GSK3β Negatively Regulates Oligodendrocyte Differentiation and Myelination In Vivo

KASUM AZIM AND ARTHUR M. BUTT*
Institute of Biology and Biomedical Sciences, School of Pharmacy and Biomedical Sciences, University of Portsmouth, Portsmouth, United Kingdom

KEY WORDS
oligodendrocyte; oligodendrocyte precursor; myelination; demyelination; GSK3β

ABSTRACT
Glycogen synthase kinase 3β (GSK3β) is an essential integrating molecule for multiple proliferation and differentiation signals that regulate cell fate. Here, we have examined the effects of inhibiting GSK3β on the development of oligodendrocytes (OLs) from their oligodendrocyte precursors (OP) in vivo by injection into the lateral ventricle of postnatal mice and ex vivo in organotypic cultures of isolated intact rodent optic nerve. Our results show that a range of GSK3β inhibitors (ARA-014418, lithium, indirubin, and L803-mt) increase OPs and OLs and promote myelination. Inhibition of GSK3β stimulates OP proliferation and is prosurvival and antiapoptotic. The effects of GSK3β inhibition in OPs is via the canonical Wnt signaling pathway by stimulating nuclear translocation of β-catenin. However, direct comparison of the effects of Wnt3a and GSK3β inhibition in optic nerves shows that they have opposing actions on OLs, whereby GSK3β inhibition strikingly increases OL differentiation, whereas Wnt3a inhibits OL differentiation. Notably, GSK3β inhibition overrides the negative effects of Wnt3a on OLs, indicating novel GSK3β signaling mechanisms that negatively regulate OL differentiation. We identify that two mechanisms of GSK3β inhibition are to stimulate cAMP response element binding (CREB) and decrease Notch1 signaling, which positively and negatively regulate OL differentiation and myelination, respectively. A key finding is that GSK3β inhibition has equivalent effects in the adult and stimulates the regeneration of OLs and remyelination following chemically induced demyelination. This study identifies GSK3β as a profound negative regulator of OL differentiation that contributes to inefficient regeneration of OLs and myelin repair in demyelination.

INTRODUCTION
The differentiation of oligodendrocytes (OLs) from oligodendrocyte precursors (OPs) depends on a highly coordinated series of events in which multiple cell-intrinsic and extracellular factors regulate the proliferation and survival of OPs and their timely differentiation into myelinating OLs (Miller, 2002). Glycogen synthase kinase 3β (GSK3β) is a multifactorial negative regulator of cell fate that is a target of many receptor-mediated signaling pathways (Cothen and Goedert, 2004; Griffin and Jope 2001b). The functions of GSK3β signaling in OLs have not been previously determined, but GSK3β has high expression and activity in developing white matter (Coyle-Rink et al., 2002; Takahashi et al., 1994), and inhibition of GSK3β is a major effect of receptor kinase activation that mediates the prosurvival effects of insulin-like growth factor 1 (IGF-1) and fibroblast growth factor 2 (FGF-2) on OPs (Frederick et al., 2007; Ye et al., 2010). In addition, GSK3β is a key regulatory factor in the Wnt signaling pathway, which is a potent negative regulator of OL differentiation and myelination (Fancy et al., 2009; Feigelson et al., 2009) and is involved in the mitogenic actions of IGF-1 in OPs (Ye et al., 2010).

Several receptor-mediated pathways regulated by GSK3β are upregulated in multiple sclerosis (MS) lesions, such as Wnt, IGF-1, FGF-2, and Notch (Bansal, 2002; Fancy et al., 2009; John et al., 2002). Hence, identifying the mechanisms by which GSK3β signaling pathways regulate OP differentiation may facilitate the development of therapies aimed at promoting OL regeneration and myelin repair in the central nervous system. However, a major issue is to translate targets identified in vitro and in genetic studies into treatments that promote OL regeneration in vivo. Here, we have used intraventricular administration of pharmacological GSK3β inhibitors to examine the role of GSK3β in OL differentiation in vivo for the first time. This technique has been used successfully to target neurons in vivo and delay neuronal death in Parkinson’s and Alzheimer’s disease models (Chen et al., 2004; Wang et al., 2007). In addition, systemic administration of lithium has been shown to prevent and ameliorate experimental autoimmune encephalomyelitis (EAE), an animal model of MS (De Sarno et al., 2008). From these reports, we selected a range of GSK3β inhibitors that have been shown to be effective in neurons, namely, ARA-014418, lithium, indirubin, and L803-mts. Our study shows that inhibition of GSK3β not only dramatically stimulates OL generation in the developing brain but also promotes repair in a toxin-induced model of demyelination. The positive effects of GSK3β inhibitors oppose and override the additional negative effects of Wnt3a and GSK3β inhibition.
negative effects of Wnt signaling on terminal OL differentiation and myelination. The results indicate that GSK3β is a key negative regulator of OL differentiation that contributes to inefficient regeneration of OLs and myelin repair in demyelination and is a potential therapeutic target in MS.

**MATERIALS AND METHODS**

**Animals**

Mice and rats aged between postnatal day (P)7 and P11, or adults, were used throughout. Rats were of the Wistar strain, and the wild-type mouse strains used were C57/BL6 or C57/BL10 strains. Transgenic mouse lines were used in which fluorescent reporters DsRed or green fluorescent protein (GFP) are under control of the glial-specific promoters proteolipid protein (PLP) or Sox10 (Hirrlinger et al., 2005; Stolt et al., 2006). All research involving animals was approved by the University of Portsmouth Ethics Committee and by the Home Office Animals Scientific Procedures Act (1986). Unless otherwise stated, animals were killed humanely by cervical dislocation, and brains were removed rapidly and placed in ice-cold saline or fixative.

**Agents**

ARA-014418 (Sigma-Aldrich), L803-mts (Calbiochem), indirubin-3-monoxim (Calbiochem), and the Wnt3a agonist 2-amino-4-(3,4-(methylenedioxy) benzylamino)-6- (3-methoxyphenyl) pyrimidine (Calbiochem) were stored in dimethyl sulfoxide (DMSO) and diluted in sterile saline vehicle; sterile saline/DMSO vehicle was used as controls in dimethyl sulfoxide (DMSO) and diluted in sterile saline vehicle; sterile saline/DMSO vehicle was used as controls for these agents. Lithium chloride (Sigma-Aldrich) was dissolved directly in sterile saline, and sterile saline vehicle was used as controls.

**In Vivo Injections and Induction of Demyelination**

Mice were deeply anesthetized under isofluorane, and agents were delivered in a volume of 2 μL into the cerebrospinal fluid (CSF) of the lateral ventricle using a Hamilton syringe, at a point 2 mm from the midline for two cycles. For pβ-catenin and Tyr216-pGSK3β, PBS was replaced by Tris-buffered saline (TBS 0.5 M Tris Base, 9% NaCl, pH 8.4) throughout to reduce nonspecific labeling of antiphospho antibodies, and sections were subjected to antigen retrieval as above. For BrdU labeling, mice were given a single intraperitoneal injection of BrdU (Sigma-Aldrich) at 50 μg/g body weight 2 h prior to sampling, and prior to immunolabeling, sections were incubated in 2 N HCl for 1 h to denature nuclear DNA, followed by three washes with 0.1 M sodium borate (pH 8.5) to neutralize HCl. In some cases, sections were incubated for up to 2 h in 0.1 mg/mL propidium iodide (PI; Sigma-Aldrich), as a marker for cell death. After

**Immunohistochemistry**

Brains were immersion fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), either for 3 h at room temperature or overnight at 4°C. Following fixation, brains were washed in PBS, and coronal vibratome sections of 30–100 μm thickness were cut through the forebrain. Sections containing the posterior lateral ventricles were selected for immunohistochemistry. Following washes in PBS, a blocking stage was performed by incubation for 2 h at room temperature or overnight at 4°C in 10% normal goat serum (NGS; Biosera) or normal donkey serum (NDS; Invitrogen) in 0.3% Triton-X-100 in PBS (PBST). After blocking, sections were incubated for 2–3 h at room temperature with agitation or overnight at 4°C in primary antibodies diluted in NGS- or NDS-PBST as appropriate: rabbit anti-PDGFαR (1:400; gift from Prof Stallcup); goat anti-PDGFβR (1:200; R&D Systems); mouse anti-NeuN (1:300; Millipore); mouse anti-bromodeoxyuridine (BrdU; 1:300; Millipore); rat anti-myelin basic protein (MBP) (1:300; Millipore); mouse anti-adenomatous polyposis coli (APC) (1:300; Millipore); rabbit anti-NF200; rabbit anti-Olig2 (1:500; Millipore); rabbit anti-glial fibrillary acidic protein (GFAP) (1:300; DAKO); mouse antinuclear pβ-Catenin (1:300; Abcam); goat anti-Tyr216-pGSK3β (1:100; Santa Cruz); and mouse anti-proliferating cell nuclear antigen (PCNA) (1:400; Sigma-Aldrich). After washes in PBST, sections were incubated for 2 h at room temperature or overnight at 4°C in the dark with the appropriate secondary antibodies conjugated with Alexafluor 488, 568, or 405 (1:500; Molecular Probes). Primary antibodies of different origin were diluted together in blocking buffer, and codilutions of the appropriate secondary antibodies were used. Control experiments were performed using appropriate blocking peptides where available or otherwise by omission of the primary antibody. For PCNA labeling, antigen retrieval was performed (Jiao et al., 1999), whereby free-floating sections were pretreated with PBST and NP-40 1% for 45 min to permeate the sections, and following washes in PBS, sections were immersed in preboiled citric acid and heated in a commercial microwave pressure cooker at full power for 30 s for two cycles. For pβ-catenin and Tyr216-pGSK3β, PBS was replaced by Tris-buffered saline (TBS 0.5 M Tris Base, 9% NaCl, pH 8.4) throughout to reduce nonspecific labeling of antiphospho antibodies, and sections were subjected to antigen retrieval as above. For BrdU labeling, sections were incubated in 2 N HCl for 1 h to denature nuclear DNA, followed by three washes with 0.1 M sodium borate (pH 8.5) to neutralize HCl. In some cases, sections were incubated for up to 2 h in 0.1 mg/mL propidium iodide (PI; Sigma-Aldrich), as a marker for cell death. After
final washes in PBS, tissues were mounted on poly-ly- 
sine-coated glass slides with Vectashield mounting 
media (Vector Laboratories) and sealed with coverslips.
Images were acquired using a Zeiss LSM 510 or 710 
confocal microscope (Zeiss). Fluorescence was visualized 
at 488 nm (green), 568 nm (red), and 405 nm (blue) 
using argon, HeNe1, and diode lasers, respectively, 
using a 40× oil immersion lens with high numerical 
aperture (1.3 nm).

Optic Nerve Tissue Culture

Mice aged P10 or rats aged P7 were killed humanely 
by cervical dislocation, and optic nerves were removed 
with the eyeball attached and placed immediately in 
ice-cold oxygenated artificial CSF (aCSF), composed of 
NaCl 133 mM, KCl 3 mM, CaCl2 1.5 mM, NaH2PO4 
25% CO2 for up to 6 days

NaCl 133 mM, KCl 3 mM, CaCl2 1.5 mM, NaH2PO4 
and with a solution of penicillin-strepto-

1.000 

cose (Sigma), and with a solution of penicillin-strepto-

1.2 mM, D-glucose 10 mM, HEPES buffer 10 mM, pH 
7.3. Residual tissue was removed, and the optic nerve-
retina unit was maintained in culture on semiporous 
membrane inserts (0.4 μm; Millipore). The inserts were 
transferred into six-well culture plates with 1 mL cul-
ture medium per well and incubated at 37°C in 95% O2, 
5% CO2 for up to 6 days in vitro (DIV). The culture me-
dium was composed of 50% Opti-MEM™ (Invitrogen), 
25% horse serum (Invitrogen), 25% Hank’s Balanced 
Salt Solution (Sigma), supplemented with 25 mM d-gluc-
cose (Sigma), and with a solution of penicillin-strepto-
ymycin (penicillin G sodium 10,000 U/mL, streptomycin 
sulfate 1,000 μg/mL; Invitrogen) diluted to 1:500. To 

promote interaction between media and the optic nerve–retina unit, 50 μL of culture medium was added 
directly over the tissue. The effects of the GSK3β inhibi-
tors ARA-014418 (20 μM) and LiCl (30 mM), or the specific 
Wnt3a agonist 2-Amino-4-(3,4-(methylenedioxy)-
benzylamino)-6-(3-methoxyphenyl)pyrimidine (3 μM) 
were determined by direct application in the culture medium. At the end of culture period, optic nerves were 
dissected free from the retina and either treated for 
Western blot or confocal microscopy. For confocal micro-
scopic examination, optic nerves from transgenic PLP-
DsRed and Sox10-GFP mice were used, and at the end 
of the culture period, nerves were immersion fixed in 
4% PFA for 30 min at room temperature, prior to whole-
mounting on slides with Vectashield and microscopic ex-
mamination of glial cells. For Western blot analysis, rat 
opic nerves were used to improve protein yields, and at 
the end of the culture period, nerves were transferred to 

ice-cold lysis buffer, prior to homogenization.

Cell Counts

Coronal sections containing the posterior lateral ven-
tricle were analyzed (≥3 sections per brain); cell counts 
confirmed that there were no significant differences 
between the sections used for analyses. Cell counts of 
OLs and OPs in the PVWM and intact optic nerves were 
performed on confocal images processed with Zeiss LSM 

Image Examiner (V. 5.2.0.121), maintaining the acquisi-
tion parameters constant to allow comparison between 
samples. In brain sections, cell counts were performed 
on flattened confocal z-stacks of 230 μm² × 230 μm² in 
the x- and y-plane and of 30 μm in the z-plane, with a 
field of view (FOV) volume of 1.6 × 10⁶ μm³. In mouse 
opic nerves, cell counts were performed on flattened 
z-stacks taken from the middle of the nerve with a FOV 
volume of 5.3 × 10⁵ μm³ for Sox10/GFP+ cells and 1 × 
10⁵ μm³ for less dense PLP/DsRed+ OLs. Cell counts are 
expressed as mean (±SEM, n ≥ 4) cells per FOV, 
where the “n” value represents the number of mice. Cell 
counts were tested for significance using GraphPad 
Prism v302 for multiple variables using either Dunnett’s 
multiple comparisons test or one-way analysis of var-
iance (ANOVA), followed by Bonferroni’s posthoc test, 
and for two variables using unpaired t-tests.

Western Blot

Rat optic nerves were placed immediately in ice-cold 
Ca²⁺ free lysis buffer containing 200 μM ethylene glycol 
tetraacetic acid (EGTA) and 200 μM ethylene diamine 
tetraacetic acid (EDTA) (pH 7.4) and protease/phospha-
tase inhibitors to stop any further phosphorylation or 
dephosphorylation [50 mM Tris-HCl, pH 7.5, 150 mM 
NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1% 
NP-40, Calbiochem protease/phosphatase cocktail set II 
(2.5 mM sodium pyrophosphate, 1 mM beta-glycerophos-
phate, 1 mM Na3VO4, 50 mM NaF); Calbiochem protease-
phosphatase cocktail set III (1 mM PMSF, 1 
mM bestatin, 14 
μM leupeptin, 0.3 
μM apronitin, 130 
μM bestatin, 14 
μM E-
64); 50 
μM Okadaic acid (LC Labs)]. Four nerves per 
sample were rapidly homogenized in lysis buffer on ice 
for 5 min, and samples were transferred to Ultrafree 
MC centrifugal spin columns (Millipore) for separation 
of protein extracts above 20 kDa and Bradford protein 
assay determination. Remaining samples were immedi-
ately frozen at −20°C until required. For protein analy-

sis, samples were then solubilized and denatured in 
Lamelli sample buffer (Biorad) with 
μM mercaptoethanol (Sigma-Aldrich) for 5 min at 95°C and were placed on 

ice until loading. 30 
μg was loaded onto the gel with 
Lamelli sample buffer. The solubilized denatured pro-
teins were then separated through a sodium dodecyl sul-
fate polyacrylamide gel electrophoresis (SDS-PAGE) gel 
and transferred to a PVDF membrane (GE Healthcare, 
Amersham). The PDVF membrane was washed in TBS 
and placed in blocking buffer for 1 h at room tempera-
ture or overnight at 4°C in blocking solution (either 5% 
BSA in TBS and 0.1% Tween or 5% skimmed milk pow-
der in TBS and 0.1% Tween). Following washes in TBS 
with 0.1% Tween (TTBS), the membrane was incubated 
with the primary antibodies diluted in TTBS containing 
1% w/v skimmed milk powder to prevent nonspecific 
binding for 1 h at room temperature or overnight at 
4°C: mouse anti-β-actin (1:10,000; Sigma-Aldrich); 
mouse antitotal GSK3β (1:2,000; BD Biosciences); goat 
anti-Tyr216-pGSK3β (1:500; Santa Cruz); goat anti-
Ser9-pGSK3β (1:500; Santa Cruz); mouse antinuclear pβ-Catenin (1:1,000; Abcam); mouse anti-PCNA (1:1,000; Sigma-Aldrich); goat anti-pCREB (1:500; Santa Cruz); mouse anti-antiNotch1 (1:1,000; Millipore); goat anti-Jagged1 (1:500; Santa Cruz). The membrane was then washed in TTBS and incubated for 1 h at room temperature in the appropriate HRP-conjugated secondary antibodies (1:1,000; DAKO). Proteins were visualized by enhanced chemiluminescent detection (Amersham Biosciences), and signal intensities were measured using ImageJ software (NIH). Experiments were repeated independently at least three times, and band densitometry values were compared by using ANOVA followed Bonferroni’s posthoc test for significance.

RESULTS

GSK3β Inhibition Increases OL Lineage Cell Numbers and Myelination In Vivo

The aim of this study was to assess the functions of GSK3β in OL differentiation by administering GSK3β inhibitors into the CSF of the lateral ventricle and examining the effects on OPs and OLs in the PVWM (Supp. Info. Fig. 1A, inset). We focused on the developmental stage of P8–P11 in the corpus callosum (CC), which is a key period of OL differentiation characterized by a developmental decrease in OPs and increase in differentiated OLs, with the commencement of active myelination (Supp. Info. Fig. 1B; Hartman et al., 1982). In controls, treatment with sterile vehicle (saline/DMSO) had no effect on the normal pattern of OL differentiation or myelination between P8 and P11 (Supp. Info. Fig. 1B). Notably, we show that the bioactive concentration of GSK3β inhibitors in the PVWM is diluted by 20-fold within the first 15 min and then remains relatively stable at a 30-fold dilution for over 5 h (Supp. Info. Fig. 1A). To account for this dilution effect, we used a range of concentrations of a number of GSK3β inhibitors selected on the basis of the concentrations used in cultures, and the 20- to 30-fold dilution of agents when injected into the ventricle (see Fig. 1 and Supp. Info.). In all cases, coronal sections were carefully taken from the same area of the CC over the posterior ventricle for analyses. All the GSK3β inhibitors had equivalent effects, namely, markedly increasing OLs and resulting in a striking increase in myelination when compared with controls (Fig. 1A,C) and increasing the number of OPs (Fig. 1B,D). Cell counts of PLP/DsRed+ OLs and PDGFαR+ OPs show that ARA-014418, lithium, and indirubin were more effective than L803-mts at the concentrations tested (Fig. 1C,D). The maximal effects on OLs (Fig. 1C) and OPs (Fig. 1D) were observed at injected concentrations of 100 μM ARA-014418 (6 μM dilution corrected), 300 mM lithium (15 mM dilution corrected), 200 μM indirubin (12 μM dilution corrected), and 80 μM L803-mts (5 μM dilution corrected). PLP/DsRed+ OL cell counts were 9.75 ± 0.25 in controls (n = 8) and increased significantly (P < 0.001, Dunnett’s multiple comparisons test) to 21.1 ± 0.8 (n = 8) in 100 μM ARA-014418, 21.15 ± 0.5 (n = 4) in 300 mM lithium, 18.8 ± 0.8 (n = 4) in 200 μM indirubin, and 14.9 ± 0.7 (n = 4) in 80 μM L803-mts (Fig. 1C); PDGFαR+ cell counts in controls were 22.1 ± 0.3 (n = 8) and increased significantly by all the GSK3β inhibitors (P < 0.001, Dunnett’s multiple comparisons test) to 50 ± 1.8 in 200 μM ARA-014418, 45 ± 0.8 in 300 mM lithium, 34 ± 2 in 100 μM indirubin, and 34 ± 0.8 in 100 μM L803-mts (Fig. 1D). A notable effect of all the GSK3β inhibitors was that the density of OPs increased markedly both within the axon tracts of the CC and in the surrounding tissues, where OPs are normally fewer in number at P11 (Fig. 1B). The morphology of OLs and OPs generated by treatment with GSK3β inhibitors appeared normal when compared with controls.

The GSK3β inhibitors also increased myelination in the CC, with ARA-014418, lithium, and indirubin appearing more striking (Fig. 1A). The density of myelin precluded accurate quantification in the CC, and so this was measured in the periventricular cortex (Cx) (Fig. 2). Immunostaining for APC (CC1) was used as a definitive marker for differentiated OLs, and immunostaining for MBP was used to label myelin. As shown above in PLP/DsRed mice, ARA-014418 doubled the number of APC+ OLs and the extent of MBP staining in the CC (Fig. 2A,B). The effects of ARA-014418 are more prominent in the Cx, because there is little myelination in controls at P11, and ARA-014418 advances the progress of myelination toward the pial surface (Fig. 2C); the mean (±SEM) distance between the myelin and the pial surface was decreased significantly (P < 0.01, unrelated t-test) from 747 ± 43 μm (n = 6) in controls to 458 ± 41 μm (n = 7) after ARA-014418 treatment. In addition, because of the lower density of OLs in the Cx, it is possible to distinguish between myelinating and premyelinating OLs, which do and do not support myelin sheaths, respectively (Fig. 2D, insets; Butt and Dinsdale, 2005). ARA-014418 resulted in significant increases (P < 0.001) in both premyelinating and myelinating OLs, although myelinating OLs were by far the most numerous in the Cx after treatment with ARA-014418 (Fig. 2E).

There was a suggestion that we may not have reached the maximal effect for ARA-014418 in the PVWM (Fig. 1), and therefore, we also examined the higher concentrations of 600 μM injected ARA-014418 (36 μM dilution corrected); however, there was no further increase in OLs or OPs when compared with 100 μM injected ARA-014418 (Fig. 2E–G). The concentration of 100 μM ARA-014418 effectively doubled OPs, OLs, and myelination, but had no effect on the density of axons, neurons, or astrocytes (Supp. Info. Fig. 3).

ARA-014418 and Lithium Inhibit GSK3β in OL Lineage Cells

The calculated bioactive concentrations of the GSK3β inhibitors that are effective in the PVWM correlate well with concentrations that are effective in vitro. To examine this further, we investigated the effects of
Fig. 1. Effects of GSK3β inhibitors on oligodendrocyte lineage cells in the corpus callosum. The GSK3β inhibitors, ARA-014418, lithium, indirubin, and L803-mts, were injected at a range of concentrations into the lateral ventricle for 3 days commencing at P8, and the periventricular corpus callosum was examined at P11. (A and B) Confocal micrographs of the periventricular corpus callosum from PLP/DsRed mice, following treatment with saline/DMSO in controls or GSK3β inhibitors, as indicated, and immunolabeled for MBP (A) and PDGFαR (B) to identify myelin sheaths and OPs, respectively; scale bar represents 10 µm in lower panels and 20 µm in top panels. (C and D) Cell counts of OLs (C) and OPs (D) in the corpus callosum showing the concentration effects of GSK3β inhibitors, as indicated. Concentrations are given as injected and dilution corrected based on the measured 20-fold dilution in the CSF (see Supp. Info. Fig. 1A). Values are expressed as a mean number of cells per FOV ± SEM (n ≥ 4). **P < 0.01 Dunnett’s multiple comparisons test. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
ARA-014418 and lithium on OLs in optic nerve organotypic cultures, in which nerves are incubated in the inhibitors and have immediate cellular access (Fig. 3). Incubation of nerves in 20 μM ARA-014418 or 20 mM lithium more than doubled PLP+ OLs and Sox10+ OL lineage cells in situ. Moreover, although some of the inhibitors used can have multiple cellular actions, the key common factor is that they all inhibit GSK3β, and at the concentration used in this study, ARA-014418 is considered an ultraspecific GSK3β inhibitor (Bhat et al., 2003). This was confirmed ex vivo in the optic nerve by Western blot to quantify changes in protein levels (Fig. 3C) and in vivo in the CC by immunostaining.
GSK3β activity is regulated by phosphorylation at the “on-state” Tyr216 and “off-state” Ser9 on GSK3β (Cohen and Goedert, 2004), and the results demonstrate that basal levels of active Tyr216-pGSK3β were high in controls (Fig. 3C,D), which is consistent with other studies on the developing brain and white matter. At the same doses that doubled OPs and OLs (Fig. 3A,B), treatment of optic nerves with ARA-014418 or lithium caused a sevenfold decrease (P < 0.001) in on-state Tyr216-pGSK3β activity, and a corresponding sevenfold increase (P < 0.001) in off-state Ser9-pGSK3β (Fig. 3C). Similarly, immunostaining in the CC demonstrates that both PDGFαR+ OPs and Olig2+ OL lineage cells express high levels of on-state GSK3β activity in controls and this is inhibited by intraventricular injection of ARA-014418 (Fig. 3D). These findings are consistent with in vitro studies, where 20 μM ARA-014418 effectively and specifically inhibits GSK3β (Bhat et al., 2003), and show that an equivalent concentration is achieved in the PVWM to inhibit GSK3β in OL lineage cells in vivo.
The results presented above show that inhibition of GSK3β markedly increases OPs and differentiated OLs.

To determine if this reflects altered proliferation and cell death, we examined PCNA/BrdU and PI labeling in vivo in the CC and Western blot analysis of proliferation and cell death markers in vivo in the optic nerve (Fig. 4). Double immunolabeling for PDGFαR with PCNA (Fig. 4A) and BrdU (Fig. 4A, insets) indicated an increase in proliferation in the CC, and the vast majority of proliferating cells were PDGFαR+ OPs. Cell counts demonstrated that local proliferation of OPs in the CC and Western blot analysis of proliferation and cell death markers in vivo in the optic nerve (Fig. 4).
the CC was increased by over fivefold ($P < 0.001$, unpaired $t$-test), which explains their observed expansion in the face of enhanced differentiation into myelinating OLs. We also examined PI labeling for cell death, and although there appeared to be less labeling following treatment with ARA-014418, there were too few PI+OLs in controls or treated groups for meaningful analysis (not illustrated). We therefore used the ex vivo optic nerve for further analysis of cell death and proliferation markers using Western blot (Fig. 4B). Inhibition of GSK3β with ARA-014418 resulted in significant increases ($P < 0.001$) in the proliferative marker PCNA by 10-fold and the prosurvival factor Bcl-2 by fivefold and a significant decrease in the apoptosis marker caspase-3 by threefold (Fig. 4B). These results indicate that GSK3β inhibitors promote proliferation and are prosurvival in OL lineage cells, consistent with other studies in neurons and glia (Chin et al., 2005; Levine et al., 2000).

**GSK3β Inhibition Stimulates Nuclear Translocation of β-Catenin in OL Lineage Cells**

GSK3β inhibitors are used as Wnt mimetics, because they prevent phosphorylation of β-catenin, allowing its rapid nuclear translocation and initiation of the canonical Wnt signaling cascade. Western blot analysis of optic nerves demonstrates that ARA-014418 caused a sixfold increase ($P < 0.001$) in nuclear pβ-catenin (Fig. 5A), and immunohistochemical analysis of the CC in Sox10/GFP mice shows that ARA-014418 induces nuclear translocation of β-catenin in OL lineage cells (Fig. 5B). There is little β-catenin immunostaining in the control CC (Fig. 5B), consistent with a postnatal loss of Wnt-β-catenin signaling in developing white matter (Coyle-Rink et al., 2002; Takahashi et al., 1994). In contrast, cellular β-catenin is evident following ARA-014418 treatment, and confocal analysis in single z-sections shows that nuclear translocation of β-catenin is localized to Sox10+ OL lineage cells (Fig. 5B). The evidence that ARA-014418 specifically inhibits GSK3β (Fig. 4) and induces nuclear β-catenin (Fig. 5) in OL lineage cells is the strongest evidence that GSK3β inhibitors directly target OLs in the PVWM.

**GSK3β and Wnt3a Differentially Regulate OL Differentiation**

Fig. 5. Inhibition of GSK3β increases nuclear β-catenin in oligodendrocyte lineage cells in the periventricular forebrain. The effects of ARA-014418 on β-catenin expression were examined ex vivo in optic nerve organotypic cultures (A) and in vivo in the corpus callosum (B). (A) Western blot analysis of P10 rat optic nerves incubated in control medium or medium containing ARA-014418 (20 μM) illustrate the increase in expression of nuclear active pβ-catenin. Data are mean densitometric values ± SEM from $n \geq 3$ replicates (**P < 0.001, ANOVA followed by Bonferroni’s post hoc test). (B) Sox10/GFP transgenic mice aged P8 were injected twice daily for 2 days with saline/DMSO vehicle in controls or the GSK3β inhibitor ARA-014418. Brains were examined at P9 1 h after the final injection by coronal sections of periventricular corpus callosum, immunostained for β-catenin (the mouse anti-β-catenin antibody also nonspecifically labels blood vessels). Arrows indicate expression of β-catenin in Sox10+ cells following ARA-014418 treatment, and lower panels illustrate confocal single z-section analysis (1 μm thick) of nuclear translocation of β-catenin in Sox10+ cells. Scale bars in B represent 20 μm in upper panels and 5 μm in lower panels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The results indicate that nuclear translocation of β-catenin and initiation of the canonical Wnt signaling cascade may be a key event in the expansion of OL lineage cells following treatment with GSK3β inhibitors. The Sox10+ cells expressing β-catenin are most likely OPs, as mature OLs express APC and Olig2, which segregate differentially with β-catenin (Fancy et al., 2009; Feigenson et al., 2009). Furthermore, the stimulatory effects of GSK3β inhibition on OLs are at odds with the effects of Wnt-β-catenin, which inhibits terminal OL differentiation and myelination (Fancy et al., 2009; Feigenson et al., 2009). To test this directly, we compared the effects of lithium and a Wnt3a agonist on OLs in P10 mouse optic nerves from Sox10/GFP and PLP/DsRed transgenic mice (Fig. 6). When compared with controls, both lithium and Wnt3a significantly increased ($P < 0.001$, ANOVA) Sox10+ cells, which comprise OPs and OLs (Fig. 6A). In contrast, lithium and Wnt3a differen-
tially affected PLP+ OLs (Fig. 6B), the former more than doubling OLs (P < 0.001), whereas Wnt3a caused a significant (P < 0.01) decrease. Furthermore, combined treatment of lithium with Wnt3a increased Sox10+ cells to levels seen with lithium alone (Fig. 6A) and blocked the negative effects of Wnt3a on PLP+ OLs (Fig. 6B).

The bipartite actions of Wnt3a on Sox10+ and PLP+ cells are consistent with increased generation of OPs and inhibition of their differentiation into myelinating OLs via the canonical Wnt-GSK3β-β-catenin pathway (Fancy et al., 2009; Feigenson et al., 2009). In contrast, the divergent effects of lithium and Wnt3a on mature OLs demonstrate that terminal OL differentiation and subsequent timely myelination are negatively regulated by GSK3β pathways that are distinct from and predominate over the inhibitory Wnt pathway. Two key signaling pathways in OL differentiation that are regulated by GSK3β are CREB and Jagged-Notch. CREB stimulates
Inhibition of GSK3β Stimulates Recruitment of OPs and Remyelination in the Adult

The effects of ARA-014418 on OLs in developing white matter raised the possibility that inhibiting GSK3β may enhance remyelination following demyelination in the adult. To examine this, we used injection of 1% lysolecithin that induces demyelination in the CC after 3 days postinjection (dpi), followed by progressive remyelination after 7 dpi, as previously described (Nait-Oumesmar et al., 1999). At 7 dpi, when compared with ipsilateral untreated CC, lysolecithin induced prominent demyelination in the CC (Fig. 7A) and the neighboring Cx (Fig. 7B), whereas treatment with ARA-014418 markedly increased myelination (Fig. 7A,B). Cell counts show that ARA-014418 significantly increased the generation of Sox10+/APC− OPs (P < 0.001) and differentiation of Sox10+/APC+ OLs (P < 0.01) when compared with lysolecithin treatment alone (Fig. 7C,D); we did not observe any detrimental or negative effects on progenitors of the subventricular zone (SVZ) (not illustrated), which is consistent with studies indicating that these are an important source of OPs in this model of demyelination (Nait-Oumesmar et al., 1999). It is likely that GSK3β inhibition enhanced OL regeneration and remyelination, since ARA-014418 was administered after demyelination occurred (3 dpi). In addition, the results presented above suggest OL survival and OP proliferation will also be enhanced and are likely to be important effects of inhibiting GSK3β. These findings establish that GSK3β inhibits the recruitment and differentiation of OPs after demyelination, retarding repair and remyelination.

DISCUSSION

Multiple extracellular and axon-derived activators and inhibitors precisely control the differentiation of OPs into OLs and regulate the subsequent timing of myelina-
tion. Here, we have identified GSK3β as a profound negative regulator of OL differentiation in vivo. Inhibition of GSK3β not only stimulated proliferation and survival of OPs but also enhanced OL differentiation and myelination via multiple mechanisms. Moreover, GSK3β inhibition had equivalent effects in the adult and stimulated regeneration of OLs and remyelination following demyelination.

GSK3β Inhibitors Injected into the Lateral Ventricle Affect OLs

Agents were delivered to the lateral ventricle of postnatal mouse, and the results show that they achieved bioactive concentrations in the PVWM to act directly on OL lineage cells. Analysis of lithium concentration in the PVWM by atomic absorption demonstrated that injected agents are diluted 20- to 30-fold following intraventricular injection. This is due to the direct dilution of the injectate in the volume of the CSF and the rapid turnover of CSF and drainage into the subarachnoid spaces, and our findings are entirely consistent with measurements of a range of small and large molecular weight agents (Nagaraja et al., 2005; Parandoosh and Johanson, 1982). We tested diverse GSK3β inhibitors and they all had equivalent effects, increasing OPs and OLs and promoting myelination in the PVWM. Calculation of the bioactive concentrations of the agents in the PVWM following intraventricular injection indicated maximal effects at concentrations equivalent to those shown to be effective in neurons and glia in vitro and in vivo (Bhat et al., 2003; Chen et al., 2004; Goldbaum and Richter-Landsberg, 2002; Kaidanovich-Beilin et al., 2004), and we show that direct administration of GSK3β inhibitors at these concentrations had the same effect on OLs ex vivo in the optic nerve. Therefore, we conclude that the highest concentrations of GSK3β inhibitors used in this study are in the same range as those used in vitro, in agreement with our previous findings on the actions of FGF-2 in vitro (Goddard et al., 1999; Butt and Dinsdale, 2005).

Inhibition of GSK3β Activity in OLs

The diverse range of inhibitors used had similar effects, indicating that they acted specifically and directly to inhibit GSK3β in OL lineage cells to increase their numbers and stimulate differentiation. In the case of ARA-014418, it is proven to be specific in inhibiting GSK3β at the concentrations used in our study (Bhat et al., 2004). We demonstrate that ARA-014418 inhibits GSK3β activity in OLs, and the concentrations of 6 μM in the PVWM and 20 μM in optic nerves are within the range of 4–50 μM used in vitro to specifically inhibit GSK3β in neurons (Bhat et al., 2003; Wang et al., 2007). Moreover, ARA-014418 induced nuclear translocation of β-catenin in OL lineage cells, which is a reported specific effect of ARA-014418 and is dependent on GSK3β
inhibition (Cohen and Goedert, 2004; Grimes and Jope, 2001b). Hence, the effects of ARA-014418 on OLs are unlikely to be due to off-target effects. In addition, we showed that OLs were similarly increased by lithium, indirubin, and L803-mts. Although these agents have diverse modes of action, they have in common that they inhibit GSK3β, providing evidence that GSK3β was the specific target mediating the changes in OLs. Our results are consistent with the reported actions of these agents. In unstimulated cells, GSK3β is phosphorylated

Fig. 7. GSK3β inhibition promotes regeneration of oligodendrocytes and remyelination. Demyelinated lesions were produced by injection of 1% lysolecithin in the periventricular white matter of adult Sox10/GFP transgenic mice. At 3 days postlesion (dpl), mice were treated with saline/DMSO vehicle in controls or ARA-014418 by injection into the lateral ventricle, and brains were examined at 7 dpl by coronal sections of periventricular forebrain. (A and B) Immunostaining for APC for differentiated OLs and MBP for myelin in the corpus callosum (A) and cortex (B) from the contralateral untreated forebrain, and from the ipsilateral forebrain following lysolecithin mediated demyelination and treatment with saline/DMSO (controls) or ARA-014418, as indicated; asterisks indicate demyelinated lesions in mice treated with saline/DMSO vehicle, which are absent in mice treated with ARA-014418. Insets in (A) illustrate the loss of coexpression of APC and Sox10 within demyelinated lesions in saline/DMSO-treated mice controls when compared with extensive coexpression of APC and the Sox10 reporter in controls and remyelinated lesions following treatment with ARA-014418. Photomicrographs are flattened confocal images of 15 μm thickness in main panels, and 5 μm thickness in insets. Scale bars represent 20 μm in main panels and 5 μm in insets. (C) Quantification of Sox10+/APC− OLs and Sox10+/APC+ OLs in the corpus callosum and cortex following lysolecithin mediated demyelination and treatment with saline/DMSO (controls) or ARA-014418. Data are mean number of cells in a constant volume (FOV), as detailed in Materials and Methods section; error bars represent SEM; n = 4 animals (*P < 0.05, **P < 0.01, ***P < 0.001, unpaired t-tests). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
by tyrosine phosphatases at the Tyr216 site to render GSK3β active (Hughes et al., 1993), and GSK3β is inactivated by phosphorylation to the Ser9 residue by several upstream serine kinases under stimulated or growth factor-induced conditions (Grimes and Jope, 2001b). Consistent with our findings, Ser9-GSK3β levels peak around 30 min of lithium incubation in brain organotypic studies and then fall over a 24-h period (Lee et al., 2008), whereas the Tyr216-pGSK3β form is reduced over longer periods (Yoshida et al., 2006). Other GSK3β inhibitors promote the Ser9-GSK3β form after approximately 60 min (Nassif et al., 2007), and ARA-011418 inhibits GSK3β at the ATP binding site in the kinase domain by conformational alteration to indirectly increase the inhibitory phosphorylation at the Ser9 site by upstream kinases (Bhat et al., 2003, 2004). Furthermore, we did not observe effects of ARA-014418 on neurons, axons or astrocytes, indicating that ARA-014418 acts directly on OPs and OLs and that GSK3β is a key negative regulator of OL differentiation.

**GSK3β and Wnt3a Differentially Regulate OL Lineage Cells**

GSK3β inhibition increased the proliferation and survival of OPs and promoted their differentiation into OLs. The effects of GSK3β inhibition on OPs may be primarily via canonical Wnt-β-catenin, as we show that ARA-014418 increased nuclear translocation of β-catenin in Sox10+ cells and that OPs are regulated by the canonical Wnt-β-catenin pathway (Fancy et al., 2009; Feigenson et al., 2009). In addition, ARA-014418 was prosurvival and mediated increased proliferation in OPs, which are key effects of Wnt-β-catenin signaling (Adachi et al., 2007; Kalani et al., 2008; Lie et al., 2005). Although the effects of GSK3β inhibition on OPs may be primarily via canonical Wnt-β-catenin, we show that GSK3β inhibition and Wnt3a have opposing effects on OL differentiation. Wnt3a signaling functions in a bipartite manner to increase OPs, but to restrict their differentiation into myelinating OLs, consistent with genetic studies on embryonic and postnatal development (Fancy et al., 2009; Feigenson et al., 2009). This is in direct contrast to the effects of GSK3β inhibition, which promotes OL generation via multiple pathways, including CREB and Notch. Inhibition of GSK3β increased CREB activity, which is a positive regulator of OL differentiation and myelination, and is able to overcome inhibition of OL differentiation in vitro (Grimes and Jope, 2001a). CREB-induced transcription also activates Bcl2 gene expression directly to prevent cell death in OLs (Saini et al., 2004). The observed reciprocal increase in active CREB, Bcl2, and PCNA following treatment in ARA-014418 indicates that GSK-3β regulated changes in OLs are via CREB. In addition, the timely progression of OL differentiation and myelination is dependent on the negative regulatory factor Notch (Wang et al., 1998), and we show that inhibition of GSK3β decreased Notch and promoted OL differentiation. Thus, our results demonstrate that GSK3β controls multiple positive and negative regulators of OL differentiation to promote OL maturation and myelination. Importantly, these GSK3β-dependent mechanisms override the negative effects of Wnt3a signaling.

**GSK3β Inhibition Stimulates OL Regeneration and Remyelination**

In the autoimmune mouse model of demyelination (EAE), systemic lithium treatment has been shown to increase remyelination (De Sarno et al., 2008). In our study, we show that direct inhibition of GSK3β in OLs significantly stimulates their regeneration within demyelinating lesions and dramatically improves remyelination. Hence, endogenous GSK3β is retarding the repopulation of demyelinated lesions and their subsequent remyelination. These GSK3β-mediated effects are consistent with the effects we observed in the postnatal brain and show that the generation of OPs and their differentiation into OLs are negatively regulated by GSK3β. The failure of remyelination in adults is due in part to upregulation of negative regulatory factors within lesions, including Wnt and Notch (Fancy et al., 2009; John et al., 2002). Our results show that inhibiting GSK3β overcomes the negative effects of Wnt3a and down-regulates Notch signaling to stimulate OP proliferation and survival, as well as promoting OL differentiation and myelination via CREB. This study indicates that targeting GSK3β may be an adjunct to therapeutic approaches for promoting OL regeneration and remyelination.

**ACKNOWLEDGMENTS**

The authors thank Professor Stallcup for antibodies against PDGFαR; Professor Kirchhoff for the PLP-DsRed transgenic mouse line; and Professor Richardson for the Sox10-GFP transgenic mouse line.

**REFERENCES**


GLIA