.26 ± 0.12 a.u. RARα siRNA: vehicle 0.72 ± 0.05 a.u.; BAPTA-AM, 0.6 ± 0.01 a.u.; BAPTA-AM+ retinal, 0.43 ± 0.02 a.u.) (Fig. 6D,E). This suggests that RARα plays a crucial role in the differentiation of NG2+ cells into oligodendrocytes.

Raldh2 activity is required for remyelination in vivo

Our results thus far suggest that the differentiation of NG2+ cells into myelin-producing oligodendrocytes is reliant upon the synthesis of RA as the endogenous ligand of RARα. We took a pharmacological and functional approach to confirm this. We performed the DRA as described above and knocked down Raldh2 by injecting at the DREZ a GFP-tagged lentivirus with an shRNA to silence Raldh2 (LV shRNA Raldh2) or an LV-overexpressing a scrambled shRNA (LV scrambled) at the time of the injury and then treated both groups with the RARβ agonist as before for 4 weeks. A separate set of avulsed rats were given a Raldh2 inhibitor, disulfiram, together with the RARβ agonist or vehicle for 4 weeks after the injury (Fig. 7A). For the LV-injected rats, we first confirmed LV transduction by assessing GFP and Raldh2 expression in the lesioned area after 2 weeks of LV injections. As a control we have included a lesioned, vehicle-treated rat which had not been injected with the LV. In LV shRNA Raldh2 RARβ-agonist-treated rats there was hardly any colocalization of Raldh2+ staining with GFP, unlike the LV scrambled shRNA RARβ-agonist-treated rats where several areas of colocalization could be seen at the DREZ (Fig. 7B), suggesting a successful LV transduction. Next, to evaluate the effect of Raldh2 on myelination, MAG, NG2, and Olig1 expression at the DREZ were assessed at the end of the treatment period (Fig. 7C). Both the LV shRNA Raldh2+ RARβ agonist and RARβ agonist + disulfiram-treated groups showed significantly less MAG and Olig1 than the LV-scrambled shRNA + RARβ agonist and RARβ-agonist-treated groups (fluorescence intensity for MAG: LV scrambled shRNA+ RARβ agonist, 56.43 ± 1.93 a.u.; LV shRNA Raldh2+ RARβ agonist 25.98 ± 3.75 a.u.; RARβ agonist, 65.90 ± 5.55 a.u.; RARβ agonist + disulfiram 25.16 ± 5.14 a.u. fluorescence intensity for Olig1: LV scrambled shRNA+ RARβ agonist, 34.95 ± 4.12 a.u.; LV shRNA Raldh2+ RARβ agonist, 9.41 ± 0.44 a.u.; RARβ agonist, 34.41 ± 4.37 a.u.; RARβ agonist + disulfiram 9.71 ± 3 a.u.) (Fig. 7D). Conversely, the groups in which Raldh2 had been suppressed showed a higher expression of NG2+ cells at the injury site (fluorescence intensity: LV scrambled shRNA+ RARβ agonist, 20.27 ± 1.55 a.u.; LV shRNA Raldh2+ RARβ agonist, 34.38 ± 4.44 a.u.; RARβ agonist, 24.56 ± 2.48 a.u.; RARβ agonist + disulfiram 37.29 ± 3.63 a.u.) (Fig. 7D), suggesting that these cells remained at an early lineage stage of development and did not differentiate into oligodendrocytes.

Our results support a model (Fig. 8) whereby RARβ activation in neurons leads to the increased synthesis and secretion of decorin. The decorin has a twofold promyelinating effect: (1) it...
acts as a scavenger of the CSPGs produced by reactive astrocytes, thus eliminating a major obstacle to OPCs differentiation and migration, and (2) its action on NG2⁺ cells that are synthesizing RA is to suppress EGFR and reduce intracellular calcium concentrations. This restricts exosome release and generates an intracellular pool of RA, which activates RARα signaling and promotes differentiation into myelinating OLs.

**Discussion**

We show here that RA signaling promotes remyelination after SCI through the synergic interaction of neuron–glia network pathways. In this context, we identify decorin as an important downstream target of RARβ in neurons. The well documented effect of decorin on CSPGs is at least one of the ways by which the RARβ agonist induces CSPG clearance. The inhibitory effect of CSPGs on OPC migration and differentiation has been associated with the downregulation of the β1-integrin pathway (Sun et al., 2017). Several approaches to neutralizing CSPGs after injury have included digestion of CSPGs with the enzyme chondroitinase ABC (Moon and Fawcett, 2001; Bradbury et al., 2002), RNA interference of chain polymerization enzyme (Laabs et al., 2007), and peptide blocking of protein tyrosine phosphatase θ (Lang et al., 2015). However, modulating CSPGs via the activation of an endogenous pathway offers clear therapeutic advantages.

Additionally, we found that the RARβ-decorin downstream cascades are not limited to astrocytes, but interestingly and crucially for remyelination, extend to NG2⁺ cells. Treatment with RARβ agonist leads to the upregulation of Raldh2 in the NG2⁺ cells that populate the injury site (Gonçalves et al., 2018). During axonal regeneration, which precedes myelination, the RA is preferentially secreted in association with exosomes to act as an axonal pathfinder (Gonçalves et al., 2018). We found that decorin significantly suppresses RA release via an EGFR-mediated decrease in intracellular calcium. In agreement with our data, decorin has been shown to attenuate the EGFR-mediated mobilization of intracellular calcium (Courdás et al., 2000). Analysis of EGFR in the neonatal SVZ progenitor cells showed that a loss of the receptor occurs as the cell lineage is established and very few NG2⁺ and Olig2⁺ cells expressed EGFR (Cesetti et al., 2009).

We found that the reduction of secretion of exosome-associated RA results in the activation of RARα in the NG2⁺ cells and in an increase in oligodendrocyte biogenesis. RARα has been shown to be constitutively present in OPCs and to be transiently increased by RA (Laeng et al., 1994), probably RARα2, because only this isoform has been reported to be induced by RA (Leroy et al., 1991), whereas RARβ is not constitutively expressed in OPCs (Laeng et al., 1994). However, prolonged treatment of human-derived OPCs with RA results in RARβ upregulation and in the upregulation of two known transcriptional inhibitors of oligodendrocyte differentiation, Hes5 and Id4 (Kim et al., 2017). In embryonic SC, where relatively high levels of retinoids are present, RA was found to inhibit oligodendrocyte differentiation during early embryonic development, permitting OPCs dispersal throughout the entire SC (Noll and Miller, 1994). Although we did not directly measure intracellular RA, it is likely that a fluctuation of RA levels, tightly modulated by neuron–glia signals, will provide the stop–go instructions to the NG2⁺ cell via RAR expression to keep the OPC/OL pools under control. It is thus thought that the developmental-stage-specific actions of RA are modulated through the spatiotemporal expression of the subtype and isoforms of RARs (Takeyama et al., 1996), as well as by RA gradients (Maden et al., 1996, 1998), and a similar mechanism may apply to the adult regenerating nervous system.

Extracellular vesicles are important in neuron–glia communications (for review, see Frühbeis et al., 2013) and specifically exosome signaling has been shown to play a role in the demyelinating disease multiple sclerosis (Carandini et al., 2015; Pusici et al., 2016). We propose that, like myelination in development, the dynamic cross talk between neurons and oligodendrocytes adapts to the physiological requirements and this is reflected in the cues exchanged (Simons and Trajkovic, 2006). The current study does not specifically address the timing for the switch between release and nonrelease mechanisms of RA associated with exosomes from the NG2⁺ cells and it is possible that they are both present during the 4-week period of RARβ agonist treatment, but not in individual NG2⁺ cells, according to the state of regeneration of the axons in their vicinity.

The importance of retinoid signaling in myelination has been previously shown (Huang et al., 2011; Kazakova et al., 2006; Latasa et al., 2010) and has been proposed to be mediated by RXRα (Huang et al., 2011). However, the identification of endogenous ligands for RXRs is contentious (Wolf, 2006; Gilardi and Desvergne, 2014). RXRs are partners to a number of other nuclear receptors (Lefebvre et al., 2010), thus integrating the corresponding signaling pathways. We have identified that RARα promotes remyelination. Our findings, together with previous work (Huang et al., 2011), indicate that the RARα/RXR pathway is a promising avenue for remyelination strategies.

Collectively, our study sheds important insights into the neuron–glia networks during remyelination. Our data suggest that targeting the RA signaling may overcome the myelination-inhibitory environment that persists after SCI as well as promoting myelination per se. Various drugs have been identified that promote myelination (Mei et al., 2014; Najm et al., 2015; Keough et al., 2016), but these drugs may not overcome inhibition by extrinsic factors in the demyelinated lesions. Similarly, OPC differentiation drugs failed to rescue inhibition of their differentiation by extracellular CSPGs (Keough et al., 2016), suggesting a need for new approaches to target extrinsic inhibition of remyelination alongside oligodendrocyte genesis. Additionally, and most relevant from a therapeutic view point, we show here that an orally available RARβ agonist can induce endogenous synthesis of RA and subsequent RARα activation in NG2⁺ cells in the nervous system, leading to regenerative myelination. This offers an advantageous strategy to promote myelination in neurological diseases with CSPG deposition. The novel RARβ agonist used in the experiments described here is orally available and has excellent potency (RARβ EC₅₀ = 1.94 nm) and selectivity for RARβ (RARα/RARβ ratio = 13.4; RARγ/RARβ ratio = 5.6). C286 is currently being tested in a phase 1 trial (ISRCTN12424734), which, if successfully completed, confers a tangible translatable aspect to the work presented herein.

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