Fyn is an Intermediate Kinase that BDNF Utilizes to Promote Oligodendrocyte Myelination

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Fyn, a member of the Src family of nonreceptor tyrosine kinases, promotes central nervous system myelination during development; however the mechanisms mediating this effect remain unknown. Here we show that Fyn phosphorylation is modulated by BDNF *in vivo*. Concordant with this, we find that BDNF stimulates Fyn phosphorylation in myelinating cocultures, an effect dependent on oligodendroglial expression of TrkB. Importantly, PP2, a pharmacological inhibitor of Src family kinases, not only abrogated the promyelinating influence of BDNF *in vitro*, but also attenuated BDNF-induced phosphorylation of Erk1/2 in oligodendrocytes. Over-expression of Fyn in oligodendrocytes significantly promotes phosphorylation of Erk1/2, and promotes myelination to the extent that exogenous BDNF exerts no additive effect *in vitro*. In contrast, expression of a kinase-dead mutant of Fyn in oligodendrocytes significantly inhibited BDNF-induced activation of Erk1/2 and abrogated the promyelinating effect of BDNF. Analysis of white matter tracts *in vivo* revealed that phosphorylated Fyn primarily colocalized with mature oligodendrocytes, and was rarely observed in oligodendrocyte progenitor cells, a profile that closely parallels the detection of phosphorylated Erk1/2 in the developing central nervous system. Taken together, these data identify that Fyn kinase exerts a key role in mediating the promyelinating influence of BDNF. Here we identify a pathway in which BDNF activation of oligodendroglial TrkB receptors stimulates the phosphorylation of Fyn, a necessary step required to potentiate the phosphorylation of Erk1/2, which in turn regulates oligodendrocyte myelination.

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Key words: BDNF, ERK, Fyn, MAPK, myelination, oligodendrocyte, Src family kinase

Introduction

In the vertebrate nervous system, axons are wrapped in an insulating myelin sheath. Myelin not only increases the speed and efficiency of impulse transduction along axons, but also provides metabolic support to the axons that are myelinated (Nave and Werner, 2014; Todorich et al., 2009). Oligodendrocytes, the myelin forming cells in the central nervous system (CNS), are derived from oligodendrocyte precursor cells (OPCs), which proliferate and migrate throughout the CNS before further differentiating into postmitotic myelinating oligodendrocytes (Baumann and Pham-Dinh, 2001). The progression from an OPC to a myelinating oligodendrocyte is intrinsically regulated by a number of key transcription factors which are responsive to extracellular cues such as axonal activity and other factors or ligands either secreted or expressed on the surface of axons (Emery, 2010; Mitew et al., 2014; Taveggia et al., 2008). The networks that integrate extrinsic signals into coherent intracellular signaling cascades have not been fully elucidated.

The nonreceptor-type tyrosine kinase Fyn, a member of the Src family of kinases, is implicated in regulating oligodendrocyte myelination. Fyn kinase (isoform 1 or B) is expressed in oligodendrocytes and neurons (Umemori et al., 1992). The expression and kinase activity of Fyn is upregulated during OPC differentiation, and peaks during the initial and most active stages of myelination *in vivo* (Kramer et al., 1999). The kinase activity of Fyn is required for oligodendrocyte differentiation *in vitro* (Bauer et al., 2009; Osterhout et al., 1999; Wolf et al., 2001) and knockout studies show

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Fyn kinase is required for normal CNS myelination *in vivo* (Goto et al., 2008; Sperber et al., 2001; Umemori et al., 1999). Mice that lack the Fyn protein or that have a mutation (K296R) blocking the kinase activity of Fyn exhibit a marked reduction in myelin protein expression and the number of myelinated axons in their forebrain and corpus collosum (Goto et al., 2008; Sperber et al., 2001), and reduced myelin thickness in their spinal cord (Umemori et al., 1999). A more recent study in Zebrafish shows that oligodendrocyte-specific activation of Fyn kinase significantly increases the number of myelinated axons in the spinal cord, whereas a reduction of Fyn kinase decreases that number (Czopka et al., 2013). Collectively these data demonstrate that Fyn activity plays a key role in regulating oligodendrocyte myelination during development; however, the mechanisms that regulate Fyn activity and the targets of Fyn kinase remain unknown.

Recent evidence from other cell systems indicate that Fyn crosstalks with other signaling pathways. For example, BDNF stimulation of cortical and hippocampal neurons *in vitro* results in an association between endogenous TrkB and Fyn, and Fyn-dependent translocation of TrkB receptors to lipid rafts (Pereira and Chao, 2007). Interestingly, BDNF also exerts a strong promyelinating influence both *in vitro* and *in vivo* (Cellerino et al., 1997; Vondran et al., 2010; Xiao et al., 2010), an effect shown to be mediated through oligodendroglial-expressed TrkB receptors and activation of extracellular signal-regulated kinases (Erk) 1/2 (Wong et al., 2013; Xiao et al., 2010, 2012). It is notable that Fyn kinase is able to interact with multiple components of the MAPK signaling pathway either directly upstream of Erk1/2 (Chao, 2003; Huang and Reichardt, 2003), or indirectly via GTPase activating proteins (Liang et al., 2004). Together, these findings suggest that Fyn kinase could be an important intermediary between TrkB receptors at the cell membrane and the cytoplasmic MAPK signaling pathway components Erk1/2 to regulate oligodendrocyte myelination.

In this study, we demonstrate that Fyn kinase acts upstream of Erk1/2 to mediate the promyelinating effect that BDNF exerts upon oligodendrocytes. Our data also suggest that the hypomyelinating phenotype of Fyn knockout mice could at least in part be due to a contextual loss of Erk1/2 signaling in oligodendroglia during myelination.

Materials and Methods

Animals and Reagents

Postnatal day 2 (P2) or P7-8 Sprague-Dawley (S/D) rats were used for primary dorsal root ganglion (DRG) neuron and OPC culture. BDNF+/− (Jackson laboratory, #002266, B6.129S4-BDNF$^{<tm1Jae>/J}$) and wild-type (BDNF+/+) littermates were used for Western blot analysis, and C57/B6 wild-type mice for immunohistochemistry. All animals used for this study were of mixed sex and bred at the Animal Facilities of the Biomedical Sciences Academic Centre and Florey Institute of Neuroscience and Mental Health at the University of Melbourne. All animal procedures were approved by Animal Experimentation Ethics Committees at the University of Melbourne. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

DRG Neuron Culture

The culture of DRG neurons has been described previously (Peckham et al., 2015; Xiao et al., 2009). Briefly, DRG neurons were isolated from P2 S/D rats and plated on to polyornithine (P3655) and laminin (23017015 Life Technologies) coated coverslips. Following dissection neurons were cultured in alternate rounds of M1 and M2 for 2/3 days beginning with M2 serum-free media containing DMEM (10313039 Life Technologies), rat transferrin (10 mg/L, T1147), insulin (5 mg/L, I6634), progesterone (20 nM, P8783), and putrescine (100 μm, P5780) in the presence of antimitotic reagents (10 μM 5-fluoro-2′-deoxyuridine F0503 and 10 μM uridine, U3003), and M1 media (MEM 10370088 Life Technologies containing 10% heat-inactivated fetal calf serum (12003C), 0.4% glucose (G7528) and 2 nM L-glutamate (G1626) with both M1 and M2 containing nerve growth factor (100 ng/mL, #N100 Alomome Labs) for 2–3 weeks.

OPC Culture

OPCs were isolated from P7–8 S/D rat brain by sequential immunopanning as previously described (Peckham et al., 2015; Xiao et al., 2010, 2012). Briefly, cerebral hemispheres were diced and digested with papain (20 U/mL, LS003126 Worthington Biochemical Cooperation) at 37°C. Cells were isolated through three sequential immunopanning dishes: Ran-2 (TIB119 ATCC), anti-O1 (MAB344 Millipore) and anti-O4 (MAB345 Millipore). O4 + O1 − OPCs were released from the final panning dish with trypsin EDTA (25300-054 Life Technologies) and cultured in SATO medium as previously described (Peckham et al., 2015).

Lentivirus Preparation and OPC Infection

The original kinase dead (KD)-Fyn (K299M) and wild-type (WT)-Fyn plasmids (Takeuchi et al., 1993) were subcloned into the lentiviral plasmid 2K7 as previously described (Peckham et al., 2015; Xiao et al., 2012). Lentivirus was prepared from the final lentiviral plasmids (2K7-CMV-IRES-EGFP Amp [control empty vector], 2K7-CMV-Flag-KD-Fyn-IRES-EGFP Amp and 2K7-CMV-Flag-WT-Fyn-IRES-EGFP Amp) by transfection into HEK293T cells as previously described (Peckham et al., 2015; Xiao et al., 2012). The concentrated lentiviral supernatant was titered on HEK293T cells and O4 + O1 − OPCs were infected with a virus concentration that maximized protein expression with no obvious cell death. For the myelination assay, O4 + O1 − OPCs were infected with lentivirus for 48 h prior to seeding on to DRGs. To confirm the infection efficiency of the OPCs in coculture settings, 50% of infected OPCs were seeded on to DRG neurons for the myelination assay, the remaining sister OPCs were cultured for additional 72 h and then used to verify the level of Fyn/Flag expression by Western blot analysis.

Purified DRG-OPC Cocultures

DRG-OPC cocultures were established based on published techniques (Peckham et al., 2015; Xiao et al., 2010, 2012). Briefly, OPCs were seeded onto coverslips (200,000 OPCs/22-mm coverslip) containing purified DRGs under the same approximate axon density. The cocultures were maintained for 14 days in a defined coculture media as previously described (Peckham et al., 2015; Xiao et al., 2012) in the presence of factors as indicated. Cocultures were re-fed with the coculture medium and treated with factors (BDNF, PP2, or PP3) as indicated every 2–3 days. For some experiments, DRG neurons were cocultured with OPCs infected with lentiviral particles that overexpress empty (GFP control), WT-Fyn or KD-Fyn. These cocultures were treated with or without BDNF (100 ng/mL), with media changes every 2–3 days. Cocultures were analyzed by Western blot for myelin protein expression and immunocytochemistry for visualizing the formation of myelinated axonal segments as described previously (Peckham et al., 2015; Xiao et al., 2010, 2012).

Western Blot Analysis

DRG-OPC cocultures, OPC cultures, or mice spinal cord and brain tissues were lysed, separated by SDS-PAGE, transferred to PVDF membrane, and variously probed with antibodies against 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase, MAB326, Chemicon), myelin-associated glycoprotein (MAG, MAB381, Chemicon), myelin basic protein (MBP, AB980, Chemicon), p44/42MAPK (Erk1/2, #9102; phospho-Erk1/2, #9101) (Cell Signaling Technology), Fyn (sc-434, Santa Cruz Biotech), Src[pY418] (#44660G, Invitrogen), Src[pY529] (#44662G, Invitrogen), TrkB (#sc-8316 Santa Cruz Biotech), Flag (#3165, Sigma), or β-actin (#5541, Sigma). All blots shown are representative of at least three independent experiments/cultures or three mice per genotype. The optical density value for each band was determined using Biorad Image Lab or NIH ImageJ, corrected to a loading control, and normalized as indicated. Quantification of phosphorylated proteins was normalized against the native form of each protein. Statistical analyses were performed using one-way or two-way ANOVA tests with Bonferroni's post hoc tests in Graphpad Prism 5.

Immunocytochemistry

Myelinating cocultures were fixed with 4% paraformaldehyde (PFA) and myelinated axonal segments were visualized with anti-MBP (MAB 381 Millipore) antibody followed by incubation with fluorophore-conjugated secondary antibodies (Molecular Probes, A11008 and A11005). An antineurofilament antibody was used to ensure that myelination occurred under the same approximate axonal density. Between six and eight fields/images per condition per experiment were captured by fluorescent or confocal microscopy (Carl Zeiss) and the number of MBP+ myelinated axonal segments in each field were counted, blinded to condition. A minimum of three independent experiments/cultures were conducted, and the data were statistically analyzed using one-way or two-way ANOVA tests with Bonferroni's post hoc tests in Graphpad Prism 5.

Live/Dead, Proliferation, and Differentiation Assays

The effect of PP2 on OPC survival, proliferation, and differentiation was examined as previously described (Xiao et al., 2010). Briefly, OPCs (10,000 cells/coverslip) were seeded on to DRG neurons and treated with PP2 (10 μM) or PP3 (10 μM, a control peptide of PP2) for 48 or 96 h. For the live and dead assay, ethidium homodimer-1 (4 μM; Invitrogen) and calcein AM (4 μM; Invitrogen) were added to each well followed by visualization under a fluorescent microscope (Carl Zeiss). The number of live (calcein AM positive) and dead (ethidium homodimer-1 positive) cells per field ware counted and the percentage of dead cells (relative to total number of cells) calculated. For the proliferation assay, cells were double-labelled with anti-BrdU (BD Biosciences, #347580, 1:200) and anti-A2B5 IgG hybridoma supernatant (1:2, a general marker of OPCs), followed by fluorophore-conjugated secondary antibodies (Molecular Probes) as well as DAPI to label the nuclei. The number of BrdU and A2B5 double-positive proliferating OPCs were counted and their percentage calculated relative to the total number of DAPI+ cells. For the differentiation assay, cells were stained with an anti-MBP antibody to mark postmitotic oligodendrocytes as well as DAPI to label nuclei. The number of MBP-positive cells was counted and their percentage calculated relative to the total number of DAPI+ cells. Groups were statistically analyzed and all data presented as mean ± SEM.

Immunohistochemistry

C57/B6 mice aged between P9 and P30 were perfused transcardially with 4% PFA, the lumbar spinal cord and brain dissected, 10 μm cryosections separated by at least 80 μm collected as previously described (Binder et al., 2011). Sections were triple-immunostained with anti-PDGFRα (#558774, BD Pharmingen™, 1:200), anti-CC1 (#OP80, CalBioChem, 1:500), and Src[pY418] (#44660G, Invitrogen, 1:200) antibodies followed by the appropriate fluorophore-conjugated secondary antibodies (Molecular Probes). Sections were washed in PBS, and mounted in DAKO mounting medium with DAPI. Three to six sections per mouse from three mice were analyzed, and images captured by confocal microscopy.

Results

Fyn Kinase is Detected in Oligodendrocytes In Vivo

It has been previously shown that Fyn KO mice exhibit hypomyelination in the brain and spinal cord (Goto et al., 2008; Sperber et al., 2001; Umemori et al., 1999). To determine the mechanisms that Fyn kinase utilizes to promote CNS myelination, we first assessed Fyn kinase phosphorylation in oligodendroglial lineage cells within the brain and spinal cord *in vivo*. We performed triple immunostaining for PDGFRα, CC1 and phospho-Fyn (catalytic autophosphorylation of Fyn at site Y418) (Colognato et al., 2004) in mice from P9 to P30, and analyzed PDGFRα+ OPCs and CC1+ postmitotic oligodendrocytes that were also positive for activated Fyn in the corpus callosum and white matter tracts of the lumbar spinal cord. Analysis of PDGFRα+ OPCs identified that the vast majority of these cells were negative for phospho-Fyn in

both regions from P9 to P30. Qualitative immunohistochemical analysis of the corpus callosum (Fig. 1A, higher magnification in A′) and lumbar spinal cord lateral column (Fig. 1B, higher magnification in B′) revealed very few PDGFRα+/phospho-Fyn+ cells at ~P9 (Fig. 1). In contrast, analysis of adjacent sections double labelled for pY418 and CC1 revealed

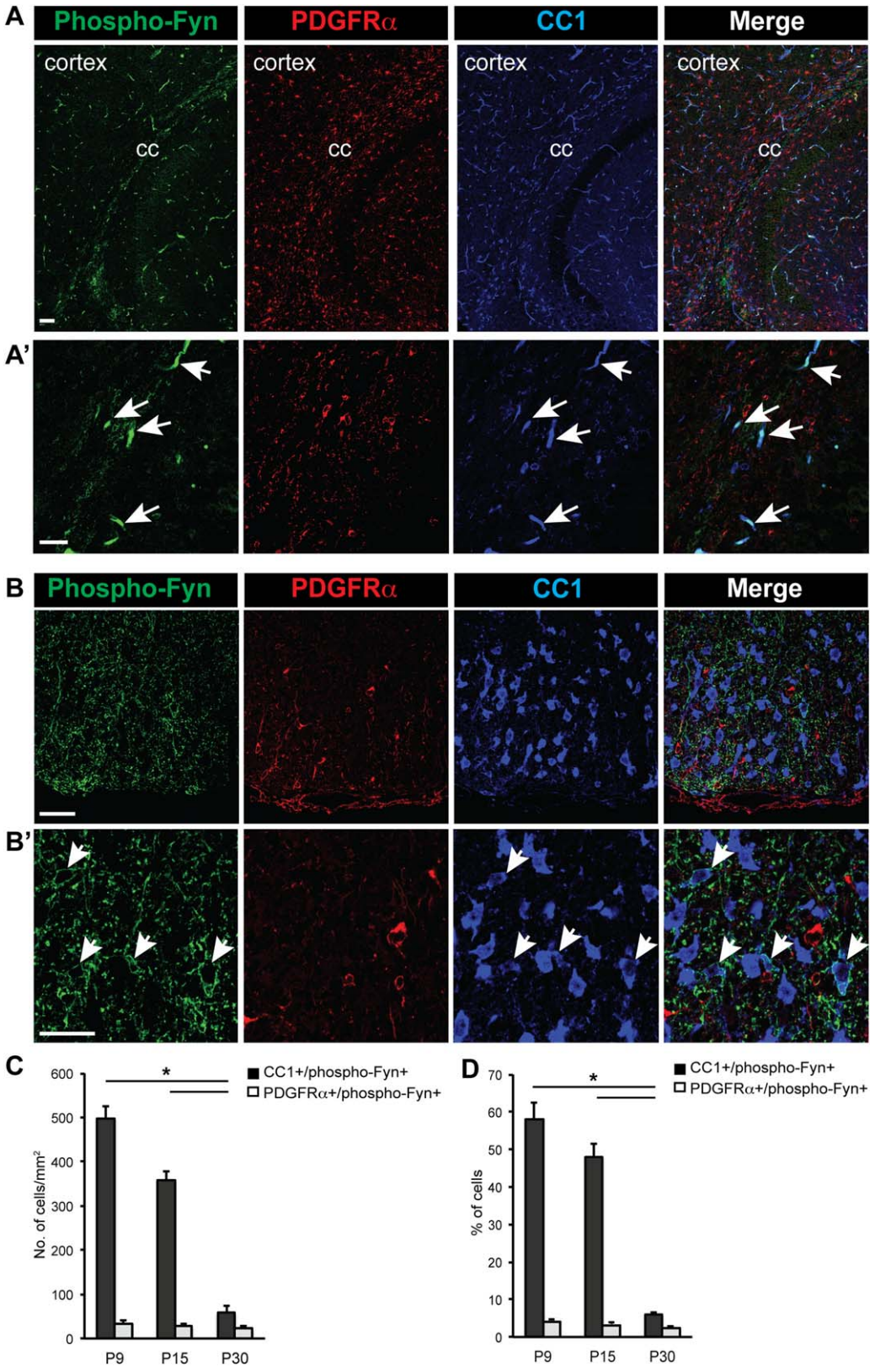

FIGURE 1

the majority of phospho-Fyn+ cells were co-localized with CC1+ postmitotic oligodendrocytes in the corpus callosum (Fig. 1A, higher magnification in A′) and lumbar spinal cord lateral column (Fig. 1B, higher magnification in B′) at ∼P9. Similar results were also found at ∼P15 (data not shown). However, phospho-Fyn immunoreactivity was rarely detectable in CC1+ cells at P30 in both regions (data not shown). Noticeably, the expression of phospho-Fyn, at both P9 and P15, is detected within the oligodendrocyte cell bodies in the corpus collosum (Fig. 1A′, arrows), but around the oligodendrocyte cell bodies in the spinal cord white matter tracts (Fig. 1B′, arrows), suggesting different subcellular locations of Fyn activity within oligodendrocytes between brain and spinal cord. Quantitative analysis of brain sections from P9 to P30 revealed that, in the midline corpus collosum, there is a significant reduction in the number of CC1+/phospho-Fyn+ mature oligodendrocytes from P9 to P30, whereas the density of PDGFRα+/phospho-Fyn+ OPCs remains unchanged over the same period (Fig. 1C,D, $^*P < 0.05$). This suggests that Fyn kinase activity peaks during the initial stages of myelination and is subsequently rapidly downregulated later postnatally. This finding is consistent with a previous study showing that Fyn kinase phosphorylation peaks during the initial phase of myelination and rapidly downregulated from the third postnatal week *in vivo* (Kramer et al., 1999). Collectively, our data demonstrate that Fyn kinase activity is predominantly present in postmitotic oligodendrocytes and its activity within oligodendrocytes peaks during early myelination *in vivo*, suggesting it exerts a key influence upon mature myelinating oligodendrocytes and their subsequent myelination.

Phosphorylation of Fyn Kinase is Modulated by BDNF During Postnatal Development In Vivo

To determine whether BDNF regulates Fyn kinase activity to promote oligodendrocyte myelination, we first examined myelin protein expression and Fyn kinase phosphorylation in both brain and spinal cord lysates generated from BDNF heterozygous (+/−) and littermate wild-type (+/+) mice. Consistent with previous reports (Cellerino et al., 1997; Vondran et al., 2010; Xiao et al., 2010), BDNF+/− mice exhibited significantly reduced expression of the myelin protein MBP at P9 and P15 compared to wild-type littermate controls, which normalized by P30 in both the brain (Fig. 2A, quantitated in B) and spinal cord (Fig. 2E, quantitated in F). The reduction in MBP expression correlated with a significant reduction in the level of active Fyn kinase (autophosphorylated at Y418) at the same time points in both the brain (Fig. 2A, quantitated in C) and spinal cord (Fig. 2E, quantitated in G). In addition, the level of inactive Fyn kinase, as assessed by inhibitory phosphorylation at Y529, was significantly increased in BDNF+/− mice compared to wild-type littermate controls at P9 and P15 in the brain (Fig. 2A, quantitated in D), but only at P9 in the spinal cord (Fig. 2E, quantitated in H). Importantly, the expression of total Fyn was unaffected between genotypes, whereas the level of BDNF expression was consistently reduced in BDNF+/− mice (Fig. 2A,E). These data therefore further demonstrate that a high level of Fyn kinase activity is detected in mice from P9 to P15, a period when the CNS is initiating and actively myelinating *in vivo*. Later, at P30, when myelination is almost complete, Fyn activity is rapidly downregulated. These findings are consistent with our data (Fig. 1C,D) and a previous report showing that Fyn kinase activity peaks during the early postnatal weeks *in vivo* also by Western blot analysis (Kramer et al., 1999). Importantly, our data suggest that the Fyn kinase activity is regulated by BDNF *in vivo*, and that this correlates with the level of myelination during development.

BDNF Stimulates Fyn Phosphorylation in Oligodendrocytes in a TrkB-Dependent Manner

We next sought to determine whether BDNF modulates Fyn kinase phosphorylation specifically in oligodendrocytes isolated from the brain. Differentiated oligodendrocytes were treated with BDNF (100 ng/mL for 0–60 min), lysed, and analyzed by Western blot (Fig. 3). Analysis of Western blot bands indicate that BDNF rapidly and significantly increases Y418 phosphorylation of Fyn kinase, however phosphorylation of Y529 is unaffected (Fig. 3A, quantitated in B and C, $^*P < 0.05$).

FIGURE 1: Fyn kinase activity is presented in oligodendrocytes *in vivo*. Representative images of phospho-Fyn, CC1, and PDGFRα triple-immunostaining in sagittal brain sections (A, corpus callosum shown in A′ with higher magnification) and in transverse sections of the lumbar spinal cord (B, higher magnification in B′) from P9 mice. In the cortex, corpus callosum (cc) and lumbar spinal cord white matter tracts, the majority of CC1+ oligodendrocytes are positive for phospho-Fyn immunoreactivity (arrows = colocalized cells in corpus callosum [A′] and lumbar spinal cord ventral column [B′]), whereas phospho-Fyn is absent in the vast majority of PDGFRα+ OPCs (Scale bars: 50 μm). (C) Quantification of the density of PDGFRα+/phospho-Fyn+ OPCs and CC1+/phospho-Fyn+ mature oligodendrocytes in the midline corpus callosum between P9 and P30. There is a significant reduction in the density of CC1+/phospho-Fyn+ mature oligodendrocytes from P9 to P30, whereas the density of PDGFRα+/phospho-Fyn+ OPCs remains unchanged during early postnatal development ($n = 3$ mice/age group, $^*P < 0.05$, one-way ANOVA followed by Bonferroni's post hoc tests). (D) Quantification of the percentage of PDGFRα+/phospho-Fyn+ OPCs and CC1+/phospho-Fyn+ mature oligodendrocytes in the lumbar spinal cord lateral column between P9 and P30. There is a significant reduction in the proportion of CC1+/phospho-Fyn+ mature oligodendrocytes from P9 to P30, whereas the proportion of PDGFRα+/phospho-Fyn+ OPCs remains unchanged during early postnatal development ($n = 3$ mice/age group, $^*P < 0.05$, one-way ANOVA followed by Bonferroni's post hoc tests).

Phosphorylation of Y418 results in activation of Fyn kinase, whereas phosphorylation of Y529 inactivates Fyn. These data indicate that in oligodendrocytes, BDNF stimulates the kinase activity of Fyn. To verify that oligodendroglial-expressed TrkB receptors mediate Fyn kinase phosphorylation, we infected primary OPC cultures with lentivirus containing either scrambled (control) or TrkB-shRNA for 48 h, allowed the OPCs to differentiate for a further 72 h and then stimulated with BDNF (100

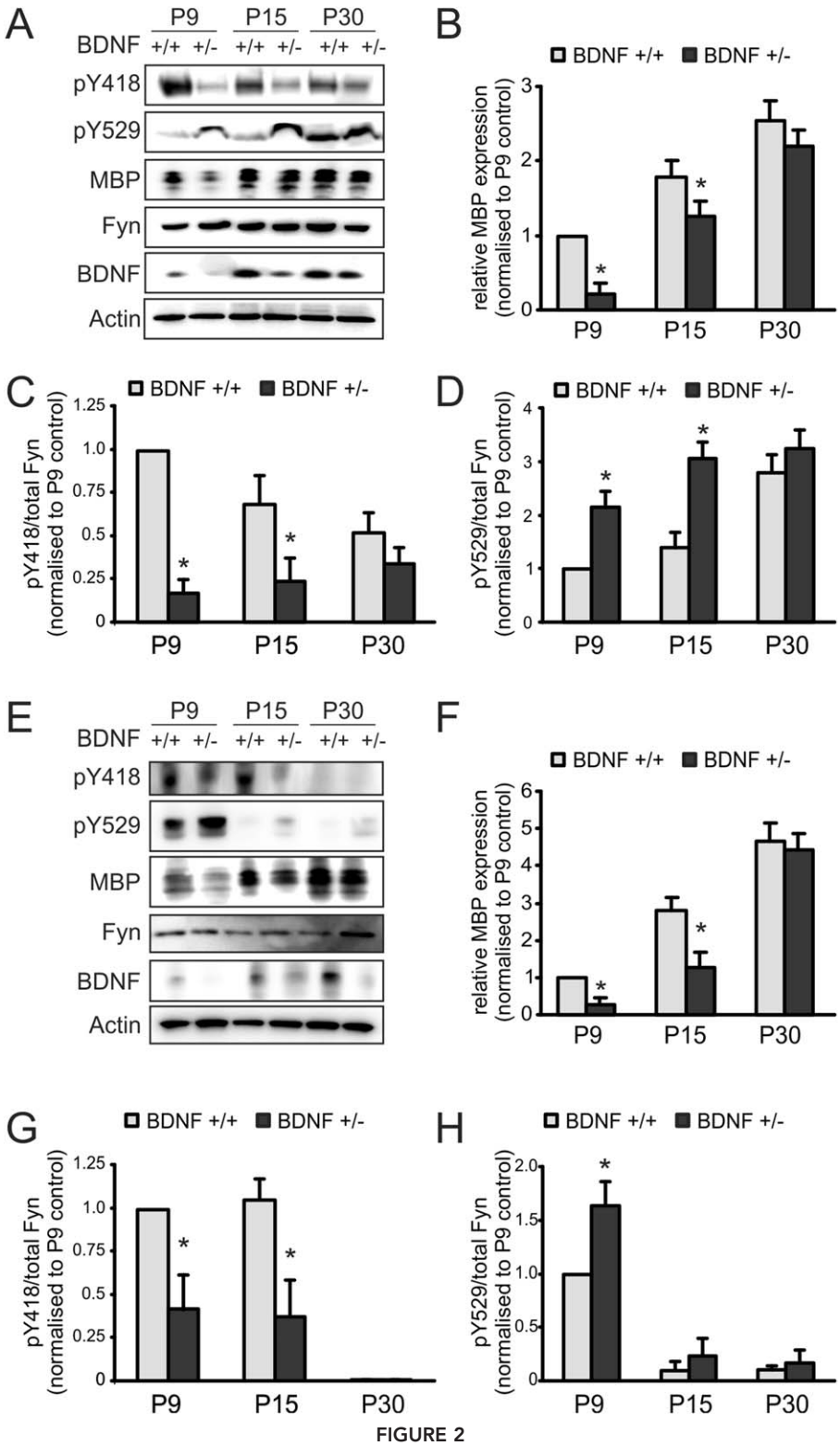

FIGURE 2

ng/mL) for 10 min before analyzing cell lysates by Western blot. The TrkB-shRNA appreciably decreased TrkB expression in oligodendrocytes, but exerted no effect upon Fyn expression (Fig. 3D). The addition of BDNF significantly increased Y418 phosphorylation of Fyn in the control (scramble RNA) oligodendrocytes, but this effect was significantly abrogated in oligodendrocytes whose TrkB expression was knocked down via TrkB-shRNA (Fig. 3D, quantitated in E). In contrast, the inhibitory phosphorylation at Y529 was not affected by either knockdown of TrkB expression or the addition of BDNF (Fig. 3D). Together these results demonstrate that BDNF acts on oligodendroglial TrkB receptors to stimulate the phosphorylation of Fyn kinase at Y418.

Fyn Phosphorylation is Increased during BDNF-Induced Myelination In Vitro

We next investigated if the activity of Fyn kinase was modulated by BDNF in the context of myelination, using *in vitro* myelinating cocultures. Myelinating cocultures were treated with BDNF (0–100 ng/mL) over 14 days, lysed, and analyzed by Western blot. BDNF increased the Y418 phosphorylation of Fyn in a dose-dependent manner (Fig. 4A, quantitated in B, $^*P < 0.05$), whereas the phosphorylation of Y529 was unaffected by BDNF in the myelinating coculture settings (Fig. 4A, quantitated in C). Densitometric analyses of Western blots bands showed that BDNF significantly increased Y418 phosphorylation of Fyn at concentrations of 10 and 100 ng/mL (Fig. 4B, $^*P < 0.05$). Taken together, we have demonstrated that BDNF promotes Fyn kinase phosphorylation within oligodendrocytes *in vitro*, and also in the context of myelination. Our data show that BDNF selectively stimulates phosphorylation of the site that activates Fyn kinase, rather than suppressing the phosphorylation of the site that inhibits Fyn.

Inhibition of Src Family Kinase Signaling Abrogates BDNF-Induced Myelination In Vitro

Having demonstrated BDNF modulated Fyn kinase activity *in vivo* and *in vitro*, we next investigated the influence that Fyn kinase activity exerted upon the promyelinating influence of BDNF. To address this, we first utilized pharmacological inhibitors of Src family kinases in *in vitro* myelination assays. Myelinating cocultures were treated with either BDNF (100 ng/mL), PP2 (a peptide inhibitor of Src family kinases, 10 μM), BDNF + PP2, or the inactive control peptide PP3 (10 μM) for 14 days. The cocultures were either lysed for Western blot analysis or fixed and immunostained. As expected, BDNF promoted myelination, as assessed by a significant increase in the expression levels of myelin proteins CNPase, MAG, and MBP (Fig. 5A, MBP quantitated in B) and the number of MBP+ myelinated axonal segments (Fig. 5C, quantitated in D, $^*P < 0.05$) compared to the untreated control (control). In isolation, the Fyn inhibitor PP2 inhibited the basal expression of myelin proteins (Fig. 5A,B, $^*P < 0.05$), and significantly reduced the formation of MBP+ myelinated axonal segments compared to the untreated control (Fig. 5C, quantitated in D, $^*P < 0.05$). The control peptide PP3 exerted no significant effect upon either myelin protein expression or the formation of myelinated axonal segments (Fig. 5A–D). This indicates that Fyn activity is critical for the relatively modest levels of myelination that occur in the untreated control condition. To assess the influence that Fyn kinase activity exerted upon the promyelinating effect of BDNF, cocultures were treated with BDNF and PP2 (PP2 + B). We found that the presence of PP2 completely abrogated the influence that BDNF exerted upon both myelin protein expression and formation of MBP+ myelinated axonal segments (PP2+B, Fig. 5A–D, $^*P < 0.05$), suggesting that PP2 not only exerted a significant inhibitory effect upon myelination, but inhibited the promyelinating effect of BDNF. To verify the inhibitory effect that PP2 exerted was direct, and not secondary to effects upon OPC survival, proliferation or differentiation, we performed live/dead, proliferation and differentiation assays in cocultures treated with PP2 or PP3 for 48 and 96 h (Fig. 5E–G). Quantitative analyses of these assays revealed that neither PP2 nor PP3 exerted any influence upon OPC survival (Fig. 5E) or the number of BrdU+/A2B5+ proliferating OPCs (Fig. 5F). Quantitative analysis of the number of MBP+ oligodendrocytes revealed that the proportion of MBP+ oligodendrocytes was significantly reduced by

FIGURE 2: Reduced phosphorylation of Fyn kinase in BDNF+/− mice during postnatal development *in vivo*. (A) Western blot and (B–D) densitometric analysis of Western blot bands from brain lysates derived from BDNF+/+ and BDNF+/− mice between P9 and P30. The lysates were probed with antibodies against two Src family kinase sites: phosphoY418 (catalytic autophosphorylation site, pY418) and phosphoY529 (COOH-terminal negative regulatory site, pY529). Compared to wild-type littermate controls, BDNF+/− mice exhibited significantly reduced levels of active Fyn phosphorylation (pY418), and significantly increased levels of inhibitory Fyn phosphorylation pY529 at P9 and P15. At these same time points, BDNF+/− mice also exhibited a significant reduction in myelin protein (MBP) expression. All data normalized to control levels at P30 (data = mean ± SEM, $n = 3$ mice/genotype/age, $^*P < 0.05$, Student's *t* test). (E) Western blot and (F–H) densitometric analysis of Western blot bands rom spinal cord lysates derived from BDNF+/+ and BDNF+/− mice between P9 and P30. The lysates were probed with antibodies as described in (A–D). Compared to wild-type littermate controls, the level of active Fyn phosphorylation (pY418) was significantly reduced in BDNF+/− mice at P9 and P15, but normalized by P30. The level of Fyn phosphorylation at the inhibitory site pY529 was significantly increased in BDNF+/− mice at P9. BDNF+/− mice exhibited a significant reduction in myelin protein (MBP) expression at P9 and P15 (data = mean ± SEM, $n = 3$ mice/genotype/age, $^*P < 0.05$, Student's *t* test).

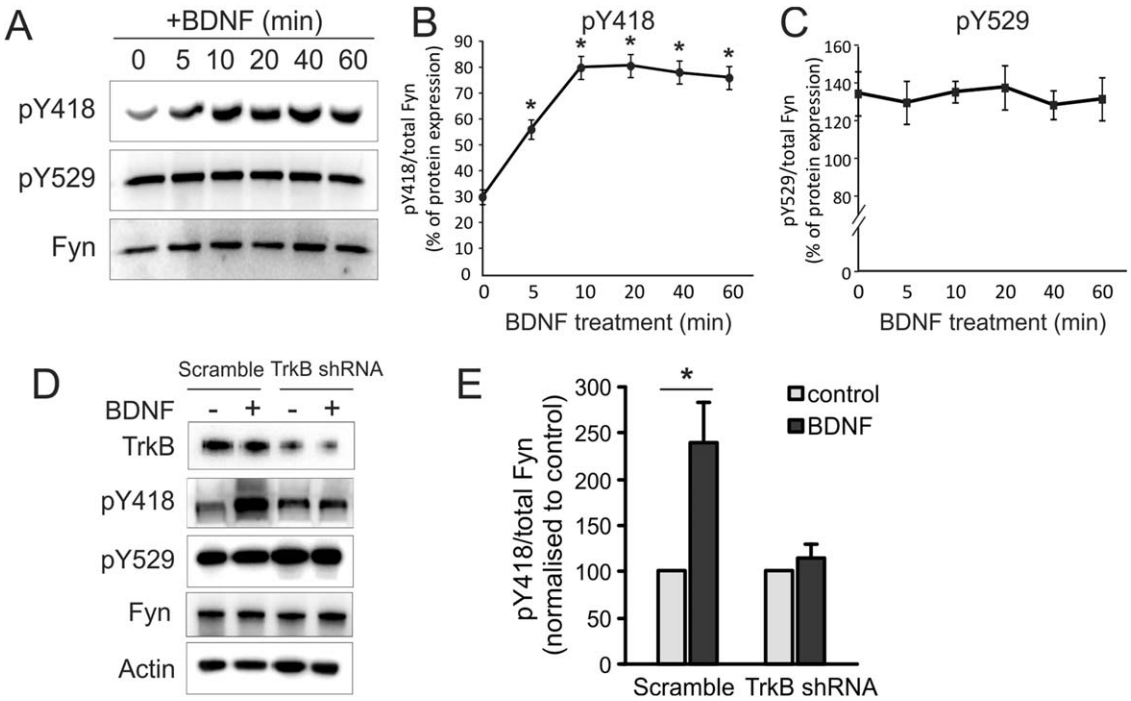

FIGURE 3: BDNF stimulates Fyn phosphorylation in oligodendrocytes in a TrkB dependent manner. (A) Western blot including (B,C) densitometric analysis of Western blot bands from oligodendrocyte lysates treated with exogenous BDNF (100 ng/mL) for the indicated time, and probed for pY418 and pY529. BDNF stimulated the active phosphorylation of Fyn (pY418) in a time-dependent manner, but exerted no effect upon phosphorylation of the inhibitory site (pY529) (data = mean ± SEM, $n = 3$ independent experiments, $^*P < 0.05$, one-way ANOVA followed by Bonferroni's post hoc tests). (D) Western blot including (E) densitometric analysis of Western blot bands from oligodendrocyte lysates. OPCs were infected with either scrambled (control) or TrkB-shRNA expressing lentivirus for 48 h and differentiated to oligodendrocytes for an additional 72 h. Cells were then treated with BDNF (100 ng/mL) for 5min. BDNF-stimulated active Fyn phosphorylation (pY418) was abrogated in oligodendrocytes following TrkB knockdown (data = mean ± SEM, $n = 3$ independent experiments, $^*P < 0.05$, one-way ANOVA followed by Bonferroni's post hoc tests).

PP2, but not PP3, at 96 h (Fig. 5G), indicating that PP2 reduced oligodendrocyte differentiation and subsequent myelination. These results are consistent with previous studies demonstrating that the kinase activity of Fyn is required for oligodendrocytes differentiation and maturation (Wolf et al., 2001; Colognato et al., 2004). Taken together our data reveal that pharmacological inhibition of Fyn signaling not only reduces the basal level of myelination, but completely abrogates the promyelinating effect of BDNF *in vitro*. These data suggest that activation of the Fyn signaling pathway is absolutely required for BDNF to exert its promyelinating influence.

Oligodendroglial Fyn Signaling Mediates the Promyelinating Effect of BDNF In Vitro

Our data suggest that activation of Fyn kinase is required for the promyelinating effect of BDNF. However, it remained possible that the Src kinase inhibitor PP2 could have exerted

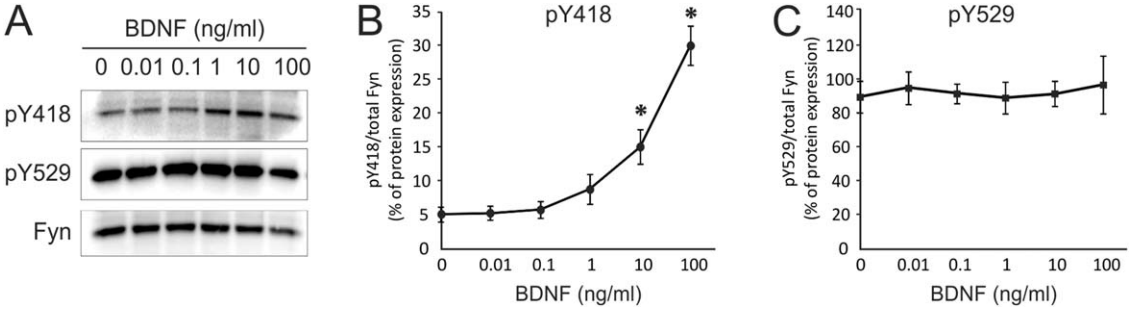

FIGURE 4: Phosphorylation of Fyn kinase is increased during BDNF-induced myelination *in vitro*. (A) Western blot and (B,C) densitometric analysis of Western blot bands from lysates derived from myelinating cocultures treated with exogenous BDNF (0–100 ng/mL) for 14 days. BDNF significantly increased the active phosphorylation (pY418) of Fyn in a concentration-dependent manner, but exerted no significant effect upon the inhibitory phosphorylation of Fyn (pY529) (data = mean ± SEM, $n = 3$ independent experiments, $^*P < 0.05$, one-way ANOVA followed by Bonferroni's post hoc tests).

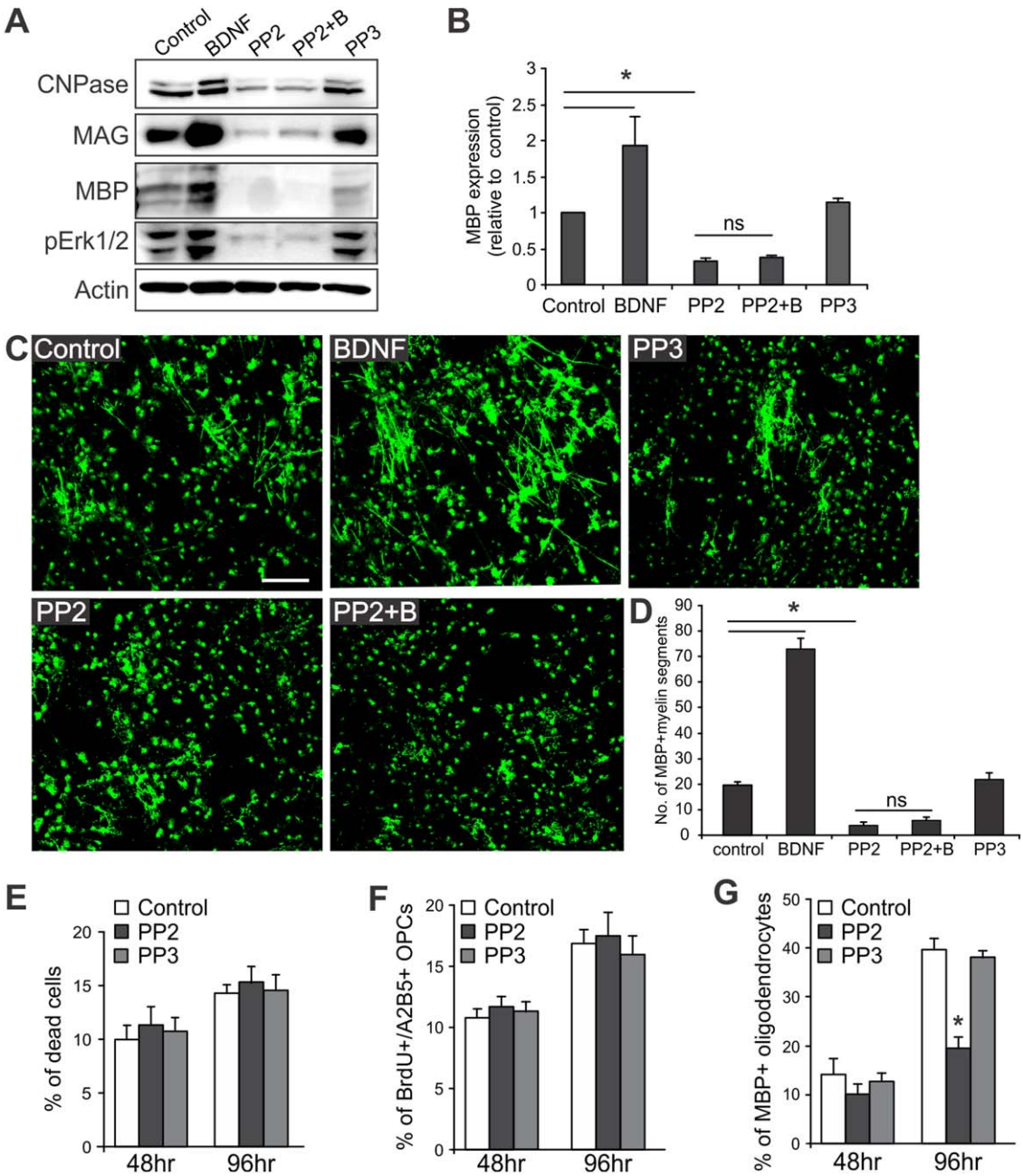

FIGURE 5: Inhibition of Fyn signaling abrogates BDNF-induced myelination *in vitro*. (A) Western blot analysis of *in vitro* myelination coculture lysates treated with BDNF (100 ng/mL), the Src family kinase inhibitor PP2 (10 μM), concurrent PP2, and BDNF (PP2 + B) treatment or the inactive control PP3 (10 μM, a control peptide of PP2). PP2 substantially decreased the basal level of myelin protein (CNPase, MAG and MBP) expression, and the levels of phosphorylated Erk1/2 (pErk1/2). In contrast, PP3 exerted no effect. (B) Densitometric analysis of MBP bands from parallel *in vitro* myelination cocultures from A. PP2 significantly decreased the basal level of myelin (MBP) protein expression, and also blocked the promyelinating effect of BDNF. In contrast, PP3 exerted no significant effect (*$P < 0.05$, ns = nonsignificant, $n = 3$ independent cocultures, data = mean ± SEM, one-way ANOVA followed by Bonferroni's post hoc tests). (C) Qualitative and (D) quantitative analysis of the number of MBP+ myelinated axonal segments of parallel *in vitro* myelination cocultures treated as in (A). Significantly more myelinated axonal segments were observed following addition of BDNF. Treatment with PP2 significantly inhibited the formation myelinated axonal segments at the basal level (PP2), and also inhibited the promyelinating effect of BDNF (PP2 + B). In contrast, PP3 exerted no significant effect upon the formation myelinated axonal segments at the basal level (*$P < 0.01$, ns = nonsignificant, $n = 3$ independent cocultures, data = mean ± SEM, one-way ANOVA followed by Bonferroni's post hoc tests). Scale bar: 100 μm. (E–G) Quantification of the percentage of ethidium homodimer-1 positive dead cells (E), BrdU+/A2B5+ proliferating OPCs (F), and MBP+ oligodendrocytes (G) treated with PP2 (10 μM) or PP3 (10 μM) at the indicated time points. There is no significant change in cell death or the proportion of proliferating OPCs treated with PP2 or PP3. PP2, but not PP3, significantly reduced the number of MBP+ cells after 96 h (*$P < 0.05$, $n = 3$ independent experiments, data = mean ± SEM, one-way ANOVA followed by Bonferroni's post hoc tests).

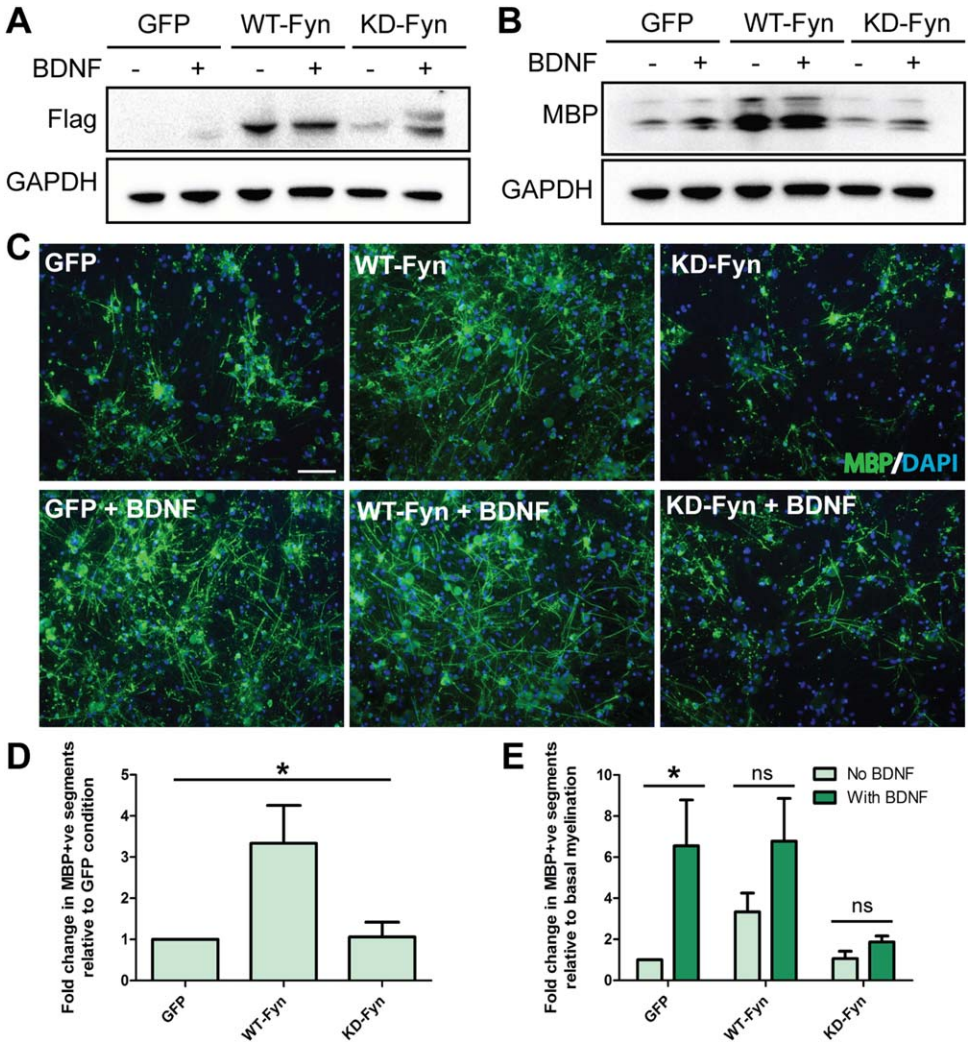

FIGURE 6: Selectively inhibiting Fyn kinase activity in oligodendrocytes blocked the promyelinating effect of BDNF *in vitro*. (A) Western blot analysis of OPC lysates derived from sister-cultures infected with lentivirus expressing GFP (control), Flag-WT-Fyn or Flag-kinase-dead (KD)-Fyn then treated with BDNF (100 ng/mL). Probing with Flag confirmed expression of the constructs. (B) Western blot analysis of *in vitro* myelination cocultures seeded with GFP (control), WT-Fyn or KD-Fyn infected OPCs, and maintained for 14 days +/−BDNF (100 ng/mL). The over-expression of WT-Fyn substantially increased the basal level of myelin (MBP) protein expression such that BDNF exerted no additional effect. Overexpression of KD-Fyn exerted amodest effect on the basal level of myelination, but abrogated the promyelinating effect of BDNF. (C) MBP immunostaining (green) and (D–E) quantitation of the number of myelinated axonal segments from the *in vitro* myelination cocultures from (B). (D) The overexpression of WT-Fyn within oligodendrocytes significantly increased the basal myelination (one-way ANOVA, followed by Bonferroni's post hoc tests *$P < 0.05$). (E) BDNF significantly increased the number of MBP+ myelinated axonal segments in the GFP (control) condition. In contrast, the promyelinating effect of BDNF was lost following over-expression of WT-Fyn or KD-Fyn (two-way ANOVA followed by Bonferroni's post hoc tests, *$P < 0.05$) (data = mean ± SEM, $n = 5$ independent cocultures, ns = nonsignificant).

effects upon the DRG neurons that were inhibitory to myelination. To unequivocally confirm that oligodendroglial Fyn signaling regulates myelination, we infected primary OPC cultures with lentivirus over-expressing GFP (control empty vector), a Flag-tagged wild-type Fyn (WT-Fyn), or a Flag-tagged kinase-dead Fyn (KD-Fyn) mutant that carries a K299M mutation in the ATP binding site. These virally infected OPCs were seeded onto DRG neurons and the resulting cocultures were maintained in the presence or absence of BDNF (100 ng/mL) for 14 days. The cocultures were either analyzed by Western blot, or fixed and immunostained. Western blot analyses of lysates generated from sister OPCs confirmed the expression of the Flag-tagged Fyn constructs following lentiviral infection (Fig. 6A). In control (GFP) infected cocultures, addition of BDNF promoted myelination as assessed by an increase in the expression of MBP (Fig. 6B), as well as a significant increase in the formation of MBP+ myelinated axonal segments (Fig. 6C, quantitated in E, *$P < 0.05$). Expression of WT-Fyn substantially increased MBP expression (Fig. 6B), and significantly increased the

number of MBP+ myelinated axonal segments, independent of the provision of BDNF (Fig. 6C, quantitated in D, $^*P < 0.05$). Treatment of WT-Fyn expressing cocultures with BDNF modestly increased MBP expression (Fig. 6B) and the number of MBP+ myelinated axonal segments (Fig. 6C, quantitated in E), but did not exert a significant additive influence. Expression of KD-Fyn in oligodendrocytes exerted no effect upon either MBP expression (Fig. 6B) or the formation of MBP+ myelinated axonal segments compared to control basal levels (Fig. 6C, quantitated in D), but did however significantly reduce the promyelinating influence of BDNF, as assessed by the formation of MBP+ myelinated axonal segments (Fig. 6C, quantitated in E). These results show that oligodendroglial expression of WT-Fyn promotes myelination to the extent that BDNF exerts no additive effect, and that oligodendroglial expression of KD-Fyn completely abrogates the promyelinating effect of BDNF *in vitro*. Collectively our data suggest that oligodendroglial Fyn signaling is absolutely required for BDNF to promote myelination *in vitro*.

Oligodendroglial Fyn Signaling is Required for BDNF-Dependent Activation of Erk1/2

We next sought to determine whether Fyn signaling influenced Erk1/2 activation, a key mediator of the promyelinating influence of BDNF. We first assessed the lysates from *in vitro* myelination cocultures treated with BDNF and PP2 (Fig. 5). Western blot analyses revealed that PP2 clearly blocked the BDNF-dependent activation of Erk1/2 in the cocultures (Fig. 5A), suggesting that Fyn kinase regulates Erk1/2 activation in BDNF-induced myelination *in vitro*. In order to dissect this pathway specifically within oligodendrocytes, these cells were starved and subsequently treated with either the Src family kinase inhibitor PP2 or control peptide PP3 (both at 10 μM) in the presence or absence of BDNF (100 ng/mL) for 10 min. Western blot analyses of oligodendrocyte lysates revealed that BDNF significantly promoted Erk1/2 phosphorylation (pErk1/2) in control cultures (Fig. 7A, pErk1 and pErk2 independently quantitated in B and C, $^*P < 0.05$). Inhibiting Src family kinase activity using PP2 significantly reduced the basal level of pErk1/2, but importantly also abrogated the BDNF-dependent phosphorylation of Erk1/2 (Fig. 7A, quantitated in B and C, $^*P < 0.05$). In contrast, the control peptide PP3 exerted no effect upon the profile of BDNF-dependent phosphorylation of Erk1/2 (Fig. 7A, quantitated in B and C, $^*P < 0.05$). These data indicate that Src family kinases mediate the BDNF-dependent activation of Erk1/2 within oligodendrocytes.

However, in addition to Fyn, PP2 can inhibit the activity of other Src family kinases. To unequivocally identify whether Fyn selectively mediates the BDNF-dependent activation of Erk1/2 within oligodendrocytes, OPCs were infected

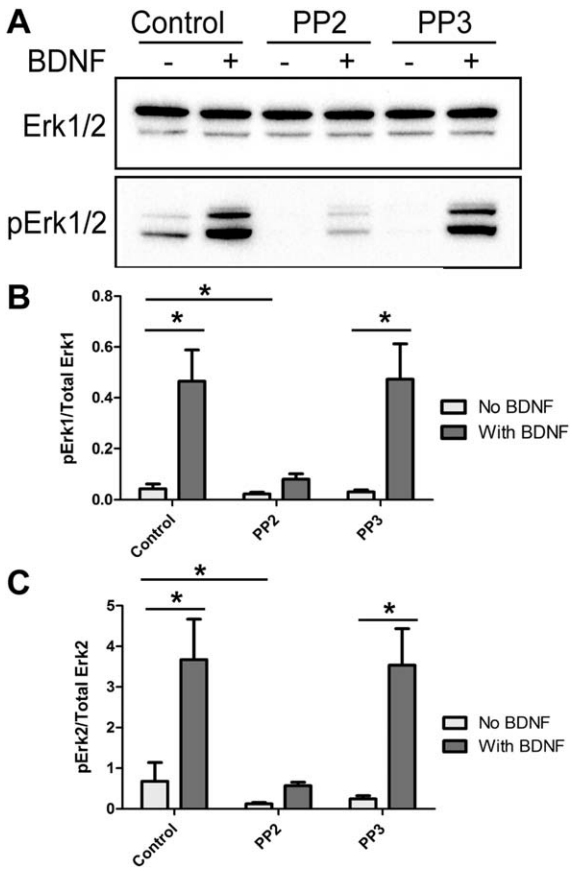

FIGURE 7: Inhibition of Fyn signaling in oligodendrocytes abrogates the activation of Erk1/2 by BDNF. (A) Western blot including (B,C) densitometric analysis of Western blot bands from oligodendrocyte lysates. OPCs were allowed to differentiate into oligodendrocytes for 72 h, and either untreated (control), or treated with PP2 (10 μM) or the control peptide PP3 (10 μM) for 1 h prior to the addition of BDNF (100 ng/mL) for another 10 min. The PP2, but not PP3, significantly abrogated BDNF-induced activation of Erk1 (B) and Erk2 (C) ($^*P < 0.05$, $n = 5$ independent cultures, data = mean ± SEM, two-way ANOVA followed by Bonferroni's post hoc tests).

with lentivirus over-expressing GFP (control condition), Flag-WT-Fyn or Flag-KD-Fyn, differentiated into oligodendrocytes, starved, and stimulated with BDNF (100 ng/mL) for 10 min, then lysed for Western blot analysis. The expression of the Flag-tag within the lysates infected with WT-Fyn or KD-Fyn lentivirus confirmed the efficacy of infection (Fig. 8A). Infection with the lentivirus exerted no effect upon the constitutive expression of Erk1/2 (Fig. 8A). Addition of BDNF to the control (GFP) cultures significantly increased pErk1/2 (Fig. 8A, pErk1 and pErk2 independently quantitated in B and C, $^*P < 0.01$). Expression of WT-Fyn in oligodendrocytes significantly increased the basal level of both pErk1 and pErk2 (Fig. 8A, quantitated in B and C, $^*P < 0.01$). The addition of BDNF to these cultures resulted in a further significant increase in Erk1/2 phosphorylation

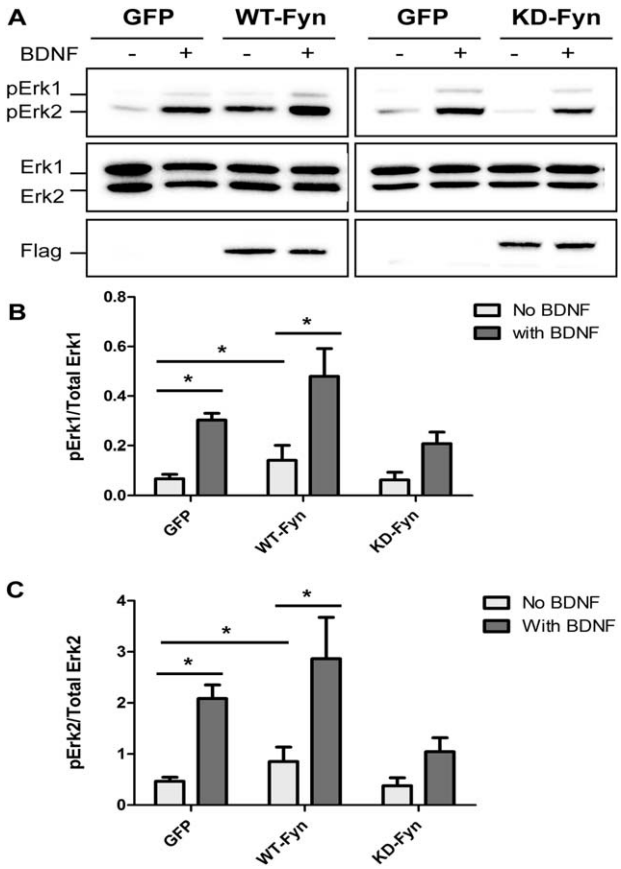

FIGURE 8: Selectively inhibiting Fyn kinase activity in oligodendrocytes abrogates the activation of Erk1/2 by BDNF. (A) Western blot including (B,C) densitometric analysis of Western blot bands from oligodendrocyte lysates. OPCs were infected with lentivirus for 48 h forcing expression of GFP (control), Flag-WT-Fyn or Flag-KD-Fyn. OPCs were allowed to differentiate into oligodendrocytes for 72 h, then challenged with exogenous BDNF (100 ng/mL) for 10 min. The overexpression of WT-Fyn significantly enhanced the basal level of phosphorylated Erk 1 (pErk1, B) and Erk 2 (pErk2, C). BDNF also significantly increased (C) Erk1 and (D) Erk2 phosphorylation in WT-Fyn infected oligodendrocytes, but exerted no effect in KD-Fyn infected oligodendrocytes (data = mean ± SEM, $n = 5$ independent cocultures, two-way ANOVA followed by Bonferroni's post hoc tests, $*P < 0.01$).

(Fig. 8A, quantitated in B and C, $*P < 0.01$), but the total level of Erk1/2 phosphorylation was not significantly different to the BDNF treated control cultures (Fig. 8A, quantitated in B–C, $P = 0.12$ [pErk1] and $P = 0.23$ [pErk2]). Interestingly, the expression of KD-Fyn in oligodendrocytes exerted no significant influence upon the basal level of Erk1/2 phosphorylation compared to the control GFP condition (Fig. 8A, quantitated in B and C, $P = 0.09$ [pErk1] and $P = 0.11$ [pErk2]), and the addition of BDNF to these cultures exerted no significant influence upon the level of Erk1/2 phosphorylation (Fig. 8B,C, $P = 0.079$ [pErk1] and $P = 0.085$ [pErk2] vs KD-Fyn without BDNF). Importantly, the expression of KD-Fyn significantly attenuated the level of BDNF-induced Erk1/2 phosphorylation compared to both control GFP and WT-Fyn expressing oligodendrocytes (Fig. 8A, quantitated in B and C).

Collectively, our data consistently demonstrate that Fyn kinase plays a key role in transducing the intracellular signaling cascade that mediates the BDNF-dependent activation of Erk1/2 signaling within oligodendrocytes. These results suggest that oligodendroglial Fyn kinase mediates the promyelinating effect of BDNF via regulating Erk1/2 phosphorylation.

Discussion

Here we have identified a novel role for Fyn kinase in regulating oligodendrocyte myelination, firstly as a mediator of the promyelinating effect that BDNF has upon oligodendrocytes, and secondly as an upstream activator of Erk1/2. Through analyses of BDNF+/− mice, we found that levels of Fyn phosphorylation strongly correlated with myelination during early postnatal development *in vivo*. This was supported by analyses of cultured oligodendrocytes and *in vitro* myelination assays, in which BDNF stimulated Fyn kinase activity, an affect dependent on oligodendroglial TrkB expression. Through pharmacological inhibition of Src family kinases and selectively manipulating Fyn activity within oligodendrocytes, we have established that Fyn kinase regulates basal levels of myelination and importantly mediates the promyelinating effect of BDNF via regulating Erk1/2 activity within oligodendrocytes. Collectively, these findings suggest that Fyn is an oligodendrocyte expressed kinase that mediates the promyelinating effect of BDNF.

Considerable evidence indicates that Fyn plays a key role in regulating oligodendrocyte differentiation *in vitro* and myelination *in vivo* (Bauer et al., 2009; Czopka et al., 2013; Gonsalvez et al., 2015; Goto et al., 2008; Kramer et al., 1999; Osterhout et al., 1999; Sperber et al., 2001; Umemori et al., 1999; Wolf et al., 2001). Previous studies have shown that mice lacking Fyn or Fyn kinase activity exhibit myelin deficits in the brain and spinal cord *in vivo* (Goto et al., 2008; Sperber et al., 2001; Umemori et al., 1999). A more recent study in Zebrafish shows that manipulation of Fyn kinase activity specifically within oligodendrocytes alters the number of myelinated axons and the number of myelin sheaths within the spinal cord (Czopka et al., 2013). We found that Fyn kinase activity is present predominantly within postmitotic oligodendrocytes, but not OPCs, in both the brain and spinal cord white matter tracts. More importantly, both our data and others (Kramer et al., 1999) have shown Fyn kinase activity peaks early in postnatal development when myelination is commencing, and then is rapidly downregulated later postnatally, when myelination is almost complete, while total Fyn protein expression remains unchanged. These together suggest that Fyn kinase activity

within oligodendrocytes exerts a key influence upon the maturation of oligodendrocytes and initial stages of myelination.

Both *in vivo* and *in vitro* studies have shown that Fyn kinase activity within oligodendrocytes controls the formation of the myelin membrane (Osterhout et al., 1999) and determines the number of myelin sheaths per oligodendrocyte (Czopka et al., 2013). However the factors upstream of Fyn kinase, and the downstream mechanisms that Fyn kinase influences to regulate myelination remain to be elucidated. We have found that Fyn phosphorylation is regulated by BDNF levels *in vivo* and *in vitro*. Analyses of BDNF heterozygous mice show that there is a reduced level of Y418 phosphorylated Fyn, and an increased level of Y529 phosphorylated Fyn early in postnatal development. The Y418 is the catalytic autophosphorylation site, with its phosphorylation leading to activation of Fyn's kinase activity, whereas the Y529 is the COOH-terminal negative regulatory site, the phosphorylation of which leads to inactivation of Fyn kinase (Colognato et al., 2002). Interestingly, in the *in vitro* myelination assay, BDNF regulated the phosphorylation of Y418, but exerted no influence on the phosphorylation of Y529, suggesting that in the context of myelination, BDNF preferentially stimulates Fyn kinase activity that leads to phosphorylation of its downstream effectors, rather than suppressing the inhibitory phosphorylation of Fyn. It is of note that in the BDNF+/− mice, both the activation (Y418) and inactivation (Y529) phosphorylation sites on Fyn are regulated *in vivo*, but in our *in vitro* model of myelination only the activation phosphorylation site (Y418) of Fyn was regulated by BDNF. The reason for this is currently unclear. The difference in our *in vitro* and *in vivo* findings illustrates the complexity and contextual nature of the regulatory pathways that link BDNF to the level of Fyn phosphorylation *in vivo*, which are yet to be fully elucidated. Collectively, we have identified that BDNF acts as an upstream factor that stimulates the autophosphorylation of Fyn kinase during myelination both *in vitro* and *in vivo*.

BDNF acts via oligodendendroglial TrkB receptors to promote oligodendrocyte myelination *in vitro* and *in vivo* (Wong et al., 2013; Xiao et al., 2010). A BDNF-dependent association between, and mutual activation of, Fyn and TrkB has been demonstrated in other cell systems, such as cortical neurons (Pereira and Chao, 2007; Iwasaki et al., 1998). Our data show that in oligodendrocytes, the BDNF dependent phosphorylation of Fyn depends on the expression of TrkB, suggesting BDNF acts on oligodendroglial TrkB receptors to provoke Fyn kinase activity, which influences downstram signaling pathways and ultimately myelination. Interestingly, Fyn kinase has also been found to transactivate receptor tyrosine kinases, such as TrkB, in response to GPCR activation (Asimaki and Mangoura, 2011; Rajagopal and Chao, 2006). Whether Fyn can transactivate TrkB in oligodendrocytes is unclear, however perhaps more interesting is whether activation of oligodendrocyte-expressed GPCRs can directly regulate Fyn phosphorylation and ultimately myelination. Context is critical in cell signaling, and these data suggest that further assessment of GPCR signaling in oligodendrocytes is warranted.

We have previously shown that the promyelinating effect of BDNF is dependent upon Erk1/2 phosphorylation (Xiao et al., 2012). Indeed, it is widely appreciated that Erk1/2 phosphorylation plays a key role in promoting oligodendrocyte myelination *in vitro* and *in vivo* (Dai et al., 2014; Ishii et al., 2012, 2013; Xiao et al., 2012). In the in *vitro* myelination assay, over-expression of WT-Fyn in oligodendrocytes significantly promotes phosphorylation of Erk1/2, and promotes myelination to the extent that exogenous BDNF exerts no additive effect *in vitro*. In contrast, expression of a KD-Fyn in oligodendrocytes significantly inhibited the BDNF-induced Erk1/2 activation and abrogated the promyelinating effect of BDNF, demonstrating that Fyn is required for Erk1/2 activation in oligodendrocytes and suggests that the Fyn and MAPK pathways are mechanistically linked. This is also supported by *in vivo* evidence. Our previous analyses of mice with TrkB-specific deletion in oligodendrocytes suggested that TrkB signaling within oligodendrocytes exerts a specific influence upon myelin wrapping during development (Wong et al., 2013). We have subsequently shown that BDNF/TrkB signals via Erk1/2 kinases within oligodendrocytes to promote myelination *in vitro* (Xiao et al., 2012), and other studies show that mice with Erk1/2 activation in oligodendroglia display thicker myelin during CNS development *in vivo* (Fyffe-Maricich et al., 2013; Ishii et al., 2012, 2013). Importantly, and relevant to the phenotype of both the TrkB and Erk1/2 transgenic mice, the loss of Fyn also resulted in reduced myelin thickness in the spinal cord *in vivo* (Umemori et al., 1999). Concordant with this finding, another recent study has shown that Fyn kinase activity within oligodendrocytes regulates myelin sheath formation in the developing spinal cord of Zebrafish (Czopka et al., 2013). Therefore, our data together with these studies *in vivo* evidence suggest that the Fyn and MAPK pathways are mechanistically linked, and that Fyn acts as an intermediate kinase to regulate BDNF-induced myelination by influencing Erk1/2 activation. These also indicate that the myelin phenotype of Fyn knockout mice (Goto et al., 2008; Sperber et al., 2001; Umemori et al., 1999) and Zebrafish with the Fyn kinase mutation (Czopka et al., 2013) could at least in part be ultimately due to a contextual loss and activation of Erk1/2 signaling in oligodendroglia during myelination.

Precisely how Fyn ultimately influences Erk1/2 phosphorylation remains unclear, however there are a number of

ways Fyn could achieve this (Gonsalvez et al., 2015). For example, Fyn has been shown to bind to and phosphorylate the p190 RhoGAP in oligodendrocytes to regulate their differentiation (Liang et al., 2004; Wolf et al., 2001). p190 RhoGAP is a GTPase activating protein that regulates G-proteins Rac1, Rho, and Cdc42, which can influence the Erk1/2 pathway. Fyn also associates with, phosphorylates and increases the kinase activity of Raf (Cleghon and Morrison, 1994), the first kinase in the MAPK cascade, and known to promote oligodendrocyte differentiation (Galabova-Kovacs et al., 2008). In addition, the phosphatase Shp2 regulates the inhibitory phosphorylation of Fyn, which in turn positively regulates Fyn dependent Ras-MAPK/Erk activation via PLC-γ (Zhang et al., 2004). Whilst we have identified that Fyn kinase is required for the promyelinating effect of BDNF, the role that it plays in regulating the promyelinating influence of other factors remains unclear. For example, laminin (Colognato and Ffrench-Constant, 2004) and the β1 integrin subunit (Lee et al., 2006) also exert promyelinating influences upon oligodendrocytes, and are also known to activate Fyn and the Ras-MAPK/Erk pathway, but whether Fyn plays a necessary role in this process is unclear. The fact that pharmacological inhibition of Fyn signaling significantly abrogated the basal levels of myelination in our cocultures suggests that Fyn does play a key role in the process and is likely to be utilized via a number of promyelinating factors.

In summary, our data have identified Fyn kinase as a molecular mediator of BDNF signaling that activates Erk1/2 and promotes oligodendrocyte myelination. These observations identify BDNF as a novel ligand that stimulates Fyn activity within oligodendrocytes, and identify Erk1/2 as the target of Fyn activity that regulates myelination. This finding provides further insight into the networks of intracellular signaling cascades that regulate CNS myelination. Further elucidation of the networks that integrate extrinsic signals into the intracellular signaling cascades that converge on to the transcriptional regulators that promote myelination is required in order to allow development of targeted therapeutics that will specifically enhance myelin repair and ultimately resolve the effects and symptoms of demyelinating diseases such as multiple sclerosis.

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