GSK3β Regulates Oligodendrogenesis in the Dorsal Microdomain of the Subventricular Zone via Wnt-β-catenin Signaling

Kasum Azim,1,2 Andrea Rivera,1 Olivier Raineteau,2,3 and Arthur M. Butt1

Oligodendrocytes, the myelinating cells of the CNS, are derived postnatally from oligodendrocyte precursors (OPs) of the subventricular zone (SVZ). However, the mechanisms that regulate their generation from SVZ neural stem cells (NSC) are poorly understood. Here, we have examined the role of glycogen synthase kinase 3β (GSK3β), an effector of multiple converging signaling pathways in postnatal mice. The expression of GSK3β by rt-qPCR was most prominent in the SVZ and in the developing white matter, around the first 1–2 weeks of postnatal life, coinciding with the peak periods of OP differentiation. Intraventricular infusion of the GSK3β inhibitor ARA-014418 in mice aged postnatal day (P) 8–11 significantly increased generation of OPs in the dorsal microdomain of the SVZ, as shown by expression of cell specific markers using rt-qPCR and immunolabelling. Analysis of stage specific markers revealed that the augmentation of OPs occurred via increased specification from earlier SVZ cell types. These effects of GSK3β inhibition on the dorsal SVZ were largely attributable to stimulation of the canonical Wnt/β-catenin signaling pathway over other pathways. The results indicate GSK3β is a key endogenous factor for specifically regulating oligodendrogenesis from the dorsal SVZ microdomain under the control of Wnt-signaling.

Key words: oligodendrocyte precursor, subventricular zone, glycogen synthase kinase 3β, Wnt, neural stem cells

Introduction

Oligodendrocytes (OLs) are the myelinating cells of the CNS and are essential for normal salutatory conduction. OLs in the postnatal forebrain are derived from oligodendrocyte precursors (OPs) that originate from neural stem cells (NSCs) of the subventricular zone (SVZ) of the lateral ventricle (LV). NSCs first give rise to cycling neural progenitors (NPs) (also known as Type-C cells), identified by their expression of the transcription factor (TF) Mash1 and S-phase markers, which then differentiate into OPs or NPs destined for the olfactory bulb (Kessaris et al., 2006; Marshall et al., 2005; Menn et al., 2006; Nakatani et al., 2013; Richardson et al., 2006). The mechanisms regulating SVZ-derived OP specification from Type-C cells are unresolved, but are believed to involve specific environmental and transcriptional cascades (He and Lu, 2013). Postnatally, there is evidence that OPs are derived primarily from the dorsal SVZ (dSVZ), whereas the lateral SVZ (lSVZ) has the greatest neurogenic potential for olfactory neurons (Brill et al., 2009; Kessaris et al., 2006; Marshall et al., 2005; Menn et al., 2006; Young et al., 2007). Recent studies shed new light on the instructive basis of NSC identities in dSVZ and lSVZ microdomains. For example, FGF2 increases OP proliferation in the dSVZ and ectopically in the lSVZ microdomains during development and into adulthood (Azim et al., 2012b). These findings complement our recent study demonstrating an early postnatal role for Wnt-β-catenin signaling in regulating NSC specification and oligodendrogenesis in the dorsal SVZ (Azim et al., 2014), whereas in the adult SVZ Wnt3a strongly increases OL proliferation in the dSVZ without affecting lineage choice or proliferation within neurogenic clones (Ortega et al., 2013).

Glycogen synthase kinase 3β (GSK3β) is a potent negative regulator of multiple signaling pathways in neuronal
development, most notably Wnt/β-catenin, as well as Shh and Notch1 pathways (Grimes and Jope, 2001; Kim et al., 2011). In unstimulated cells, GSK3β primes β-catenin for destruction and in the presence of canonical Wnt ligands, β-catenin translocates to the nucleus to target specific sets of genes associated with Wnt-signaling (Doble and Woodgett, 2003). Because canonical Wnt/β-catenin signaling appears to regulate OL and NP generation in the early postnatal SVZ (Azim et al., 2014), but only OLs in the adult SVZ (Ortega et al., 2003). Because canonical Wnt/β-catenin signaling regulates OL and NP generation in the early postnatal SVZ in the second week postnatal. The results indicate that at P8–11, the major period of oligodendrogenesis from the postnatal SVZ in the second week, and accounting for the dilution effects of injected agents were used at 2.5 mg L⁻¹ in accordance with Lie et al., 2012b). In unstimulated cells, GSK3β/Wnt/β-catenin is a key determinant of OL generation specifically in the dorsal microdomain of the SVZ.

Materials and Methods

Animals

Mice aged postnatal day (P)8–11 were used throughout. All animal procedures were performed in accordance with the UK Home Office Animals Scientific Procedures Act (1984). Wild-type mice (C57/BL6) were used together with transgenic mice of C57/BL6 background in which fluorescent reporters were driven by Sox10–EGFP mouse to identify OPs and OLs as previously characterized (Azim et al., 2012b).

In Vivo Injections

Pups of similar size litters were used throughout. Intraventricular injections of the GSK3β inhibitor ARA-014418 (Bhat et al., 2003) were performed daily for 3 days commencing at P8 and brains examined at P11, as previously described (Azim and Butt, 2011). In brief, mice were deeply anaesthetized under isofluorane and ARA-014418 (Sigma–Aldrich) was delivered into the cerebrospinal fluid of the LV using a Hamilton syringe, at a point 2 mm from the midline along the Bregma, and to a depth of 2 mm. ARA-014418 was stored in DMSO, and diluted in sterile saline vehicle and delivered in a volume of 2 µL; sterile saline/DMSO vehicle was used as controls, referred to as controls henceforth. The growth factors PDGF-AA, IGF1 and FGF2 were used at 5 µg mL⁻¹ in volumes of 2 µL, as in our previous studies (Azim et al., 2012b; Butt et al., 1997; Goddard et al., 1999). The growth factors Wnt3a, EGF and BMP4 were used at 2.5 µg mL⁻¹ in volumes of 2 µL, in accordance with their effective in vitro concentrations in NSC cultures (Lie et al., 2005), and accounting for the dilution effects of injected agents (Azim and Butt, 2011). Shh was injected at 100 µg mL⁻¹ in a volume of 2 µL, as previously reported (Jiao and Chen, 2008). Wnt3a, BMP4, IGF1, PDGF-AA, and EGF were obtained from R&D Systems. Shh was obtained from eBiosciences and FGF2 obtained from Peprotech, USA.

Immunohistochemistry

Published protocols were followed for immunolabelling (Azim and Butt, 2011; Azim et al., 2012b). In brief, brains were immersion fixed in 4% paraformaldehyde and coronal vibratome sections of 20–100 µm thickness were cut through the periventricular forebrain. Primary antibodies used were: rabbit anti-PDGFβR (1:400, gift from Prof Stallcup; or 1:100, Abcam); goat anti-PDGFβRα(1:200, R&D Systems); rat anti-MBP (1:300, Millipore); mouse anti-Nestin (1:300, BD Biosciences); rabbit anti-Nestin (1:300, R&D Systems); rabbit anti-GFAP (1:300, DAKO); mouse anti-nuclear β-Catenin (1:300, Abcam); goat anti-Tyr216-pGSK3β (1:100, Santa Cruz); goat anti-Ser9-pGSK3β (1:100, Santa Cruz); mouse anti-PCNA (1:400, Sigma–Aldrich); mouse anti-Mash1 (1:200, BD Biosciences). Appropriate species-matched secondary antibodies conjugated with Alexafluor 488, 568 or 405 (1:500, Molecular Probes) were used. 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 or Mash1 labeling, antigen retrieval was performed, whereby free-floating sections were pretreated with PBST and NP-40 1% for 20 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 nuclear β-catenin, Ser9-pGSK3β, and Tyr216-pGSK3β, treated brains were fixed for 3 h in Histochioce Fixative (Sigma–Aldrich). PBS was replaced by TBS (0.5M Tris Base, 9% NaCl, pH 8.4) throughout, to reduce nonspecific labeling of anti-phospho antibodies. For pGSK3β detection, sections were incubated in Proteinase K (20 µg mL⁻¹ in TBS, Invitrogen) for 20 min at 37°C followed by incubation in 1% SDS for 1 hr at RT and several washing steps. After final washes in PBS, tissues were mounted on poly-lysine coated glass slides with Vectashield mounting media (Vector Laboratories) and sealed with coverslips. Images were acquired using an LSM 5 Pascal Axioskop2 or LSM 510 meta 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).

Cell Counts

Rostral periventricular coronal sections containing the LV were analyzed (>3 sections per brain, n > 4 animals per group) at rostrocaudal axis 0.6–0.9 relative to the Bregma; counts of OL and OP cell numbers confirmed that there were no significant differences between sections in untreated controls (Azim and Butt, 2011; Azim et al., 2012b). Images were processed with Zeiss LSM Image Examiner (V. 5.2.0.121), maintaining the acquisition parameters constant to allow comparison between samples. Coronal sections were used throughout and cell counts performed in the dSVZ, lSVZ and corpus callosum/periventricular white matter on flattened confocal z-stacks, of 230 µm² × 230 µm² in the x-y-plane, and 30 µm in the z-plane. Extracellular markers were quantified via a nuclear counterstain (propidium iodide (Sigma–Aldrich) or DAPI (Molecular Probes/Invitrogen)). Counts are expressed as mean (SEM, n ≥ 4) cells per field of view volume (FOV) of 1.6 × 10⁶ µm³, where the "n" value represents the number of mice and the standard error of the mean (SEM). Cell counts were tested for significance using
GraphPad Prism v302, for unpaired t test (referred to as t test) or ANOVA followed by Bonferroni’s post hoc test as appropriate.

**SVZ Microdissection and Gene Expression Profiling**

Published protocols were followed for microdissection of the SVZ microdomains and subsequent RNA procedures (Azim et al., 2012b). ARA-014418 or saline/DMSO was injected into the LV as above at P8 and at P9 animals were sacrificed ~2.5 h after last treatment by cervical dislocation. A litter of pups were pooled to yield 1 “n” value. Primers used were: GAPDH, forward caaggtcgctgccgctgagcct reverse tggactggttagacg; MASH1 forward, GACATTGGAGGCA GGTAGG, reverse GCTGCTGGTTTTCCTTTTCT; Axin2: forward GGGGAAAACACACCTTACA, reverse ACTGGGTCCG TCTCCTGGA; Fzd1: forward CAAAGTTTACGGGCTCATGT, reverse GTAAACAGGGGACAGAA; GSK3β: forward GTGG TACCTTGGTCCATC, reverse GACCGAGAAACACTTCTTT; ACG2 forward, CTCAACCTGCCCATTTCAAGTGC; Reverse GTGGGA AGTGCAGGCTGTGAGA; Nestin ForwardTACAA AGTCCTGGTCAAGAGGTAGTCTC, Reverse GGTGGT CGTTATGCCTTACCGAG; Hes1 Forward AGGAAATCCGATACCGGAG, Reverse GGTGGTT GCTGCTTCTTCTTCT; Hes3 Forward CGCTGGGATGCTGAATTAGT , Reverse TAGTCCTGGTGCAGGCTCTT , Hes5 Forward CGGAAAAGCAAGCGTAATCT; Reverse GCTGCTGGAGTTCCTGGG, Reverse CGGAAAAGCAAGCGTAATCT; Hes5 Forward CGGAAAAGCAAGCGTAATCT, Reverse GCTGCTGGAGTTCCTGGG, Reverse CGGAAAAGCAAGCGTAATCT; Hes5 Forward CGGAAAAGCAAGCGTAATCT, Reverse GCTGCTGGAGTTCCTGGG, Reverse CGGAAAAGCAAGCGTAATCT; Hes5 Forward CGGAAAAGCAAGCGTAATCT, Reverse GCTGCTGGAGTTCCTGGG, Reverse CGGAAAAGCAAGCGTAATCT.

**Results**

**Regional Expression and Activity of GSK3β in SVZ Microdomains**

We have shown that GSK3β is implicated in OL differentiation in the developing white matter (Azim and Butt, 2011), but it is not clear whether GSK3β regulates the generation of OPs in the postnatal SVZ. To examine this, we determined spatiotemporal differences in GSK3β expression by qPCR of microdissected dSVZ and lSVZ compared with corpus callosum (CC) at differing postnatal ages and in the adult. Transcript levels were normalized against GAPDH housekeeping gene by the comparative AA−CT method (Azim et al., 2012b) and expression values compared for statistical significance by ANOVA followed by Bonferroni’s test (Fig. 1A,B). Overall, spatial expression was greatest in the dSVZ at P4 (Fig. 1A; P < 0.05), and in the corpus callosum at P8-P11 (Fig. 1A; P < 0.05). Levels in the corpus callosum fell markedly in the adult (Fig. 1B, P < 0.01), whereas overall levels were significantly greatest at P8 in both microdomains of the SVZ (Fig. 1B; P < 0.01), although they remained fairly constant at all ages. The results are consistent with GSK3β being most active during the peak period of OL generation in the postnatal forebrain. We tested this directly by measuring GSK3β activity in the SVZ following infusion of the GSK3β inhibitor ARA-014418, as detailed previously (Azim and Butt, 2011; Azim et al., 2014). GSK3β activity was determined by immunostaining for Ser9-pGSK3β as a marker for the inactive form of GSK3β and Tyr216-GSK3β as a marker for the active form (Cohen and Goedert, 2004). Immunostaining demonstrates GSK3β activity in both dSVZ and lSVZ (Fig. 1C–F) in dSVZ OPs (Fig. 1G–J), and western extracts above 15KDa. Purification of cytoplasmic and nuclear extracts were obtained using the “NE-PER Nuclear and Cytoplasmic Extraction” kit (Thermo Fisher Scientific, IL) and protein concentration determined by Bradford protein assay. Subsequent standard procedures were followed for western blot. Primary antibodies used were: mouse anti-total GSK3β (1:2,000, BD Biosciences); mouse anti-nuclear pβ-Catenin (1:1,000, Abcam); mouse anti-β-Catenin (1:2,000, Abcam); mouse anti-β-actin (1:10,000, Sigma–Aldrich); goat anti-Tyr216-pGSK3β (1:500, Santa Cruz); goat anti-Ser9-pGSK3β (1:500, Santa Cruz); mouse anti-Notch1 intracellular cleaved component (NICD) (1:1,000, Millipore); rabbit Anti-phospho-STAT1/MAD1/5/8 (1:1,000, cell signaling); Anti-Gli1 (1:1,000, cell signaling); rabbit Anti-Histone H3 (1:4,000; Millipore); rabbit anti-Phospho-Erk1/2 (1:1,000, cell signaling); mouse anti-Total Erk1/2 (1:2,000, cell signaling). Proteins were visualized by enhanced chemiluminescent detection (Amersham Biosciences), and signal intensities measured via ImageJ software (NIH) and normalized to β-actin or H3 internal controls as appropriate. Experiments were repeated independently at least three times and band densitometry values tested for significance using unpaired t test or ANOVA followed by Bonferroni’s post hoc test.
FIGURE 1: Functional expression of GSK3β in SVZ microdomains and corpus callosum. (A, B) GSK3β expression was measured by qPCR in microdissected microdomains of the P8 SVZ (Azim et al., 2012b) and corpus callosum (CC) at different ages. GAPDH normalized expression values were compared for statistical significance by ANOVA followed by Bonferroni’s test. (C–F) Effects of ARA-014418 on GSK3β activity were examined by immunostaining for the inactive form Ser9-pGSK3β in the SVZ microdomains in combination with PI labeling. (G–J) Expression of both Ser9-pGSK3β and the active form Tyr216-pGSK3β in OPs, identified by immunostaining for PDGFRα following ARA-014418 treatment. Arrows in C–F show examples of pronounced endogenous GSK3β activity in the SVZ before (C, E) and after ARA-014418 (D, F). Arrows in G–J indicate increased inactivity in OPs following ARA-014418 infusion (G, H) as well as reduction in the active forms of GSK3β activities within OPs (I, J). (K, L) Western blot analysis of inactive (ser9) pGSK3β and total GSK3β; (L) Data are mean densitometric % changes in values ± %SEM (error bars) from n>3 replicates (**P<0.01; *P<0.05). Grey bars represent ARA-014418 and white bars represent controls. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
blot shows inhibition was significantly greater in the dSVZ microdomain (Fig. 1K,L; \( P < 0.05 \), ANOVA followed by Bonferroni’s post hoc test). Two-thirds of OPs in the corpus callosum expressed “active” Tyr216-pGSK3\( \beta \) in controls, and this was significantly decreased following treatment with ARA-014418 (from 64\% \pm 8\% of PDGFR\( \alpha \) cells \((n = 4)\) to 33.8\% \pm 6.5\% of PDGFR\( \alpha \) cells \((n = 5); P < 0.01, t\) test), with a concomitant increase in the “inactive” form Ser9-pGSK3\( \beta \) (from 27.5\% \pm 3.3\% of PDGFR\( \alpha \)+ cells in controls \((n = 4)\) to 72.3\% \pm 7.6\% of PDGFR\( \alpha \)+ cells in ARA-014418 \((n = 5); P < 0.01, t\) test). These data show that GSK3\( \beta \) is effectively inhibited by LV infusion of ARA-014418 and demonstrate prominent endogenous GSK3\( \beta \) activity in the SVZ, with greatest activity at P8–11 in the dSVZ microdomain, the time and site of greatest OP generation postnatally. ARA-014418 is not known to have off-target effects on other kinases (Bhat et al., 2003) as described previously (Azim and Butt, 2011; Azim et al., 2014).

**GSK3\( \beta \) Regulates Generation of OPs Predominantly from the dSVZ Microdomain**

The data presented above indicates that GSK3\( \beta \) activity is greatest postnatally in the dSVZ microdomain, which a number of studies suggest is the primary source of OPs that migrate to populate the forebrain during this period (Azim et al., 2014; Kessaris et al., 2006). We therefore examined whether GSK3\( \beta \) specifically regulates the generation of OPs in the dSVZ, using transgenic mice in which the lineage-specific transcription factor Sox10 drives the expression of EGFP (Fig. 2). Inhibition of GSK3\( \beta \) with ARA-014418 markedly increased the density of Sox10-EGFP+ cells in the SVZ and surrounding periventricular white matter (PVWM), compared with controls (Fig. 2A,B). Detailed examination of the dSVZ and ISVZ microdomains showed that by far the predominant effect of GSK3\( \beta \) is on the dSVZ, where Sox10-EGFP+ cells were significantly increased threefold (Fig. 2C,D,G; \( P < 0.01, t\) test), and in the adjacent PVWM (Fig. 2I), compared with...
a small although statistically significant effect on the lSVZ (Fig. 2E,F,H). These findings are consistent with GSK3\(\text{b}\) activity being greatest in the dSVZ microdomain and having a primary role in regulating the generation of OPs from this microdomain, resulting in an increase in the number of OLs in the overlying PVWM.

**GSK3\(\text{b}\) Negatively Regulates the Generation of OPs and NPs Within Specific SVZ Microdomains**

The results presented above demonstrate GSK3\(\text{b}\) activity in the ISVZ, but that its inhibition did not markedly alter OP numbers in this microdomain, suggesting GSK3\(\text{b}\) mainly regulates OP generation from the dSVZ. Therefore, we next examined the effects of GSK3\(\text{b}\) inhibition on NSCs and their progeny by qPCR of microdissected microdomains (Fig. 3A–C) and immunostaining (Fig. 4A–D). As described previously (Azim et al., 2012a,b), the SVZ is delineated up to 70 \(\mu\)m from the ependymal wall, based on labeling and aided by nuclear markers. Transcripts enriched in early SVZ cells, ABCG2 and Nestin, were significantly increased by ARA-014418 in both SVZ microdomains, indicative of an increase in NSCs (Fig. 3A; \(P < 0.05\), t test), whereas the NP marker Mash1, and the OP markers Olig2 and PDGFR\(\alpha\) were differentially altered in the two microdomains (Fig. 3B,C). Transcripts for Mash1, which is expressed by NPs that generate both neurons and OLs, were significantly increased in both microdomains, but the effect was greatest in the dSVZ, where it was barely detectable in controls and reached expression levels similar to the control ISVZ following GSK3\(\text{b}\) inhibition (Fig. 3B). In contrast, GSK3\(\text{b}\) inhibition markedly increased the OL lineage and OP transcripts Olig2 and PDGFR\(\alpha\) by over threefold in the dSVZ (Fig. 3C; \(P < 0.01\), t test), with levels in the ISVZ being very low and in the case of PDGFR\(\alpha\) not statistically affected by GSK3\(\text{b}\) inhibition. Immunohistochemical examination of the potential origins of OPs from the dSVZ indicates GFAP+ NSCs and Sox10-EGFP+ cells were segregated populations (Fig. 4A,B). In contrast, GSK3\(\text{b}\) inhibition resulted in striking increases in the dSVZ of Sox10-EGFP coexpression with the NSC/NP marker Nestin (Doetsch et al., 1997) (Fig. 4C–E) and the NP marker Mash1 (Fig. 4F–H), consistent with previous reports of early or new OPs expressing Mash1 (Nakatani et al., 2013).

---

**FIGURE 3:** Inhibition of GSK3\(\text{b}\) up-regulates markers for NSCs, NPs and OPs predominantly in the dSVZ. Real-time qPCR analysis of the SVZ microdomain showing the effects of ARA-014418 on markers for (A) NSCs (ABCG2 and Nestin), (B) NPs (Mash1), and (C) OPs (Olig2 and PDGFR\(\alpha\)). Expression was normalized to the house keeping gene GAPDH. White bars are saline/DMSO treated controls and grey bars ARA-014418 treated. Error bars show the SEM (*\(P < 0.05\), **\(P < 0.01\); t test).
Notably, GSK3β inhibition induced a massive expansion in Mash1+ cells in the dSVZ, and a significant ($P < 0.001; t$ test) three-fold increase in the proportion of Mash1+/Sox10-cells (arrows; Fig. 4G,H). Mash1 expression in OPs is confined to the earliest stage of their differentiation and is transient, hence the large increase in Mash1+/Sox10- cells is consistent with these cells generating the increase in Sox10+/Mash1- OPs (Nakatani et al., 2013; Ortega et al., 2013). The remaining proportion of Mash1+ cells that do not express Sox10-EGFP are Type-C cells and a small fraction of Mash1
expressing neuroblasts (Azim et al., 2012a; Parras et al., 2004; Smith and Luskin, 1998). Double immunolabeling for PDGFRα with PCNA (proliferating cellular nuclear antigen) demonstrated a doubling in proliferating PDGFRα+ OPs and PDGFRα- cells within the dSVZ following GSK3β inhibition, the latter indicative of potential and likely NSC proliferation (Fig. 4I–K; P < 0.001, t test). These findings indicate that GSK3β regulates the generation of NP in both the dSVZ and ISVZ, while regulating the generation of OP in a region-specific manner. Cell death was examined using propidium iodide labeling, but in all cases was low in controls and following treatment, in line with our previous findings (Azim and Butt et al., 2011).

**GSK3β Differentially Regulates Multiple Targets in SVZ Microdomains**

GSK3β is known to modulate several signaling pathways in neural development, most notably Wnt and notch1 pathways (reviewed in (Grimes and Jope, 2001; Kim and Snider, 2011), which we previously showed were mechanisms by which GSK3β regulates OL differentiation in the optic nerve (Azim and Butt, 2011). We therefore examined this in the SVZ microdomains using western blot and qPCR (Fig. 5). In the P9 SVZ, cytosolic bound β-catenin was increased in both dSVZ and ISVZ microdomains following ARA-014418 (Fig. 5A,B), whereas nuclear levels of β-catenin were increased to a significantly greater extent in the dSVZ (Fig. 5C,D; P < 0.01, t test). Moreover, within cytoplasmic extracts, the levels of Erk1/2 were not affected by ARA-014418, indicating it did not have off target effects on other growth factor-related signaling pathways (Fig. 5A). In addition, we measured nuclear accumulation of the transcription form of Notch1 (NICD), together with BMP signaling via pSMAD1/5/8 and Gli1 for Shh-signaling (Fig. 5C–E). Nuclear levels of activated cleaved Notch1 (NCID) were reduced similarly in both SVZ microdomains (P < 0.05; t test), whereas BMP-signaling was only decreased in the dSVZ (P < 0.05; t test), and Gli1 was marginally, but significantly, increased in the ISVZ, consistent with greater Shh expression in this microdomain (P < 0.05, t test) (Azim et al., 2012b; Palma et al., 2005). These effects were further validated by qPCR of microdissected dSVZ assayed ~2.5 h following ARA-014418 infusion (Fig. 5F). Transcripts for β-catenin target genes cyclin D1, Axin2 and Lef1 were all significantly increased by almost threefold in the dSVZ following GSK3β inhibition (Fig. 5F; P < 0.01, t test). By comparison, the Notch1 target Hes3 and Hes5 genes were marginally but statistically significantly downregulated, whereas Hes1 was not statistically altered (P < 0.05; t test), as was the case for BMP signaling—Sox4, ID2, Stat1 (Fig. 5F). These findings suggest that the predominant effect of inhibiting GSK3β in the dSVZ microdomain is largely attributed to the Wnt pathway, with little effect on other pathways that regulate oligodendrogenesis.

**FGF2 and Wnt3a Act by Inhibiting GSK3β**

The effects of ARA-014418 demonstrate that endogenous GSK3β regulates the generation of OPs in the postnatal SVZ. Several trophic factors are known to regulate NSC fate and OP differentiation, including FGF2 and Wnt (Azim and Butt, 2011; Azim et al., 2012, 2014), and so we examined whether these may act to inhibit GSK3β in a manner similar to ARA-014418 (Fig. 6A,C). Growth factors were infused into the LV of P9 pups and animals sacrificed 45 min later and microdissected dSVZ analyzed by western blot of cytosolic extracts for Ser9 pGSK3β. Compared with controls, we found that only FGF2 and Wnt3a inhibited pGSK3β (Fig. 6A,C; P < 0.01, t test), and we confirmed that Wnt3a and ARA-014418 acted to increase nuclear β-catenin translocation (Fig. 6B,D; P < 0.01, t test), as did FGF2, but to a lesser extent (P < 0.05; t test). In addition, EGF appeared to reduce Ser9 pGSK3β via unknown mechanisms, but this was not examined further. Intriguingly, BMP4 and EGF reduced nuclear β-catenin (P < 0.01, t test) (Fig. 6C,D), whereas other growth factors (FGF1, PDGF-AA, and Shh) did not affect pGSK3β or nuclear β-catenin. Hence, inhibition of GSK3β with ARA-014418 primarily mimics the effects of endogenous canonical Wnts on the dSVZ and to a lesser extent FGF2, consistent with previous findings (Azim et al., 2012b, 2014; Ortega et al., 2013).

**Discussion**

Multiple cues control the numbers of OPs in the CNS, but the factors that regulate their generation from germinal SVZ cells is unclear. Here, we show that inhibition of GSK3β with ARA-014418 profoundly stimulated the expansion of NPs in the dSVZ and their differentiation into OPs derived from this microdomain. The results demonstrate that oligodendrogenesis is strongly regulated by GSK3β predominantly in the dSVZ microdomain acting via the canonical Wnt/β-catenin pathway.

The default source of SVZ-derived oligodendrogenesis under specific demyelinating or growth factor conditions appears to be dSVZ (Azim et al., 2012b, 2014), consistent with this microdomain in generating most of the forebrain derived OL lineage cells during postnatal development (Kessaris et al., 2006). Inhibiting GSK3β resulted in the preferential genesis of OL lineage cells from the dSVZ, mirroring our previous observations of Wnt signalling and FGF2 (Azim et al., 2012b, 2014). Furthermore, we provide evidence that FGF2 acts in part to inhibit GSK3β and increase nuclear translocation of β-catenin, but not to the same extent as canonical Wnt-signaling. Targeted genetic ablation of GSK3β...
in NSCs of the developing telencephalon dramatically disrupts neuronal maturation, whilst NSCs and cycling NPs are massively upregulated (Kim et al., 2009a). In comparison, targeted β-catenin expression in later OL lineage cells delays their differentiation (Fancy et al., 2009; Ye et al., 2009). In addition, inhibitors for GSK3β have been used previously in vivo to assess the role of Wnt-signaling in determining cell fate in the SVZ and in NSCs (Adachi et al., 2007; Azim et al., 2014; Kriks et al., 2011; Maurer et al., 2007). Our findings identify a key role for GSK3β and Wnt/β-catenin in regulating oligodendrogenesis in the forebrain from the proliferating Mash1+ NPs in the dSVZ microdomain. Adachi
et al. (2007) have described that the increased proliferation observed in the SVZ results in an increase migration of NPs and OPs. Our results suggest that might occur in neonates observed in the SVZ results in an increase migration of NPs, FGF2 represses SMADs to induce Olig2 expression of SVZ gliogenesis. However, in cultured dorsal spinal cord NPs, FGF2 represses SMADs to induce Olig2 expression in NSCs that promoted Shh signaling via Gli1 and Lef1 together with increases in OL lineage markers from NSCs of the dSVZ microdomain in early postnatal mice (Azim et al., 2014) and adult mice (Ortega et al., 2013). With respect to neurogenesis from the dSVZ and lSVZ, the effects of GSK3β on Notch1, BMPs, and Shh signaling pathways are likely to be important (Kim and Snider, 2011). The Shh activated transcription factor Gli1 was only induced by GSK3β inhibition in the lSVZ, consistent with higher Shh expression in this region at P8 (Azim et al., 2012b; Palma et al., 2005). A mechanism exists for GSK3β in negatively regulating downstream Shh signaling (Jia et al., 2002) and has been described in the case of GSK3β ablation in NSCs that promoted Shh signaling via Gli1 (Kim et al., 2009a). In comparison, Notch1 signaling via NICD nuclear activation was reduced dramatically by GSK3β inhibition in the dSVZ compared with the lSVZ, implying a greater role for Notch signaling in regulating oligodendrogenesis and neurogenesis from the dSVZ microdomain. Earlier studies have indicated a direct role for GSK3β in phosphorylating NICD in shortening its half-life (Foltz et al., 2002), whilst KO of GSK3β in NSCs induces the inverse of promoting Notch1 signaling and its target genes in early forebrain development (Kim et al., 2009a). In addition, Notch1 activity is required to be downregulated for the timely differentiation of early OL lineage cells (Wang et al., 1998), which fits with the results of our study where GSK3β inhibition promoted OL lineage progression, as observed previously in the optic nerve (Azim and Butt, 2011). BMPs are strong inhibitors of oligodendrogenesis and their downstream TFS work in concert with β-catenin to repress maturation in later stage OL lineage cells (Bilican et al., 2008; Weng et al., 2012). We observed a partial reduction in the downstream BMP pathway factors pSMADs1/5/8 in the dSVZ in response to GSK3β inhibition, which has not previously been reported in the context of SVZ gliogenesis. However, in cultured dorsal spinal cord NPs, FGF2 represses SMADs to induce Olig2 expression (Bilican et al., 2008; Weng et al., 2012). The results indicate GSK3β regulates multiple pathways that to a certain extent are microdomain specific and may have differential roles in regulating oligodendrogenesis and neurogenesis.

Extracellular cues that could regulate OP generation via GSK3β and β-catenin were examined in the dSVZ. Like ARA-014418, Wnt3a was equally effective in modulating GSK3β and β-catenin, providing further proof that the effects of GSK3β inhibition are mediated primarily via the canonical Wnt pathway (Azim et al., 2014). Moreover, FGF2 appears to also regulate GSK3β and β-catenin in the dSVZ, as described in cultured NSCs (Israsena et al., 2004). Intriguingly, EGF and BMP4 almost completely abolished nuclear β-catenin expression, suggesting they may exert an inhibitory effect on endogenous Wnt/β-catenin signaling in the dSVZ. Neurospheres derived from the dorsal embryonic forebrain rapidly lose Wnt-signaling in conditions containing EGF.
(Machon et al. 2005), and EGF-signalling could repress β-catenin in dorsalizing NSC phenotypes.

In summary, these findings are in line with our previous study underlying GSK3β inhibitors increase the number of OLs and promotes myelination in the developing corpus callosum and following a chemical demyelinating lesion (Azim and Butt, 2011). Our present study provides further evidence that postnatally the dSVZ is the primary source of newly generated OLs in the forebrain, and that their generation from NSC/NP is regulated by endogenous GSK3β activity and Wnt signalling in a microdomain specific manner (Azim et al., 2014). The failure of remyelination in MS is due in part due to upregulation of negative regulatory factors and a loss of positive regulatory factors (Franklin and Ffrench-Constant, 2008). For example, Notch1 signaling may be upregulated in multiple sclerosis and delay remyelination by inhibiting OPC differentiation (Blanchard et al., 2013; John et al., 2002; Zhang et al., 2009). Targeting GSK3β through the use of small molecule inhibitors may therefore complement other therapeutic approaches for stimulating OP generation from the dSVZ.

Acknowledgment

Grant sponsor: Forschungskredit of University of Zurich; Grant number: K-41211-01-01; Grant sponsor: National Research Project; Grant number: NRP63; 406340_128291; Grant sponsor: Swiss National Fund; Grant number: 31003A_127082; Grant sponsors: Multiple Sclerosis Society (UK); Anatomical Society of the UK and Ireland.

The authors thank Professor Stallcup for antibodies against PDGFRα and Professor Richardson for the Sox10-GFP transgenic mouse line. They thank Phillip Smithurst, Stefano Pino, and Samir Mistry at the University of Portsmouth for their technical assistance.

References

Adachi K, Mirzadeh Z, Sakaguchi M, Yamashita T, Nikolcheva T, Gotoh Y, Peltz G, Gong L, Kawase T, Alvarez-Buylla A, et al. 2007. Beta-catenin signaling promotes proliferation of progenitor cells in the adult mouse subventricular zone. Stem Cells 25:2827–2836.

Aguirre A, Gallo V. 2004. Postnatal neurogenesis and gliogenesis in the olfactory bulb from NG2-expressing progenitors of the subventricular zone. J Neurosci 24:10530–10541.

Azim K, Butt AM. 2011. GSK3beta negatively regulates oligodendrocyte differentiation and myelination in vivo. GLIA 59:540–553.

Azim K, Fiorelli R, Zweifel S, Hurtado-A Chong A, Yoshikawa K, Slomianka L, Raineteau O. 2012a. 3-dimensional examination of the adult mouse subventricular zone reveals lineage-specific microdomains. PLoS One 7:e49087.

Azim K, Fischer B, Hurtado-Chong A, Draganova K, Cantú C, Zemke M, Sommer L, Butt AM, Raineteau O. 2014. Persistent Wnt/JCatenin signaling determines dorsalization of the postnatal subventricular zone and neural stem cell specification into oligodendrocytes and glutamatergic neurons. Stem Cells. Advanced online publication. doi: 10.1002/stem.1639.

Bhat R, Xue Y, Berg S, Hellberg S, Ormo M, Nilsson Y, Radesater AC, Jerning E, Markgen PO, Borgegard T, et al. 2003. Structural insights and biological effects of glycogen synthase kinase 3-specifier inhibitor AR-A014418. J Biol Chem 278:45937–45945.

Bilican B, Fiore-Heriche C, Compston A, Allen ND, Chandran S. 2008. Induction of Olig2 precursors by FGF involves BMP signalling blockade at the Smad level. PLoS One 3:e2863.

Blanchard B, Heurttaux T, Garcia C, Moll NM, Caillava C, Grandbarbe L, Klospeot A, Kernina C, Frah M, Coawar D, et al. 2013. Tocopherol derivate TFA-12 promotes myelin repair in experimental models of multiple sclerosis. J Neurosci 33:11633–11642.

Brill MS, Ninkovic J, Wimperry E, Hodge RD, Ozen I, Yang R, Lepier A, Gason S, Erdelyi F, Szabo G, et al. 2009. Adult generation of glutamatergic olfactory bulb interneurons. Nat Neurosci 12:1524–1533.

Butt AM, Hornby MF, Kirvell S, Berry M. 1997. Platelet-derived growth factor delays oligodendrocyte differentiation and axonal myelination in vivo in the anterior medullary velum of the developing rat. J Neurosci Res 48:588–596.

Cohen P, Goedert M. 2004. GSK3 inhibitors: Development and therapeutic potential. Nat Rev Drug Discov 3:479–487.

Doble BW, Woodgett JR. 2003. GSK-3: Tricks of the trade for a multi-tasking kinase. J Cell Sci 116:1175–1186.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. 1999. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703–716.

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A. 1997. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J Neurosci 17:5046–5061.

Fancy SP, Baranzini SE, Zhao C, Yik DI, Irvine KA, King S, Sanasi N, Franklin RJ, Rowitch DH. 2009. Dysregulation of the Wnt pathway inhibits timely myelination and remyelination in the mammalian CNS. Genes Dev 23:1571–1585.

Fei T, Xia K, Li Z, Zhou B, Zhu S, Chen H, Zhang J, Chen Z, Xiao H, Han JD, et al. 2010. Genome-wide mapping of SMAD target genes reveals the role of BMP signaling in embryonic stem cell fate determination. Genome Res 20:36–44.

Foltz DR, Santiago MC, Berechid BE, Nye JS. 2002. Glycogen synthase kinase-3beta modulates notch signaling and stability. Curr Biol 12:1006–1016.

Franklin RJ, Ffrench-Constant C. 2008. Remyelination in the CNS: From biology to therapy. Nat Rev Neurosci 9:839–855.

Goddard DR, Berry M, Butt AM. 1999. In vivo actions of fibroblast growth factor-2 and insulin-like growth factor-I on oligodendrocyte development and myelination in the central nervous system. J Neurosci Res 57:74–85.

Grimes CA, Jope RS. 2001. The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog Neurobiol 65:391–426.

He L, Lu QR. 2013. Coordinated control of oligodendrocyte development by extrinsic and intrinsic signaling cues. Neurosci Bull 29:129–143.

Ishasena N, Hu M, Fu W, Kan L, Kessler JA. 2004. The presence of FGF2 signaling determines whether beta-catenin exerts effects on proliferation or neuronal differentiation of neural stem cells. Dev Bioi 268:220–231.

Jia J, Amanai K, Wang G, Tang J, Wang B, Jiang J. 2002. Shaggy/GSK3 antagonist Hedgehog signaling by regulating Cubitus interruptus. Nature 416:548–552.

Jiao J, Chen DF. 2008. Induction of neurogenesis in nonconventional neurogenic regions of the adult central nervous system by niche astrocyte-produced signals. Stem Cells 26:1221–1230.

John GR, Shankar SL, Shafit-Zagardo B, Massimi A, Lee SC, Raine CS, Brossan CF. 2002. Multiple sclerosis: Re-expression of a developmental pathway that restricts oligodendrocyte maturation. Nat Med 8:1115–1121.
Kessaris N, Fogarty M, Iannarelli P, Grist M, Wegner M, Richardson WD. 2006. Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. Nat Neurosci 9:173–179.

Kim WY, Snider WD. 2011. Functions of GSK-3 signaling in development of the nervous system. Front Mol Neurosci 4:44.

Kim WY, Wang X, Wu Y, Doble BW, Patel S, Woodgett JR, Snider WD. 2009a. GSK-3 is a master regulator of neural progenitor homeostasis. Nat Neurosci 12:1390–1397.

Kim Y, Comte I, Szabo G, Hoobberger P, Szol F. 2009b. Adult mouse subventricular zone stem and progenitor cells are sessile and epidermal growth factor receptor negatively regulates neuroblast migration. PLoS One 4:e8122.

Kim YT, Hur EM, Snider WD, Zhou FQ. 2011. Role of GSK3 signaling in neuronal morphogenesis. Front Mol Neurosci 4:48.

Kriks S, Shim JW, Piao J, Ganat YM, Wakeman DR, Xie Z, Carrillo-Reid L, Au Yeung G, Antonacci C, Buch A, et al. 2011. Dopamine neurons derived from human ES cells efficiently engraft in animal models of Parkinson’s disease. Nature 480:547–551.

Lie DC, Colamarino SA, Song HU, Desire L, Mira H, Consiglio A, Lein ES, Jessberger S, Lansford H, Deane AR, et al. 2005. Wnt signaling regulates adult hippocampal neurogenesis. Nature 437:1370–1375.

Machon O, Backman M, Krauss S, Kozmik Z. 2005. The cellular fate of cortical progenitors is not maintained in neurosphere cultures. Mol Cell Neurosci 30:388–397.

Marshall CA, Novitch BG, Goldman JE. 2005. Olig2 directs astrocyte and oligodendrocyte formation in postnatal subventricular zone cells. J Neurosci 25:7289–7298.

Maurer MH, Bromme JO, Feldmann RE Jr, Jarve A, Sabouri F, Burgers HF, Schelshorn DW, Kruger C, Schneider A, Kuschinsky W. 2007. Glycogen synthase kinase 3beta (GSK3beta) regulates differentiation and proliferation in neural stem cells from the rat subventricular zone. J Proteome Res 6:1198–1208.

Menn B, Garcia-Verdugo JM, Yaschine C, Gonzalez-Perez O, Rowitch D, Alvarez-Buylla A. 2006. Origin of oligodendrocytes in the subventricular zone of the adult brain. J Neurosci 26:7907–7919.

Nakatani H, Martin E, Hassani H, Clavairoly A, Maire CL, Viadieu A, Keminon C, Delmasure A, Frah M, Weber M, et al. 2013. Ascl1/Mash1 promotes brain oligodendrogenesis during myelination and remyelination. J Neurosci 33:9752–9768.

Ortega F, Gascón S, Masserotti G, Deshpande A, Simon C, Fischer J, Dimou L, Chichung Lie D, Schroeder T, Berninger B. 2013. Oligodendrogligenic and neurogenic adult subependymal zone neural stem cells constitute distinct lineages and exhibit differential responsiveness to Wnt signaling. Nat Cell Biol 15:602–613.

Ota T, Fujii M, Sugizaki T, Ishii M, Miyazawa K, Akuratani H, Miyazono K. 2002. Targets of transcriptional regulation by two distinct type I receptors for transforming growth factor-beta in human umbilical vein endothelial cells. J Cell Physiol 193:299–318.

Palma V, Lim DA, Dahmane N, Sanchez P, Brionne TC, Herzberg CD, Gitton Y, Carleton A, Alvarez-Buylla A, Ruiz J, Altaka A. 2005. Sonic hedgehog controls stem cell behavior in the postnatal and adult brain. Development 132:335–344.

Parras CM, Galli R, Britz O, Soares S, Galichet C, Battiste J, Johnson JE, Nakafuku M, Vescovi A, Guillemot F. 2004. Mash1 specifies neurons and oligodendrocyte progenitors in the postnatal brain. EMBO J 23:4495–4505.

Peretto P, Merighi A, Fasolo A, Bonfanti L. 1997. Gcial tubes in the rostral migratory stream of the adult rat. Brain Res Bull 42:9–21.

Richardson WD, Kessaris N, Pringle N. 2006. Oligodendrocyte wars. Nat Rev Neurosci 7:11–18.

Smith CM, Luakin MB. 1998. Cell cycle length of olfactory bulb neuronal progenitors in the rostral migratory stream. Dev Dyn 213:220–227.

Wang S, Sdrulla AD, diSibio G, Bush G, Nofziger D, Hicks C, Weinmaster G, Barres BA. 1998. Notch receptor activation inhibits oligodendrocyte differentiation. Neuron 21:63–75.

Weng Q, Chen Y, Wang H, Xu X, Yang B, He Q, Shou W, Higashi Y, van den Berghe V, Seuntjens E, et al. 2012. Dual-mode modulation of Smad signaling by Smad-interacting protein Sip1 is required for myelination in the central nervous system. Neuron 73:713–728.

Wu M, Hernandez M, Shen S, Sabo JK, Kelkar O, Wang J, O’Leary R, Phillips GR, Cate HS, Casaccia P. 2012. Differential modulation of the oligodendrocyte transcriptome by sonic hedgehog and bone morphogenetic protein 4 via opposing effects on histone acetylation. J Neurosci 32:6651–6661.

Ye F, Chen Y, Hoang T, Montgomery RL, Zhao XH, Bu H, Hu T, Taketo MM, van Es JH, Clevers H, et al. 2009. HDAC1 and HDAC2 regulate oligodendrocyte differentiation by disrupting the beta-catenin-TCF interaction. Nat Neurosci 12:829–838.

Young KM, Fogarty M, Kessaris N, Richardson WD. 2007. Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. J Neurosci 27:8286–8296.

Zhang Y, Argaw AT, Gurfein BT, Zameer A, Snyder BJ, Ge C, Lu QR, Rowitch DH, Raine CS, Brosnan CF, et al. 2009. Notch1 signaling plays a role in regulating precursor differentiation during CNS remyelination. Proc Natl Acad Sci USA 106:19162–19167.

Zhou YY, Armstrong RC. 2007. Interaction of fibroblast growth factor 2 (FGF2) and notch signaling components in inhibition of oligodendrocyte progenitor (OP) differentiation. Neurosci Lett 421:27–32.