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The Thrombin Receptor is a Critical Extracellular Switch Controlling Myelination

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Abstract

Hemorrhagic white matter injuries in the perinatal period are a growing cause of cerebral palsy yet no neuroprotective strategies exist to prevent the devastating motor and cognitive deficits that ensue. We demonstrate the thrombin receptor (protease activated receptor 1, PAR1) exhibits peak expression levels in the spinal cord at term and is a critical regulator of the myelination continuum from initiation to the final levels achieved. Specifically, PAR1 gene deletion resulted in earlier onset of spinal cord myelination, including substantially more Olig2-positive oligodendrocytes, more myelinated axons and higher proteolipid protein (PLP) levels at birth. *In vitro*, the highest levels of PAR1 were observed in oligodendrocyte progenitor cells (OPCs), being reduced with differentiation. In parallel, the expression of PLP and myelin basic protein (MBP), in addition to Olig2, were all significantly higher in cultures of PAR1^{-/-} oligodendroglia. Moreover, application of a small molecule inhibitor of PAR1 (SCH79797) to OPCs *in vitro*, inhibited PLP and MBP expression. Enhancements in myelination associated with PAR1 genetic deletion were also observed in adulthood as evidenced by higher amounts of myelin basic protein and thickened myelin sheaths across large, medium and small diameter axons. Enriched spinal cord myelination in PAR1^{-/-} mice was coupled to increases in extracellular-signal-regulated kinase 1/2 and AKT signaling developmentally. Nocturnal ambulation and rearing activity were also elevated in PAR1^{-/-} mice. These studies identify the thrombin receptor as a powerful extracellular regulatory switch that could be readily targeted to improve myelin production in the face of white matter injury and disease.

Introduction

Myelination in the central nervous system is achieved through a delicate balance of extrinsic and intrinsic signaling mechanisms with aberrations in the perinatal period, resulting in white matter injury and profound sensorimotor and cognitive disabilities. Normal

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myelination requires a series of well-orchestrated events, including the generation of oligodendrocyte progenitors (OPCs), their migration to specific regions of the brain or spinal cord and their differentiation into oligodendrocytes that elaborate multilamellar sheaths of plasma membrane to myelinate axons in precise relation to their diameter. Multiple factors can disrupt these key developmental mileposts, including hemorrhagic-ischemic injuries occurring perinatally (Volpe, 2009). Since myelin not only enhances axonal conduction velocity, but also provides protection and trophic support (Wilkins et al., 2003), the identification of new therapeutic targets to prevent perinatal white matter injuries provides the opportunity to improve both short and long term neurological functional outcomes.

Leakage of blood-derived serine proteases such as thrombin into the CNS is a common component of hemorrhagic, hypoxic, traumatic and infectious injuries (Gingrich and Traynelis, 2000). Thrombin can also be generated by CNS endogenous cells with elevations reported in spinal cord injury (Citron et al., 2000; Yoon et al., 2013), ischemia (Riek-Burchardt et al., 2002; Chen et al., 2012) and Alzheimers (Arai et al., 2006). In addition to its roles in thrombostasis, elevations in thrombin can serve as a powerful neurotoxic agent and possible new target for neuroprotection (Han et al., 2011; Yoon et al., 2013). Significantly with regard to potential new protective strategies for perinatal white matter injury, thrombin's cellular actions are conveyed by N-terminal cleavage of a seven transmembrane G-protein coupled receptor, protease activated receptor 1 (PAR1), also referred to as the thrombin receptor (Vu et al., 1991). PAR1 has highest affinity for thrombin, but can also be activated by other secreted serine proteases, including plasmin, activated protein C, granzyme A, MMP-1, and select kallikreins (Oikonomopoulou et al., 2006; Vandell et al., 2008; Adams et al., 2011; Burda et al., 2013; Yoon et al., 2013). We recently demonstrated a unique role for PAR1 activation in suppressing myelin gene transcription, in limiting oligodendrocyte progenitor (OPC) process elaboration, and in exacerbating the impact of neurotoxic agents *in vitro*. Moreover, we showed PAR1 mediates demyelination elicited by neurosin (kallikrein 6) in the adult murine spinal cord (Burda et al., 2013). Together these lines of evidence indicate that the thrombin receptor is an integral biological translator of microenvironmental protease activity and that this signaling axis can directly impact myelin dynamics. Here we use a murine genetic model to functionally evaluate the role of PAR1 in the process of murine spinal cord myelination at a cellular, molecular and ultrastructural level. Results demonstrate PAR1 is a key suppressor of developmental myelination, whereby its absence results in elevations in extracellular-signal-regulated kinase (ERK1/2) and AKT signaling that was coupled with hypermyelination, including more myelinated axons and higher levels of proteolipid protein (PLP) at term and the attainment of higher levels of myelin basic protein (MBP), thicker myelin sheaths and enhanced motor activity in adults.

Materials and Methods

Animal care and use

Mice genetically deficient in PAR1 (PAR1^{-/-}, B6.129S4-F2r^{tm1Ajc}/J) were obtained from Jackson (Bar Harbor, ME) and backcrossed to C57BL6/J for more than 30 generations (Burda et al., 2013; Yoon et al., 2013). PAR1^{+/+} littermates served as controls. All animal

experiments were carried out with adherence to NIH Guidelines for animal care and safety and were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Quantification of myelin protein expression using Western blot

Western blots were used to quantify myelin and signaling proteins. Whole spinal cords were harvested from three individual PAR1^{+/+} or PAR1^{-/-} mice on postnatal day (P) 0, 7, 21 or 45 (adulthood). Spinal cords at each time point were collectively homogenized in radio-immunoprecipitation assay buffer and 25 µg of protein resolved on sodium dodecyl sulfate-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). Multiple electroblotted membranes were used to sequentially probe for antigens of interest, including myelin proteins PLP (Ab28486, Abcam, Cambridge, MA), MBP (MAB386, Chemicon, Billerica, MA), and CNPase (MAB326, Millipore, Billerica, MA); oligodendrocyte proteins, Olig2 (Ab9610, Millipore); neuron specific proteins, Neurofilament H or L (N4142, N5139, Sigma, St. Louis, MO); or the phosphorylated or total protein forms of select signaling proteins, ERK1/2 (9101S, 9102S, Cell signaling, Boston, MA), protein kinase B (AKT, 4058L, 9272S, Cell signaling), or signal transducer and activator of transcription 3 (STAT3, sc-8059, sc-8019, Santa Cruz, Santa Cruz, CA). Membranes were also re-probed for β-actin (NB600-501, Novus Biological, Littleton, CO, USA) to further control for loading. The relative optical density (ROD) of each protein of interest was normalized to that of Actin or in the case of pERK1/2 or pAKT to total ERK1/2 or AKT, respectively. The mean and standard error (s.e.) of ROD readings across at least 3 independent Westerns for each antigen of interest was used for statistical comparisons (Yoon et al., 2013).

PAR1 expression by oligodendrocytes and quantification of oligodendrocyte number in the developing mouse spinal cord

To evaluate whether the PAR1-regulated changes in myelin proteins and myelin gene expression reflect changes in the number of OPCs or mature oligodendroglia, we enumerated Olig2 (Ab9610, Millipore) or CC-1/APC 1 (adenomatous polyposis coli, Ab16794, Abcam, Cambridge, MA) immunopositive cells in 5 µm paraffin sections through the dorsal columns of P0, 7, 21, or 45 spinal cords. Olig2 is a basic helix-loop-helix transcription factor expressed by OPCs and oligodendroglia at the early stages of differentiation, whereas CC-1 is associated only with the mature phenotype (Ligon et al., 2006; Kuhlmann et al., 2008; Funfschilling et al., 2012). Immunoperoxidase stained sections were cover slipped with Hardset containing DAPI (Vector, Burlingame, CA) and digitally imaged (Olympus BX51 microscope, Olympus, Center Valley, PA). Counts were made of either Olig2 or CC-1+ cells with a DAPI stained nucleus within the entire dorsal column of at least 3 mice at each time point without knowledge of genotype. The association of PAR1 with spinal cord OPCs or oligodendrocytes was evaluated by co-immunolabeling of PAR1 (sc-5606, clone H-111, Santa Cruz) with platelet derived growth factor receptor (PDGFR)-alpha chain (BD Pharmingen, San Diego, CA), or and CC-1, respectively. Co-labeling in each case was visualized using species appropriate fluorochrome conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Immunofluorescence images were captured using a LSM laser scanning confocal microscope (Carl Zeiss, Thornwood, NY).

Myelin RNA and protein expression by OPCs and oligodendroglia *in vitro*

To determine whether the absence of PAR1 directly impacts myelin expression, we used real time reverse transcription PCR to establish the level of oligodendrocyte associated gene transcripts in OPCs freshly shaken from PAR1^{+/+} or PAR1^{-/-} mixed glial cultures (0 h), or after a 72 h period of differentiation *in vitro*. Mixed glial cultures were prepared from the cortices of P1 mice according to a modified McCarthy and de Vellis protocol (Burda et al., 2013). 0 h OPC RNA was obtained from cells immediately after shaking from 10 day-*in-vitro* mixed glial cultures. Alternatively, OPCs were differentiated for 72 h prior to RNA isolation by plating at $3 \times 10^4/\text{cm}^2$ cells per well on poly-L-lysine (PLL, 10 $\mu\text{g}/\text{mL}$) coated 6-well plates in Neurobasal A media containing 1% N2, 50 U/mL penicillin/streptomycin, 2 mM Glutamax, 1 mM sodium pyruvate and 0.45% glucose.

To further understand the impact of altering PAR1 exclusively at the level of the oligodendrocyte on myelin gene expression, we examined the impact of a PAR1 antagonist, SCH79797 dihydrochloride (70 nM, Tocirs Bioscience, Minneapolis, MN) on myelin gene expression (Burda et al., 2013). In this case, freshly isolated OPCs were plated for a period of 24 h before being treated with SCH79797, or vehicle alone, for an additional 48 h culture period to permit differentiation and follow myelin gene expression.

The level of RNA encoding PAR1, MBP, PLP, NogoA or Olig2 was determined in 0.10 μg of RNA in triplicate using an iCycler iQ5 system (BioRad) with primers described in Table 1 (Burda et al., 2013). Results were repeated twice from independent cell preparations with parallel results. The relative amount of RNA at each time point was normalized to the constitutively expressed gene Rn18S. Mean expression levels in cells derived from PAR1^{-/-} mice, were expressed as a percent of the level observed in cells derived from wild type mice. Similarly, RNA expression levels seen in PAR1^{+/+} cells treated with SCH79797 were expressed as a percent of that seen in untreated cells.

The impact of PAR1 gene deletion on the expression of PLP protein *in vitro* was determined by comparing PLP-immunoreactivity (Ab28486, Abcam) in 72 h differentiated PAR1^{+/+} or PAR1^{-/-} oligodendrocytes plated at $7 \times 10^4/\text{cm}^2$ on PLL coated 12 mm glass cover slips. Five 20X fields encompassing the poles and center of each coverslip were captured digitally and Image J software was used to determine the ROD of somal PLP staining, as well as somal area. The mean number of PLP⁺ cells was also enumerated and expressed as a ratio of the number of DAPI cells present in each field.

Analysis of the number of myelinated nerve fibers and myelin thickness

The number of myelinated nerve fibers and the thickness of myelin sheaths were determined by structural and ultrastructural analysis of the spinal cord dorsal column white matter at P0 and P45. Mice were perfused with Trump's fixative (4% formaldehyde with 1% glutaraldehyde, pH 7.4) and a 1 mm segment of the cervical spinal cord was osmicated and embedded in araldite. The number of myelinated nerve fibers was counted in 1 μm semi-thin sections stained with 4% *p*-phenylenediamine to visualize the myelin sheaths. Digital images capturing the entire dorsal-ventral and lateral-medial axis of the spinal cord dorsal columns were captured at 60X. The number of myelinated nerve fibers and their diameter

was automatically quantified from digital images using a batch algorithm generated in Matlab (The Mathworks, Natick, MA) (Denic et al., 2009). For P45 spinal cords, the number of myelinated nerve fibers that were $< 4 \mu\text{m}^2$, $4\text{--}10 \mu\text{m}^2$ or $> 10 \mu\text{m}^2$ was also examined. All myelinated nerve fiber counts for each genotype were averaged across at least 3 independent animals per time point.

Myelin sheath thickness in the dorsal column of the cervical spinal cord at P0 and P45 was quantified in ultrathin ($0.1 \mu\text{m}$) sections taken from araldite blocks using a JEM-1400 Transmission Electron Microscope (JEOL USA, Inc., Peabody, MA). Images were captured at 8000X without knowledge of genotype and included 5 fields across the dorsal-ventral axis of the dorsal column at P0 and 6 fields at P45. G-ratios were calculated from all myelinated axons in each image. Across 3 animals per time point this resulted in measurement of roughly 60 myelinated fibers at P0 and 2200 at P45 for each genotype. Measurements of axon diameter (d) and myelin fiber diameter (D) were made by including axons of all diameters using Image J software and presented as mean g-ratio (d/D) or myelin thickness $\pm$ s.e. across axon diameters. Since very few axons were $> 4 \mu\text{m}$, the mean values calculated in each case were essentially identical to those when only axons $< 4 \mu\text{m}$ were included, facilitating comparison with prior studies (Ishii et al., 2013).

Evaluation of locomotor activity

Potential differences in locomotor activity between PAR1^{+/+} and PAR1^{-/-} mice were evaluated using a Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, Columbus OH). Animals were housed in the system and total activity, ambulatory activity, and rearing data collected for a period of 72 h that included a 24 h period of acclimation followed by 24 h fed and 24 h fasted periods. The mean activity across genotypes in each case (PAR^{+/+}, $n=11$ or PAR1^{-/-}, $n=12$) was analyzed for light and dark periods under both fed and fasted conditions.

Statistical comparisons

All data were expressed as mean $\pm$ s.e.. Comparisons between multiple groups were made using a One-Way Analysis of Variance (ANOVA) and the Newman Keuls post-hoc test. When multiple comparison data was found to be not normally distributed, the Kruskal-Wallis ANOVA on Ranks was applied with Dunn's method. For pairwise comparisons between two groups the Student's unpaired t-test was used. Statistical significance was set at $P < 0.05$.

Results

OPCs and oligodendroglia are PAR1-immunopositive and PAR1 RNA levels in total spinal cord are inversely correlated with the onset of myelination

To begin to address the potential significance of PAR1 to oligodendroglia, we used immunofluorescence double labeling approaches to determine its appearance on PDGFR- α -immunopositive OPCs and on mature CC-1-immunopositive oligodendrocytes in the spinal cord white matter from P0 to P45 (Figure 1A and B). PAR1 was readily detected in association with both PDGFR- α - and CC-1-immunoreactive cells at each developmental

stage examined. We then used quantitative real time PCR to quantify any changes in PAR1 expression over the course of spinal cord development. Strikingly, two-fold reductions in PAR1 RNA were observed in the spinal cord between P0 and P7 and this lower level of PAR1 expression persisted through adulthood (Figure 1C, $P < 0.001$, Newman Keuls). Perinatal reductions in PAR1 RNA, when the levels of many myelin proteins begin to surge (Figure 2), supports an emerging model in which high levels of PAR1 signaling in the spinal cord at birth engages a myelination suppressive signaling cascade (Fig. 7).

Knockout of PAR1 results in accelerated PLP expression in the perinatal period and higher levels of MBP in adults

To critically evaluate the role of PAR1 in myelin development *in vivo*, we directly compared the onset, magnitude and duration of myelin protein expression, including the two major myelin structural proteins, proteolipid protein (PLP) and myelin basic protein (MBP), in the spinal cord of PAR1^{+/+} and PAR1^{-/-} mice at P0 through P45 (adulthood) (Figure 2). Consistent with a regulatory role for PAR1 in the onset of myelin protein expression, spinal cord PLP levels were 2.4-fold higher at P0 in PAR1^{-/-} mice relative to PAR1^{+/+} mice ($P = 0.003$, Neuman Keuls). Also, peak PLP levels were achieved by P7 in the absence of PAR1, 2 weeks ahead of the P21 peak observed in the wild type spinal cord ($P = 0.03$, Newman Keuls). Supporting a unique role for PAR1 in regulating the onset of PLP production, despite the earlier commencement of spinal cord PLP protein expression in PAR1^{-/-} mice, by P21, and at P45, levels were identical across genotypes. These data highlight a key role for PAR1 in regulating the early stages of PLP protein production.

Levels of MBP protein were comparable between PAR^{+/+} and PAR1^{-/-} mice at early stages of development (Figure 2). By adulthood however, MBP protein levels were 1.7-fold higher in PAR1^{-/-} mice ($P = 0.02$, Newman Keuls). The manifestation of higher MBP protein levels in adult PAR1^{-/-} mice is consistent with the model that blocking PAR1 signaling creates a microenvironment that enhances myelin production (Figure 7) (Burda et al., 2013). By contrast to the elevated levels of PLP and MBP protein seen in the spinal cord in the absence of PAR1, levels of 2', 3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase), were reduced by 1.4-fold on P21 ($P = 0.03$, Newman Keuls). This is of interest with regard the role of CNPase as a microtubule-associated protein, promoting microtubule assembly in events leading up to myelination (Bifulco et al., 1997). No impact of PAR1 deletion was seen on the heavy or light chains of neurofilament protein (NF_H or NF_L), at any age examined.

PAR1 is a negative regulator of ERK1/2 and AKT signaling in the developing spinal cord

To determine the likely intracellular signaling cascade(s) impacted by PAR1, we evaluated extracellular-signal-related kinase (ERK1/2) and AKT (protein kinase B), since each of these signaling intermediates participate in myelin development (Czopka et al., 2010; Harrington et al., 2010; Guardiola-Diaz et al., 2012; Ishii et al., 2012; Fyffe-Maricich et al., 2013; Ishii et al., 2013). Levels of the transcription factor signal transducer and activator of transcription 3 (STAT3), which has been both indirectly (Nobuta et al., 2012) and directly (Dell'Albani et al., 1998) linked to oligodendrocyte differentiation, was also examined in parallel. Consistent with prior studies demonstrating that elevations in ERK1/2 signaling

enhance myelination, we observed significantly higher levels of activated ERK1/2 in the spinal cords of PAR1^{-/-} mice on P0 and P21, compared to wild type controls (Figure 2). Peak elevations in activated ERK1/2 in PAR1^{-/-} spinal cords were seen at P21, when levels were 1.6-fold higher than those observed in PAR1^{+/+} mice ($P = 0.001$, Newman Keuls). Elevated levels of activated ERK1/2 were also detected in PAR1^{-/-} spinal cords on P0, when levels were 2.9-fold higher than wild type ($P = 0.01$, Newman Keuls). Suggestive of a biphasic role of ERK1/2 signaling in spinal cord development, levels of activated ERK1/2 on P7 were generally lower than those observed on P0 in both PAR1^{+/+} and PAR1^{-/-} mice, with the later reaching the level of statistical significance ($P = 0.01$, Newman Keuls). Elevated levels of activated AKT were also observed in the PAR1^{-/-} spinal cord on P7 (1.2-fold, $P = 0.005$) and P21 (1.3-fold, $P = 0.002$, Newman Keuls), relative to the level detected in wild type spinal cords. By contrast, PAR1-loss-of-function had little impact on the STAT3 signaling pathway.

Knockout of PAR1 increases oligodendrocyte number in the early postnatal period

To determine whether increases in PLP and MBP protein in the spinal cord of PAR1^{-/-} mice reflect increases in myelin protein expression per cell, or alternatively, more myelin producing oligodendroglia, we quantified protein levels of oligodendrocyte transcription factor 2 (Olig2) from P0 through adulthood (Figure 2). Findings regarding overall levels of Olig2 in the spinal cord were complemented by counts of Olig2- or adenomatous polyposis coli (CC-1)-immunoreactive oligodendrocytes in the dorsal column of parallel sets of mice (Figure 3). Olig2 protein levels detected by Western blot were higher in spinal cords of PAR1^{-/-} compared to PAR1^{+/+} mice at P7 (2.6 fold, $P = 0.04$) and P21 (1.6-fold, $P = 0.02$) (Figure 2, Newman Keuls), but not in adults. In parallel, counts of Olig2⁺ cells revealed significantly greater numbers in PAR1^{-/-} at P0 (1.5-fold, $P = 0.04$) and P7 (1.3-fold, $P = 0.05$), but identical numbers thereafter (Figure 3, Newman Keuls). Also, counts of CC-1-immunoreactive oligodendrocytes indicated increased numbers in the dorsal columns of PAR1^{-/-} mice on P7 (1.6-fold, $P = 0.03$) (Figure 3, Newman Keuls). These findings link PAR1-gene deletion to enhancements in oligodendrocyte differentiation in the spinal cord during the first week postnatal week.

PAR1 gene deletion enhances myelin expression in differentiated oligodendroglia *in vitro*

PAR1 RNA levels were 2.2-fold higher in freshly shaken OPCs compared to those differentiated for 72 h *in vitro* ($P = 0.0005$, Students unpaired t-test, Fig. 4A). To determine whether reductions in PAR1 at the level of the oligodendrocyte directly impact myelin expression, we evaluated the appearance of RNA encoding myelin proteins in freshly isolated PAR1^{+/+} or PAR1^{-/-} OPCs (0 h), or after a 72 h period of differentiation *in vitro* using real-time PCR (Figure 4B and C). Consistent with a cell intrinsic role for PAR1 in suppression of the process of oligodendrocyte differentiation (Figure 7), PLP (2.7 fold, $P = 0.00001$), MBP (3.7-fold, $P = 0.0001$) and Olig2 (1.5-fold, $P = 0.00002$) RNA transcripts were each elevated in oligodendroglia lacking PAR1 after a 72 h period of differentiation *in vitro* (Students unpaired t-test). Supporting these observations, examination of PLP-immunoreactivity in parallel 72 h differentiated OPC cultures revealed that expression of PLP-protein occurred in more oligodendrocytes (1.3-fold more, $P = 0.03 \times 10^{-5}$, Students unpaired t-test), and at a higher level per cell (1.9-fold, $P = 0.02 \times 10^{-5}$), in cultures derived

from PAR1^{-/-} compared to PAR1^{+/+} mice (Figure 4D, E). By contrast to the increases in PLP and MBP, RNA transcripts encoding NogoA were reduced in PAR1^{-/-} oligodendroglia following differentiation (1.5-fold, $P = 0.0007$, Students unpaired t-test). No significant differences in the level of RNA encoding the same myelin associated proteins were observed in OPCs at the time of isolation (0 h).

A small molecule inhibitor of PAR1 (SCH79797) was applied to differentiating oligodendrocytes *in vitro* to further understand the cell intrinsic role of PAR1 in regulating oligodendrocyte differentiation towards a myelinating phenotype. Paralleling the effects of PAR1 gene deletion, treatment of wild type oligodendrocytes 24 h after plating with SCH79797 enhanced the expression of PLP (1.4-fold, $P = 0.04$) and MBP (1.5-fold, $P = 0.03$) RNA (Students unpaired t-test), and reduced levels of NogoA RNA (0.95-fold, $P = 0.02$). The SCH79797 PAR1 inhibitor also reduced levels of Olig2 RNA (0.47-fold, $P = 0.001$), an effect opposite to PAR1 genetic targeting that will need to be followed up on in future studies to determine the potential significance.

PAR1 regulates the onset of axon ensheathment and myelin thickness in adults

To determine whether the increases observed in PLP and MBP proteins in the spinal cord of PAR1^{-/-} mice were reflected in developmental changes in myelin structure, we systematically evaluated the impact of PAR1-loss-of-function on the number of myelinated axons and myelin thickness in the dorsal funiculi (columns) at P0 and P45 (Figure 5A). The number of myelinated nerve fibers and their size were determined in paraphenylenediamine stained semithin (1 μm) spinal cord sections. There were nearly two-fold more thinly myelinated nerve fibers in the dorsal funiculi of PAR1^{-/-} mice at P0 (252 ± 65) relative to wild type littermates (100 ± 19) (mean $\pm$ s.e., $P = 0.02$, Students unpaired t-test). By P45, the number of myelinated nerve fibers was no longer different, however, PAR1^{-/-} mice had significantly more myelinated nerve fibers that were $>10 \mu\text{m}^2$ ($P = 0.02$, Students unpaired t-test). To delineate whether this shift in the size distribution of myelinated nerve fibers reflected an increase in myelin thickness or a shift in the size distribution of axons, we assessed the g-ratio of dorsal column myelinated fibers at P0 and P45 in ultrathin (0.1 μm) sections using electron microscopy (Figure 5B to D). At P0, the mean g-ratio of axons was not significantly different in PAR1^{-/-} (0.83 ± 0.007) compared to PAR1^{+/+} mice (PAR1^{+/+} 0.85 ± 0.007 , $P = 0.06$, Students t-test), however a small and significant increase in mean myelin thickness was already evident (PAR1^{-/-} $= 0.22 \pm 0.007 \mu\text{m}$; PAR1^{+/+} $= 0.19 \pm 0.012 \mu\text{m}$, $P = 0.02$, Students t-test). At P45, mean g-ratios were smaller in PAR1^{-/-} (0.71 ± 0.002 compared to PAR1^{+/+} (0.75 ± 0.002) mice ($P = 0.29 \times 10^{-68}$, Students t-test). A decrease in dorsal column white matter g-ratios in the PAR1^{-/-} adult spinal cord was reflected in significantly thicker myelin sheaths (PAR1^{-/-} $= 0.37 \pm 0.004 \mu\text{m}$; PAR1^{+/+} $= 0.28 \pm 0.003 \mu\text{m}$, $P = 0.66 \times 10^{-63}$, Students t-test). Thus, not only does axon ensheathment and myelination occur earlier in the spinal cord of mice lacking the thrombin receptor, but the thickness of the myelin sheath ultimately achieved in adults is also significantly enhanced.

Motor Activity in PAR1^{-/-} mice

To link changes in spinal cord myelination observed in PAR1^{-/-} mice to functional outcomes, we evaluated overall motor activity, ambulation and rearing during diurnal and nocturnal cycles under both fed and fasted conditions (Figure 6). Overall activity of mice lacking the thrombin receptor was increased during the day under fed conditions ($P = 0.04$) and at night when fasted ($P = 0.02$) (Students unpaired t-test, Figure 6A). Also, both ambulation ($P = 0.02$) and rearing responses ($P = 0.04$) were increased in thrombin receptor-deficient mice under fasting conditions at night (Students unpaired t-test, Figure 6 B and C).

Discussion

Here we show that the thrombin receptor is an essential intrinsic suppressor of spinal cord myelination. Deletion of the gene encoding the thrombin receptor resulted in spinal cord hypermyelination, including more myelinated axons and higher PLP levels at term and the attainment of higher MBP levels, thicker myelin sheaths and enhanced motor activity in adults. The enhancements in myelination observed as a result of PAR1 loss-of-function *in vivo* were associated with elevations in the activated forms of the pro-myelination signaling intermediates ERK1/2 and AKT. These findings identify the thrombin receptor as a key translator of myelination suppressive signals, such as those mediated by serine proteases (Burda et al., 2013), and is therefore positioned to critically impact the process of developmental spinal cord myelination.

Reciprocal relationship between the expression of PAR1 and myelination

Favoring a model where PAR1 activation serves as a negative regulator of spinal cord myelination, PAR1 RNA was highest at birth with greater than 2-fold reductions thereafter. This decline in PAR1 correlated temporally with axon ensheathment and increases in myelin protein production, including Olig2, PLP, MBP and CNPase between P7 and P21. The fact that PAR1 RNA was 2-fold higher in purified OPCs compared to differentiated oligodendrocytes *in vitro*, also supports a model where developmental reductions in PAR1 facilitate oligodendrocyte differentiation towards a myelinating phenotype. Notably, PAR1 was among the genes identified as most enriched in OPCs relative to myelinating oligodendrocytes (48.2-fold) in a prior genome wide transcriptome analysis (Cahoy et al., 2008). Together, these lines of evidence demonstrate that PAR1 RNA expression in oligodendrocytes is reduced with differentiation. We also point out however that significant levels of PAR1-immunoreactivity were observed on both PDGFR- α OPCs, and CC-1 immunopositive mature oligodendrocytes, indicative of roles for this receptor at successive stages of oligodendrocyte maturity that will require additional studies to fully clarify.

The absence of PAR1 creates a permissive environment for production of myelin-associated proteins

PAR1 activation by thrombin, or the CNS expressed serine protease, neurosin (kallikrein 6), suppresses expression of the major myelin genes PLP and MBP in primary oligodendrocytes *in vitro* (Burda et al., 2013). Moreover, microinjection of PAR1 agonists into adult spinal cord white matter promotes PAR1-dependent reductions in MBP (Burda et al., 2013). Here we present findings in the context of myelin development that support a model in which

activation of PAR1 is a negative regulator of myelin production, and that its impact on the major myelin proteins, PLP and MBP, occurs in a temporally discrete manner. Deletion of the PAR1 gene results in substantially higher spinal cord PLP levels at birth and during the first postnatal week, whereas MBP levels were elevated in adulthood. Importantly, no changes were observed in the heavy or light chains of neurofilament (NF_H or NF_L) at any age, suggesting the hypermyelinating phenotype in PAR1^{-/-} mice was not dependent on, nor did it elicit, changes in axon number. The unique and parallel regulation of the two major myelin structural proteins, located on distinct chromosomes, by a single receptor points to the thrombin receptor as an integral biological node regulating unique aspects of myelination across the lifespan.

PAR1 gene deletion enhances spinal cord ERK1/2 and AKT signaling during development

ERK1/2 (Fyffe-Maricich et al., 2011; Ishii et al., 2012; Fyffe-Maricich et al., 2013; Ishii et al., 2013) and AKT (Flores et al., 2000; Narayanan et al., 2009; Tyler et al., 2009; Czopka et al., 2010; Goebbels et al., 2010; Harrington et al., 2010; Sherman et al., 2012) are key signaling intermediates playing essential roles in oligodendrocyte development and myelination (Guardiola-Diaz et al., 2012). These pathways are regulated by numerous growth factors such as BDNF, FGF, PDGF, IGF-1 and neuregulin (Canoll et al., 1996; Shi et al., 1998; Baron et al., 2000; Bansal et al., 2003; Fortin et al., 2005; Du et al., 2006; Cui and Almazan, 2007; Frederick et al., 2007; Frost et al., 2009; Van't Veer et al., 2009; Furusho et al., 2011; Xiao et al., 2012). Substantial evidence suggests ERK1/2 is a positive regulator of myelination, whereby its constitutive activation in oligodendrocyte-lineage cells *in vitro*, or *in vivo*, enhances the extent myelination without impacting oligodendrocyte differentiation, or the onset of myelination (Guardiola-Diaz et al., 2012; Ishii et al., 2012; Ishii et al., 2013). Moreover, upregulation of pERK1/2 accelerates remyelination and enhances myelin thickness in the lyssolecithin-demyelinated spinal cord (Fyffe-Maricich et al., 2013). The AKT signaling pathway represents another important positive regulator of myelination with roles in OPC differentiation, the onset of myelination and myelin thickness. The actions of AKT signaling in myelin growth extend to the development of morphological complexity, myelin protein expression, lipid synthesis, and cytoskeletal rearrangement (Narayanan et al., 2009; Tyler et al., 2009; Tyler et al., 2011; Wahl et al., 2014).

The current studies reveal that PAR1 is positioned to serve as a negative regulator of pro-myelination signaling pathways during spinal cord development and further highlight ERK1/2 and AKT as points of convergence of multiple extracellular signals that fine tune myelination. The 'pro-myelination' effect of the PAR1 antagonist, SCH79797 *in vitro*, is encouraging, singling out PAR1 as a potential therapeutic target for demyelinating diseases. However, while it is tempting to speculate that the absence of PAR1 promotes myelination by elevating the activated forms of ERK1/2 and AKT developmentally, we cannot conclude that this link is the cause alone, or that it occurs directly downstream of PAR1 deletion. For example, activation of PAR1 would be expected to increase ERK1/2 signaling (Buresi et al., 2002; Burda et al., 2013). Thus, it would be anticipated that the absence of PAR1 would lead to a decrease of ERK1/2 activation, not an increase, as we observed in spinal cords obtained from PAR1 gene knockout mice. Further studies will be needed to determine how

the absence of PAR1 leads to elevated ERK1/2 activity in the spinal cord and this is most likely to occur by an indirect mechanism. In addition, while no significant impact of PAR1 gene deletion was seen on neurofilament proteins, we cannot exclude other neuronal and non-neuronal effects that could in turn impact the production of myelin. For example, the impact of global PAR1 deletion on other cell types, including potential effects on astrocytes, microglia and endothelial cells, all of which can affect oligodendrocyte metabolism (Nave and Werner, 2014), should also be investigated to help elucidate the likely complex regulatory role exerted by PAR1 in myelin biology.

PAR1 regulates the number of oligodendrocytes available for myelination

Enhancements in PLP, MBP and Olig2 proteins in the spinal cord of PAR1^{-/-} mice across the lifespan may reflect more oligodendroglia or their progenitors, or alternatively, the production of more myelin-associated proteins per cell. To begin to distinguish between these possibilities, we made counts of Olig2- (a pan-oligodendrocyte lineage marker expressed by progenitors and early stage oligodendrocytes), or CC-1-immunopositive cells (expressed by mature oligodendrocytes), in white matter of wild type or PAR1 deficient mice. Since more Olig2-positive cells were enumerated in the spinal cord of PAR1^{-/-} mice in the perinatal period (P0 to P7), the near parallel elevations in Olig2 protein in PAR1^{-/-} spinal cord homogenates likely reflect increases in OPC and/or oligodendrocyte numbers. By the same argument, elevated levels of PLP protein seen at birth through P7 in the spinal cord of PAR1^{-/-} mice may also reflect the presence of more oligodendroglia, since significantly more CC-1-positive cells were enumerated in the PAR1^{-/-} spinal cord on P7. In addition, since PAR1^{-/-} OPCs expressed higher levels of PLP upon differentiation *in vitro*, expression of higher levels of PLP on a per cell basis may also be involved. The elevated levels of MBP in the adult spinal cord, in the absence of elevations in CC-1- or Olig2-positive oligodendroglia, suggests adult PAR1^{-/-} mice likely express more MBP per cell. The presence of thickened myelin sheaths in PAR1^{-/-} mice supports this concept. Whether elevated numbers of Olig2- and CC-1-positive cells in the developing PAR1^{-/-} spinal cord reflect the production of more oligodendrocytes, potential changes in oligodendrocyte specification, accelerated oligodendrocyte differentiation, or reduced programmed cell death will need to be addressed in future studies. The data presented here however, including evidence of earlier axon ensheathment and higher levels of PLP protein in the PAR1^{-/-} spinal cord in first postnatal week, favor a model in which the absence of PAR1 leads to accelerated oligodendrocyte differentiation.

PAR1 can influence oligodendrocyte differentiation and impact myelination by a cell-intrinsic mechanism

In these studies, we localized PAR1 to PDGFR-alpha+ and CC-1+ oligodendroglia in the spinal cord at all post-term intervals examined. However, PAR1 is also functionally linked to neurons (Hamill et al., 2009; Yoon et al., 2013), and astroglia (Nicole et al., 2005; Vandell et al., 2008; Scarisbrick et al., 2012), which may secondarily affect the pattern of myelination observed *in vivo*. Therefore, to determine whether PAR1 is capable of regulating myelination by a mechanism intrinsic to the oligodendrocyte, we examined the impact of PAR1 gene deletion on myelin production by freshly isolated OPCs (0 h), or after a 72 h period of differentiation in culture. While differences were not observed in the

expression myelin associated genes in OPCs, the absence of PAR1 enhanced myelin gene expression, including higher levels of PLP, MBP and Olig2 in cultures of differentiated oligodendroglia. Further supporting a role for PAR1 in oligodendrocyte differentiation, we observed increased numbers of PLP-immunoreactive cells in 72 h differentiated PAR1^{-/-} cultures, and increases in the amount of PLP per cell. These findings further imply that purified OPCs and oligodendrocytes secrete a PAR1 agonist capable of acting by autocrine or paracrine signaling to activate PAR1 *in vitro*. In this regard, we highlight our previous findings demonstrating that the secreted serine protease neurosin is expressed both by OPCs and mature oligodendrocytes *in vitro*, and moreover, that this protease suppresses myelin gene expression by activation of PAR1 (Burda et al., 2013).

The cell intrinsic effects of PAR1 in oligodendrocyte differentiation are also supported by experimental findings that demonstrate a small molecule inhibitor of PAR1, applied to PAR1^{+/+} oligodendrocytes *in vitro*, produces increases in the expression of PLP and MBP, parallel to those seen in OPCs lacking the PAR1 gene. Thus, while an indirect influence of PAR1 gene deletion on other cell types that in turn affect myelination *in vivo* cannot be excluded in the present study, the enhancement of myelin production seen with genetic or pharmacologic PAR1-loss-of function at the level of oligodendrocyte differentiation *in vitro* supports a model in which PAR1 signals in a cell-intrinsic manner to suppress myelin protein production. Together, these observations support the idea that reducing PAR1, genetically or pharmacologically, can accelerate oligodendroglial differentiation. Therefore PAR1, like several other receptor systems, such as TrkB, ErbB, EGF and FGF, IGF-1, AMPA, and Glutamate, is positioned to serve as a key integrator of extrinsic signals that critically influence the complex cascade of events by which OPCs transition to mature myelinating oligodendroglia.

PAR1 is a key regulator of the onset of myelination and the ultimate thickness of myelin achieved

Rapid conduction of action potentials depends on myelination with myelin thickness correlating with axon diameter to maximize velocity (Rushton, 1951). In addition, myelin plays key neuroprotective functions (Wilkins et al., 2003; Ghosh et al., 2011). Ultrastructural analysis of myelin sheaths in the PAR1^{-/-} spinal cord demonstrates earlier axon ensheathment and thickened myelin sheaths in adulthood. Thus, PAR1 impacts the timing of myelination critical for neuroprotection and electrical transmission, and the amount of myelination ultimately achieved. Notably, a characteristic of remyelinated axons in multiple sclerosis is reduced myelin sheath thickness. A key future direction therefore is to determine whether targeting PAR1 can improve remyelination, including progenitor cell generation and differentiation, the rate of myelin repair, and the final level and thickness of myelination achieved. Interestingly, while not yet directly linked to enhancements in spinal cord myelination, adult PAR1^{-/-} mice showed increased locomotor activity.

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Main Points

PAR1 gene deletion results in spinal cord hypermyelination, including more myelinated axons and higher proteolipid protein levels at term and higher levels of myelin basic protein, thicker myelin sheaths and enhanced motor activity in adults.

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Summary

Here we identify the thrombin receptor as a critical biological node that could be targeted in myelin pathologies to improve myelin health. Notably, a common feature of pre-term birth is intraventricular or intraparenchymal hemorrhage which would excessively engage the thrombin receptor leading to a functional blockade of normal myelination. In the model proposed, the absence of PAR1 during myelination, or remyelination, would enhance ERK1/2 and AKT signaling leading to improvements in myelin production. The development of new strategies to target PAR1 therapeutically, in addition to cell specific knockout mice, will be important to validate and extend the current findings and to push therapeutic targeting of PAR1 towards clinical translation in the context of developmental myelination and myelin regeneration in the adult nervous system.

Figure 1. PAR1 expression by PDGFR-alpha positive oligodendrocyte precursor cells and CC-1-positive oligodendrocytes in the early postnatal and adult spinal cord
(A) Immunofluorescence for PAR1 and PDGFR-alpha, or (B) PAR1 and CC-1, in the dorsal column white matter of the spinal cord at P0, P7, P21 and P45. Arrows show a sample of PAR1 co-labeling with PDGFR-alpha on OPCs, or on CC-1 oligodendrocytes, in (A) or (B), respectively. DAPI is shown to visualize all of the cells present in the section. (C) PAR1 RNA expression in whole spinal cord homogenates from PAR1+/+ mice was highest at P0 with nearly two-fold reductions seen by P7 through adulthood (Figure ***P < 0.001, Newman Keuls). (Scale bar = 50 μ m)

Figure 2. PAR1 gene deletion differentially increases PLP and MBP protein levels in the developing and adult spinal cord and is associated with enhanced ERK1/2 and AKT signaling Western blots and associated histograms illustrate that PAR1^{-/-} gene deletion results in significant changes in the expression of myelin-associated proteins (**A** to **E**), in addition to ERK1/2 (**H-I**), and AKT (**H-J**), in homogenates of whole spinal cord. Genetic deletion of PAR1 resulted in higher levels of PLP protein (**B**) at birth (P0) and P7, higher levels of MBP protein (**C**) at P45, and higher levels of Olig2 protein (**E**) at P7 and P21, compared to levels detected in age matched PAR1^{+/+} littermates. By contrast, CNPase levels (**D**), were slightly reduced in PAR1^{-/-} mice at P21. No significant differences in NF_H (**F**) or NF_L (**G**) were observed over the same period. (**H**), Deletion of the PAR1 gene was associated with elevated levels pERK1/2 at P0 and P21 (**I**), and elevated levels of pAKT at P7 and P21 (**J**). No significant impact of PAR1 gene deletion was observed on STAT3 signaling in the same spinal cord samples (**K, L**). ROD readings for pERK and pAKT were normalized to the total protein. Since no pSTAT3 was observed, STAT3 shown is that for the total protein normalized to Actin. Actin was probed on every membrane to control for loading and is shown for the corresponding membrane in the lower panel in (**A**) or (**H**). (*P < 0.05, ** P 0.01, ***P 0.001 Newman Keuls; ND, not detected).

Figure 3. PAR1 gene deletion results in increased numbers of spinal cord oligodendroglia
(A, B) Counts of Olig2-immunopositive cells within the dorsal columns of the spinal cord revealed higher numbers at P0 (1.5-fold, $P = 0.04$, Newman Keuls) and P7 (1.3-fold, $*P = 0.05$, Newman Keuls) in PAR1^{-/-} while the number of CC-1 immunopositive cells **(C, D)** was significantly elevated in PAR1^{-/-} at P7 (1.6-fold, $*P = 0.03$, Newman Keuls). Parallel elevations in Olig2 protein was seen by Western blot (see Figure 2A and E). (Scale bar B and D = 30 μ m).

Oligodendrocytes *In Vitro*

Figure 4. PAR1 gene deletion, or pharmacological inhibition, increases the expression of myelin-associated genes and PLP protein *in vitro*

(A) OPCs significantly down regulate the expression of PAR1 RNA after a 72 h period of differentiation *in vitro* (*** $P = 0.0005$, Students unpaired t-test). (B) Immediately after isolation (0 h) by shaking from mixed glial cultures, PAR1+/+ and PAR1-/- OPCs express similar levels of RNA encoding myelin associated proteins. (C) After a 72 h period of differentiation *in vitro*, PAR1-/- oligodendroglia express higher levels of PLP, MBP and Olig2, but lower levels of NogoA, compared to wild type oligodendroglia cultured in parallel. (*** $P = 0.001$, Students unpaired t-test). (D) Treatment of oligodendrocytes (24 h in culture) with a small molecule inhibitor of PAR1 (SCH79797, 70 nM) for 48 h, promoted a significant increase in the expression of PLP and MBP RNA and a decrease in NogoA and Olig2 RNA. (E, F), Photomicrographs show PLP-immunofluorescence in PAR1+/+ and PAR1-/- OPCs differentiated for 72 h *in vitro*. PAR1-gene deletion (PAR1-/-) was associated with a significant increase in the number of PLP-immunoreactive cells (1.3-fold more, ** $P = 0.03 \times 10^{-5}$, Students unpaired t-test) and in the amount of PLP-immunoreactivity (ROD) per somal area (1.9-fold, ** $P = 0.02 \times 10^{-5}$, Students unpaired t-test), (Scale bar = 20 μm).

Number of Myelinated Nerve Fibers

Figure 5. Myelination occurs earlier and the thickness of the myelin sheath attained is greater in the spinal cord of PAR1^{-/-} mice

(A) At P0, histograms show more myelinated axons were counted in the dorsal column white matter of paraffin-embedded (PPD) stained spinal cord sections taken from PAR1^{-/-} compared to PAR1^{+/+} mice on P0 (*P = 0.02, Students unpaired t-test). Photomicrographs show examples of PPD stained myelin sheaths in the dorsal column of PAR1^{+/+} and PAR1^{-/-} mice. At P45, while parallel numbers of PPD stained myelinated axons were observed in the spinal cord dorsal columns of PAR1^{+/+} and PAR1^{-/-} mice, the number of myelinated fibers with a diameter greater than 10 μm² was significantly increased in mice lacking PAR1 (*P = 0.02, Students unpaired t-test). (B) Representative electron micrographs taken from the spinal cord dorsal column white matter of PAR1^{+/+} or PAR1^{-/-} mice are shown. These micrographs were used to calculate g-ratios and myelin thickness which are plotted relative to axon diameter at P0 and P45 (C). At P0, mean myelin thickness across all axon axons was significantly greater in PAR1^{-/-} (0.22 ± 0.007 μm) compared to PAR1^{+/+} mice (0.19 ± 0.012 μm, P = 0.02, Students t-test). At P45, mean g-ratios were significantly lower in PAR1^{-/-} mice (0.71 ± 0.002) compared to PAR1^{+/+} mice (0.75 ± 0.002, P=0.29x10⁻⁶⁸, Students t-test) and myelin thickness was significantly greater (PAR1^{-/-} = 0.37 ± 0.004 μm; PAR1^{+/+} = 0.28 ± 0.003 μm; P=0.66x10⁻⁶³, Students t-test). (D) Electron micrographs from the spinal cord dorsal column of PAR1^{+/+} or PAR1^{-/-} mice show representative images demonstrating the relative thickness of myelin sheaths wrapping 2, 1.3 or 0.5 μm axons. (Scale bar, A = 10 μm; B = 2 μm, D = 0.2 μm).

Figure 6. PAR1 gene deletion results in increased locomotor activity in adult mice

A comprehensive laboratory animal monitoring system was used to demonstrate that PAR1^{-/-} mice have (A), higher total activity under fed day (*P = 0.04) or fasted night conditions (*P = 0.02) and (B), higher ambulation (*P = 0.02) and (C), rearing (*P = 0.04) under fasted night conditions (Students unpaired t-test).

Figure 7. Reductions in PAR1 enhance developmental myelination

Findings presented suggest a model in which high levels of OPC PAR1 expression limit their capacity to differentiate towards a myelinating phenotype. PAR1 gene deletion is associated with an earlier onset of PLP protein expression and elevated levels of MBP in the adult spinal cord. Co-ordinate early elevations in the pro-myelination signaling intermediates ERK1/2 (Fyffe-Maricich et al., 2011; Ishii et al., 2013) and AKT (Flores et al., 2000; Narayanan et al., 2009; Tyler et al., 2009) were also observed. OPCs lacking the PAR1 gene, or treated with a PAR1 small molecule inhibitor (SCH79797), also showed enhanced expression of PLP and MBP *in vitro*. Together these data support a model where PAR1 can suppress CNS myelination and that elevated signaling through the ERK1/2 and AKT pathways are likely involved.

Table 1

Primers used for quantitative real-time PCR

Gene	Accession number	Primer Sequence Forward/Reverse
MBP	NM_001025251	CCAGTAGTCCATTCTTCAAGAACAT/ GCCGATTATAGTCGGAAGCTC
PLP	NM_011123.2	TCTTTGGCGACTACAAGACCAC/ CACAAACTGTCTGGGATGTCCTA
NogoA	NM_024226.4	Applied Biosystem, Assay ID: Mm00445861_m1
Olig2	NM_016967	Assay ID: Mm.PT.56a.42319010
PAR1	NM_010169.3	CTTGCTGATCGTCGCCC/ TTCACCGTAGCATCTGTCCT
Rn18S	NR_003278.3	Applied Biosystems, Assay ID: Mm03928990_g1

All primers were obtained from Integrated DNA Technologies (IDT) unless otherwise indicated.